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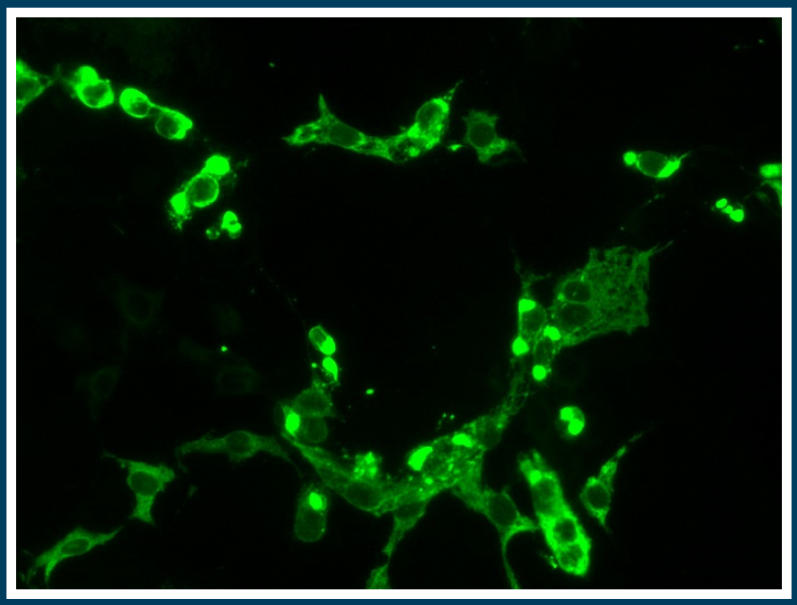
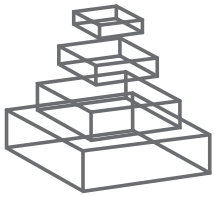


THE UNFOLDED PROTEIN RESPONSE IN VIRUS INFECTIONS

Topic Editor
Shiu-Wan Chan

frontiers in MICROBIOLOGY



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THE UNFOLDED PROTEIN RESPONSE IN VIRUS INFECTIONS

Topic Editor:
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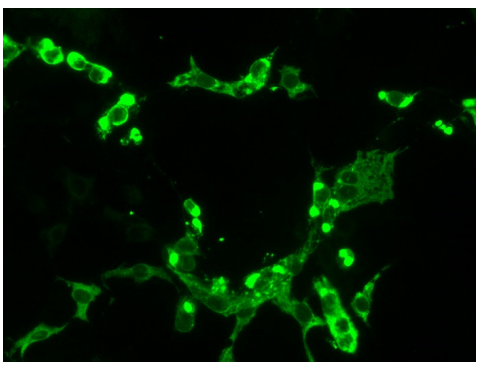

Hepatitis C virus envelope glycoprotein E2 expression in COS-1. Courtesy of Shiu-Wan Chan.

Unfolded protein response (UPR) is a cellular adaptive response for restoring endoplasmic reticulum (ER) homeostasis in response to ER stress. Perturbation of the UPR and failure to restore ER homeostasis inevitably leads to diseases. It has now become evident that perturbation of the UPR is the cause of many important human diseases such as neurodegenerative diseases, cystic fibrosis, diabetes and cancer. It has recently emerged that virus infections can trigger the UPR but the relationship between virus infections and host UPR is intriguing. On one hand, UPR is harmful to the virus and virus has developed means to subvert the UPR. On the other hand, virus exploits the host UPR to assist in its own infection, gene expression, establishment of persistence, reactivation from latency and to evade the immune response. When this delicate balance of virus-host UPR interaction is broken down, it may cause diseases. This is particularly challenging for viruses that establish a chronic infection to maintain this balance. Each virus interacts with the host UPR in a different way to suit their life style and how the virus interacts with the host UPR can define the characteristic of a particular virus infection. Understanding how a particular virus interacts with the host UPR may pave the way to the design of a new class of anti-viral that targets this particular pathway to skew the response towards anti-virus. This knowledge can also be translated into the clinics to help re-design oncolytic virotherapy and gene therapy. In this research topic we aimed to compile a collection of focused review articles, original research articles, commentary, opinion, hypothesis and methods to highlight the current advances in this burgeoning area of research, in an attempt to provide an in-depth understanding of how viruses interact with the host UPR, which may be beneficial to the future combat of viral and human diseases.

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EDITORIAL
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The unfolded protein response in virus infections

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Keywords: unfolded protein response, endoplasmic reticulum stress, ERAD, autophagy, innate immunity, gene therapy, pathogenesis, virus-host interaction

Unfolded protein response (UPR) is a cellular homeostatic response to endoplasmic reticulum (ER) stress. Increasing evidence suggests an intimate relationship between virus and UPR. This research topic collated a number of review articles and original research article, in an attempt to highlight how viruses interact with the host UPR in the establishment of acute, chronic and latent infections.

Virus infection represents an arm race between virus and the host. On one hand, the host mobilizes the UPR in an attempt to restrict virus infection. On the other hand, virus subverts or even manipulates the UPR to assist in its own infection. The consequence of this is that the UPR is often skewed during virus infections to either favor virus elimination or virus invasion. Whoever won, the outcome could be pathogenic. The relationship between virus and UPR and its associated autophagy is being addressed in three reviews focusing on RNA viruses, as their life cycles are closely associated with the ER (Blazquez et al., 2014; Fung and Liu, 2014; Jheng et al., 2014). Miguel Martin-Acebes and his group focuses on flaviviruses whereas To S. Fung and Ding X. Liu focus on coronaviruses. Jim-Tong Horng's group takes a closer look at virus interaction with autophagy and also discusses the potential of targeting UPR and autophagy as novel anti-virals.

In contrast to acute virus, one can only imagine that virus establishing a life-long chronic infection may interact with the host UPR in a completely different way to maintain an environment favorable for virus survival. Two reviews presented by Shiu-Wan Chan and Norica Branza-Nichita's group on hepatitis C virus and hepatitis B virus, respectively, shed light on how persistent virus interacts with the host UPR to benefit establishment of a chronic infection and how chronic activation of the UPR leads to diseases (Chan, 2014; Lazar et al., 2014).

UPR is prevalent in viruses establishing latent infections such as herpesviruses. Herpesvirus is an ancient virus. During its course of millions of years of co-inhabitation with its host, herpesvirus has borrowed a number of molecules from its host to be used in its life cycle. There is no exception in UPR, in which herpesviruses also share molecular mimicry with the UPR molecules and utilize UPR to set up lytic infection and to break dormancy, suggesting that interaction of virus with host UPR may be very ancient. Varicella-zoster virus (VZV) possesses the smallest genome of human herpesviruses and lacks some genes used by other herpesviruses to manipulate the UPR. The key question is therefore whether VZV UPR induction is merely a host response or a result of viral manipulation. By using a UPR PCR array, John Carpenter and Charles Grose demonstrated VZV differentially induced the UPR to expand the ER to cope with viral glycoprotein synthesis (Carpenter and Grose, 2014). This study also uncovered VZV upregulation of an unusual UPR molecule, the cAMP responsive element binding protein H. Clearly, this will pave the way to future studies to disclose the relationship between VZV and UPR.

ER-associated degradation (ERAD) is part of an UPR functioning to extract unfolded/misfolded proteins from the ER into the cytosol for proteasomal degradation. Not surprisingly, this process is also targeted by virus. Jaquelin Dudley and her group re-captures the ERAD process in details followed by an illustration of how viruses exploit this process (Byun et al., 2014). First, viruses can simply mobilize the ERAD to degrade important immune molecules or viral envelope glycoproteins to evade innate and adaptive immune responses. At a more intimate level, some viruses have actually incorporated ERAD into their life cycles for viral protein and even virion maturation. It is fascinating how naked polyomaviruses will make a *de tour* to the ER for ERAD-assisted uncoating before re-entering the cytosol *en route* to the nucleus. Lastly, viruses can interfere with ERAD tuning and hijack certain ERAD cargo into forming double membrane vesicles as sites of virus replication.

UPR has emerged to be more than a homeostatic cellular response to virus infections. UPR has been intimately linked to innate immunity; whether by modulating innate immunity or as part of the innate immunity. Innate immunity is initiated by the sensing of "danger signals" by host pattern recognition receptors (PRRs), culminating in the release of interferon, which in turn activates the professional virus killer, one of which is RNase L. One of the proximal UPR sensors, inositol-requiring enzyme 1 (IRE1), is evolutionarily related to RNase L. In the review of Sankar Bhattacharyya, he provides a structural and functional comparison between IRE1 and RNase L and comments on a potential anti-viral function of IRE1 by the creation of "danger signals" via the regulated IRE1-dependent decay (RIDD) pathway (Bhattacharyya, 2014). An important question remains as to whether UPR represents a new tool for sensing viruses or select UPR molecules are merely being co-opted in "microbial stress response." This is being addressed in Judith Smith's review, in which she provides a critique on the intersection of the UPR with the inflammatory pathways and innate immunity and offers an insight into UPR-PRR synergy as an evolutionary adaptation to ensure specificity of anti-viral responses (Smith, 2014).

It is increasingly popular to use viruses in clinical applications such as gene therapy and oncolytic virotherapy. The use of viral vectors/viruses in the clinics will not be valid without a thorough understanding of virus-host interaction. Giridhara Jayandharan and his group presents a review on the emerging impact of UPR on gene therapy and how the understanding of this will allow us to exploit and improve the use of viral vectors in gene therapy (Sen et al., 2014).

To date we are still at the sprouting stage of understanding this virus-host interaction. We hope that this selection of articles will provide a foundation to spark more interest in this research area. This will not only lead to a deeper understanding of virus infection and pathogenesis but will also unravel novel anti-viral mechanisms. Eventually it will help to unlock novel anti-viral targets and may also impact on optimizing the use of viruses in the clinics.

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Stress responses in flavivirus-infected cells: activation of unfolded protein response and autophagy

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The *Flavivirus* is a genus of RNA viruses that includes multiple long known human, animal, and zoonotic pathogens such as Dengue virus, yellow fever virus, West Nile virus, or Japanese encephalitis virus, as well as other less known viruses that represent potential threats for human and animal health such as Usutu or Zika viruses. Flavivirus replication is based on endoplasmic reticulum-derived structures. Membrane remodeling and accumulation of viral factors induce endoplasmic reticulum stress that results in activation of a cellular signaling response termed unfolded protein response (UPR), which can be modulated by the viruses for their own benefit. Concomitant with the activation of the UPR, an upregulation of the autophagic pathway in cells infected with different flaviviruses has also been described. This review addresses the current knowledge of the relationship between endoplasmic reticulum stress, UPR, and autophagy in flavivirus-infected cells and the growing evidences for an involvement of these cellular pathways in the replication and pathogenesis of these viruses.

Keywords: flavivirus, unfolded protein response, autophagy, dengue virus, West Nile virus, endoplasmic reticulum stress, virus replication

INTRODUCTION

In recent years, the knowledge of virus–host interactions has unveiled multiple connections between virus life cycle steps and a variety of cellular organelles and signaling pathways. Deciphering the complexity of these interactions will provide key information for the control of viral pathogens. This mini-review addresses the current knowledge and challenges for a deep understanding of the interactions of flaviviruses with the endoplasmic reticulum (ER) and two related cellular pathways: the unfolded protein response (UPR) and autophagy.

FLAVIVIRUS OVERVIEW

The *Flavivirus* genus comprises more than 50 distinct species of enveloped positive single strand RNA viruses. This genus is classified into the *Flaviviridae* family together with *Pestivirus*, *Hepacivirus*, and *Pegivirus* (http://www.ictvonline.org/virusTaxonomy.asp). Flaviviruses include multiple well known human, animal, and zoonotic pathogens such as yellow fever virus (YFV), dengue virus (DENV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), or West Nile virus (WNV), as well as other emerging or re-emerging pathogens such as Usutu virus (USUV) or Zika virus, which are now being considered as potential threats for human and animal health (Weissenbock et al., 2010). As arboviruses (**ar**thropod-**bo**rne **viruses**), most flaviviruses are transmitted by mosquitoes or ticks and maintained in nature through complex infectious cycles that involve different hosts. The variety of symptoms caused by flaviviruses includes jaundice (YFV), febrile illnesses (YFV, DENV, or WNV), hemorrhagic fevers (DENV), or encephalitis (JEV, SLEV, WNV, or TBEV). As a result of different factors, including globalization of travel and trade, climate warming, or changes in land use and vector behavior, different flaviviruses are currently becoming global health threats with DENV being amongst the most prominent human pathogens. In fact, DENV is responsible for up to 50 million infections each year, including 22,000 deaths, mostly among children (http://www.who.int/csr/disease/dengue/impact/en/). There are several vaccines against flaviviruses currently licensed for use in humans (YFV, JEV, TBEV) or animals (WNV, louping ill virus, Wesselsbron virus; Ishikawa et al., 2014). However, there is still a need for specific vaccines or treatments to combat many of these pathogens, i.e., DENV, and a detailed knowledge of flavivirus–host interactions is considered crucial to develop effective therapies.

ER AND FLAVIVIRUSES: AN INTIMATE RELATIONSHIP

Flavivirus replication takes place in association with intracellular membrane structures (**Figure 1**). As other positive-strand RNA viruses, flaviviruses rearrange host cell membranes to build organelle-like structures in order to establish the appropriate environment for viral replication (Paul and Bartenschlager, 2013). The main source of these membranes is provided by the ER where both viral structural and non-structural proteins accumulate (Welsch et al., 2009; Gillespie et al., 2010; Martin-Acebes et al., 2011; Miorin et al., 2013; Junjhon et al., 2014). Membrane reorganizations are driven by viral proteins. These not only induce

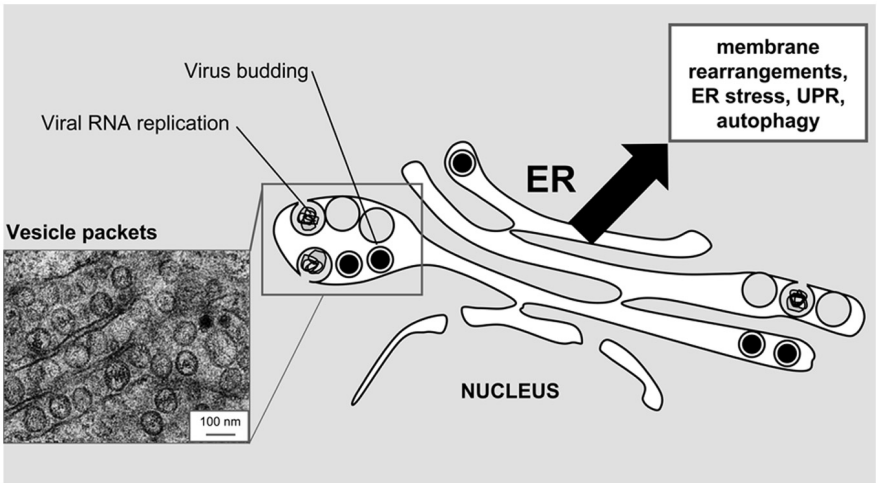

FIGURE 1 | Schematic view of flavivirus connection with the ER. The alterations in the ER architecture in flavivirus-infected cells are highlighted. A representative electron micrograph of HeLa cells infected with the flavivirus WNV is shown as an example of these alterations in the ER. Note electron dense virions and vesicle packets. Micrograph courtesy of Miguel A. Martín-Acebes.

changes in the protein composition of ER membranes but also in their lipid content (Mackenzie et al., 2007; Heaton et al., 2010; Martin-Acebes et al., 2011; Perera et al., 2012). The formation of the replication complex has been mainly associated with the expression of hydrophobic transmembrane nonstructural proteins NS4A (Roosendaal et al., 2006; Miller et al., 2007) and NS4B (Kaufusi et al., 2014) that are involved in membrane remodeling. The infection induces the formation of membrane vesicles inside the lumen of the ER (an example of WNV-infected cells is depicted in **Figure 1**). These characteristic structures usually referred to as vesicle packets (VPs) or double membrane vesicles (DMVs) have been associated with viral genome replication (Welsch et al., 2009; Gillespie et al., 2010; Miorin et al., 2013; Junjhon et al., 2014). Other flavivirus-induced membrane structures that could also be ER-related are the so-called paracrystalline arrays or convoluted membranes (Mackenzie and Westaway, 2001; Welsch et al., 2009). However, convoluted membranes are not induced in all flavivirus-infected cell types and their specific function in viral infection remains unclear (Junjhon et al., 2014). The newly synthesized viral genomes are enclosed into virions that assemble and bud into the ER, and then traffic through the Golgi complex along the secretory pathway and maturate (Mukhopadhyay et al., 2005) prior to be released from infected cell. In this way, the interaction of flaviviruses with the ER not only provides a replication platform but also the membrane components for the virions (Mukhopadhyay et al., 2005). All these findings make the ER and ER-related pathways key players during flavivirus infection.

ER, CELLULAR STRESS, AND UPR DURING FLAVIVIRUS INFECTIONS

The ER is an essential organelle involved in many cellular functions including protein folding and secretion, lipid biosynthesis, and calcium homeostasis. A quality control mechanism ensures that only properly folded proteins exit from the ER while incorrectly folded proteins are retained and degraded. The accumulation of misfolded or unfolded proteins can trigger ER stress. To cope with stress, cells activate the intracellular signaling pathway called UPR (Liu et al., 2000). The UPR includes transcriptional induction of genes, attenuation of global protein synthesis, and ER-associated degradation (ERAD). The three main branches of the UPR are the protein kinase-like ER resident kinase (PERK), the activating transcription factor 6 (ATF6), and the inositol-requiring enzyme 1 (IRE1; **Figure 2**; Liu and Kaufman, 2003). These proteins are associated with the ER chaperone BiP/Grp78, which prevents their aggregation and further activation. But the UPR is not only triggered by misfolded proteins, other perturbations can also alter the ER homeostasis such as glucose deprivation, aberrant Ca^{2+} regulation or viral infections. Related to the Ca^{2+} balance, WNV for example induces a Ca^{2+} influx early after infection of cells that has been associated with a virus-induced rearrangement of the ER membrane and activation of different cellular kinases involved in stress response and cell survival, focal adhesion kinase (FAK), mitogen-activated extracellular signal-regulated protein kinase (ERK1/2), and protein-serine kinase B alpha (Akt; Scherbik and Brinton, 2010).

Viruses have evolved to manipulate host UPR signaling pathways to promote viral translation and persistence in infected cells (Chan and Egan, 2005; Tardif et al., 2005; Ke and Chen, 2011; Ambrose and Mackenzie, 2013b; Green et al., 2013). Studies that focused on the *Flaviviridae* family have documented the activation of one or more of the three arms of the UPR. However controversial reports have been published even for the same virus. The reasons for these different results are likely due to differences in the strains or serotypes used, or derived from the use of subgenomic replicons, isolated proteins or complete viruses. For instance, it has been documented that infection by the *Hepacivirus* hepatitis C virus (HCV) leads to the activation of the three UPR signaling pathways (Shinohara et al.,

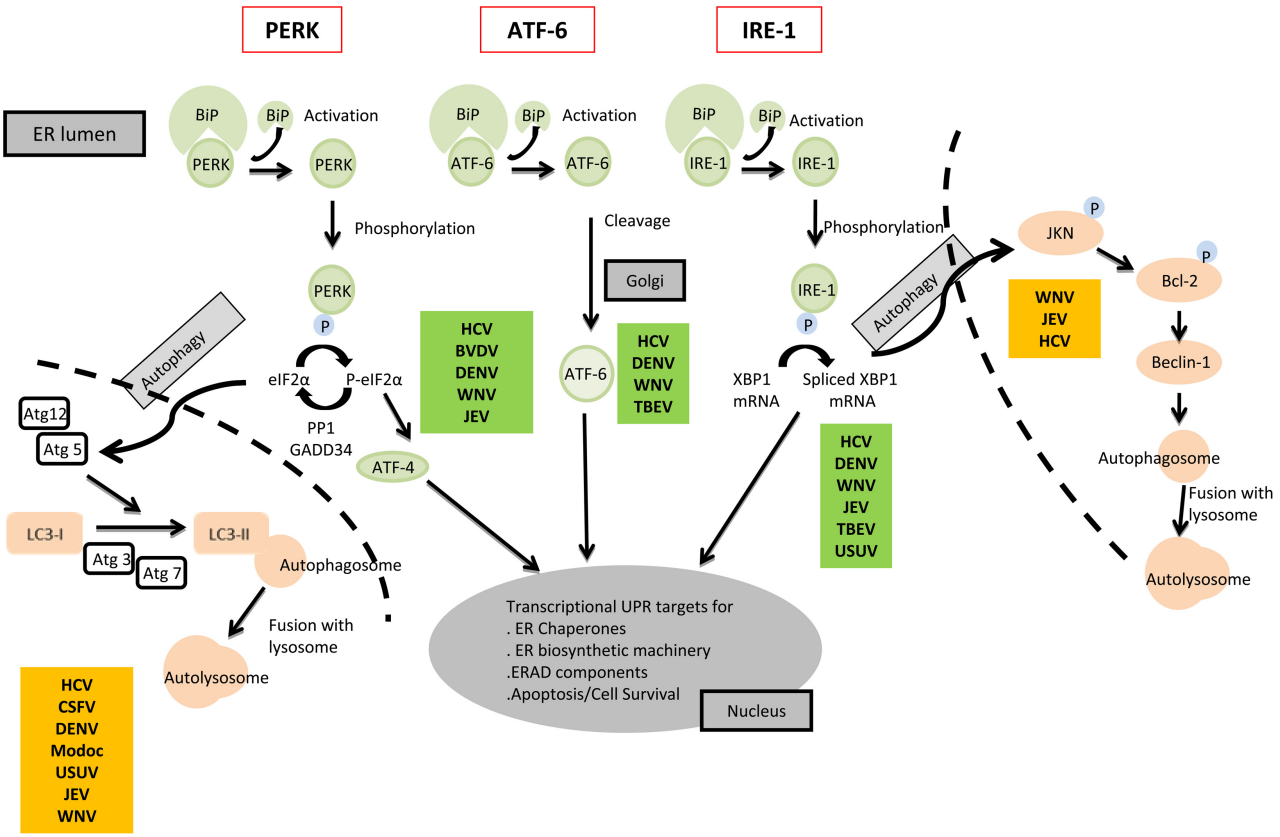

FIGURE 2 | Cell signaling pathways of the UPR, autophagy connections and flaviviruses. The three arms of UPR (PERK, ATF-6, IRE-1) are shown in the figure. The viruses from the *Flaviviridae* family whose infection has been related to each process have been noted in the figure. See the text for details.

2013) including BiP expression, IRE1 activation, and Xbp-1 splicing (Tardif et al., 2004), ATF6 cleavage (Tardif et al., 2002; Li et al., 2009), eIF2α phosphorylation, and induction of CHOP expression (Chan and Egan, 2005). In contrast, cells harboring a neomycin-adapted subgenomic replicon of HCV that express the nonstructural proteins showed a reduction of eIF2α phosphorylation (Tardif et al., 2002). For the *Pestivirus* bovine viral diarrhea virus (BVDV), the stimulation of proapoptotic effectors with high-level signaling through PERK and eIF2α phosphorylation resulting in CHOP activation and induction of apoptotic effectors caspase 12 and poly ADP ribose polymerase (PARP) has been described (Jordan et al., 2002). Specifically among *Flavivirus,* infection with DENV showed a time dependent activation of the UPR pathways, with PERK activation and eIF2α phosphorylation during early stages of replication that rapidly switched off, with IRE1 and ATF6 upregulation occurring at mid and late stages in the replication cycle, respectively (Pena and Harris, 2011). However, it has also been described the induction of Xbp-1 splicing (Yu et al., 2006; Umareddy et al., 2007; Pena and Harris, 2011), ATF6 cleavage (Umareddy et al., 2007; Pena and Harris, 2011) and activation of GADD34 and CHOP expression leading to apoptosis (Umareddy et al., 2007). In the case of WNV, UPR is activated towards chaperone production and membrane biogenesis to benefit replication (Medigeshi et al., 2007). ATF6 and IRE1 upregulation has also been demonstrated, with Xbp-1s induction, even though the IRE1–Xbp-1 pathway seems to be non-essential for its replication (Medigeshi et al., 2007). In addition to this, WNV strain specific differences regarding regulation of the PERK arm of the UPR have been described. For example, while infection with a WNV attenuated strain prevents PERK-mediated translation and CHOP transcription (Ambrose and Mackenzie, 2010), infection with the highly neurovirulent WNV NY-99 strain upregulates all three pathways of the UPR (Medigeshi et al., 2007) with an early induction of eIF2α phosphorylation and upregulation of downstream apoptotic factors such as CHOP, GADD34, caspase-3, and PARP, which may represent a host defense mechanism to limit viral replication. Other members of the *Flavivirus* genus distinct from DENV and WNV also activate different components of the UPR. For instance, the induction of Xbp-1 splicing after infection with JEV, TBEV, and USUV (Yu et al., 2006, 2013; Blazquez et al., 2013), the expression of CHOP during JEV infection, and the cleavage of ATF6 in TBEV-infected cells (Yu et al., 2006, 2013) have been described.

It is important to highlight the described relevant function of viral proteins of the *Flaviviridae* in the regulation of the UPR. For example, HCV NS4B is a strong regulator of UPR signaling (Zheng et al., 2005; Li et al., 2009), while HCV

envelope proteins activate IRE1 and Xbp-1 splicing, and upregulate Bip expression (mainly by E2; Chan and Egan, 2005). WNV NS4A and NS4B strongly induce Xbp-1 transcription and processing when individually expressed, and this ability is directly related to the number of hydrophobic segments they contain (Ambrose and Mackenzie, 2010). In the case of DENV-2, Xbp-1 splicing is induced by NS2B/3 (Yu et al., 2006). Therefore, the role of the UPR during flavivirus infections has been associated with factors contributing to the establishment of an environment more favorable for replication such as chaperone expression, membrane biogenesis, or ATF4-mediated antioxidant and amino acid transporter production. However, some downstream UPR effects such as the inhibition of translation, mRNA decay, production of degradative proteins, or induction of apoptosis are not necessarily beneficial for viral replication (Ambrose and Mackenzie, 2013b). Finally, interaction between the UPR and interferon (IFN) signaling in flaviviral infections has been reported, as ATF6 and IRE1 seem to be required for WNV Kunjin-induced STAT1 phosphorylation and nuclear translocation in response to IFN stimulation (Ambrose and Mackenzie, 2013a). All these findings provide evidence for the multifaceted roles of UPR during flavivirus infections and its connections with cellular metabolism, apoptosis, and innate immunity. These aspects remark the importance of a proper understanding of the interaction of each flavivirus with this cellular signaling pathway.

STRESS, UPR, AND AUTOPHAGY IN FLAVIVIRUS INFECTED CELLS

Autophagy is a cellular process by which cytoplasmic components are sequestered in double-membrane vesicles and degraded. Autophagy is also intrinsically linked to ER function since the ER provides the membranes involved autophagy (Lamb et al., 2013). There are multiple connections between ER, UPR, and autophagy and changes in ER architecture or composition can trigger autophagy through activation of components of the UPR (Suh et al., 2012; **Figure 2**). By facilitating the removal of damaged organelles and cytoplasmic protein aggregates, autophagy has been proven to be essential for the maintenance of cellular homeostasis (Kudchodkar and Levine, 2009). In addition, this constitutive degradation pathway also plays important roles in development, differentiation, and stress responses (Levine and Klionsky, 2004), and it is an important component of the innate and adaptive immune response elicited against a variety of viral and bacterial pathogens (reviewed in Deretic, 2005; Deretic and Levine, 2009).

The process of autophagy comprises three steps starting with the nucleation and elongation of vesicles to form the phagophore. The edges of phagophore then fuse to assemble the autophagosome. Finally, autophagosomes maturate to autolysosomes by membrane fusion with endosomes (then called amphisomes) or lysosomes (resulting in autolysosomes). Different roles for multiple cellular proteins involved in autophagy have been reported to date. One of the most widely used indicators of upregulation of autophagy is the cytoplasmic aggregation of microtubule-associated protein 1 light chain 3 (LC3), that is modified by its conjugation to phosphatidylethanolamine and targeted to autophagic membranes labeling autophagic vacuoles (Kabeya et al., 2000; Klionsky et al., 2008). An upregulation of the autophagic pathway, characterized by an increase in LC3 modification and its cytoplasmic aggregation, has been noticed following infection by members of the *Flaviviridae* including the flaviviruses DENV, Modoc virus, JEV, USUV (Khakpoor et al., 2009; Panyasrivanit et al., 2009; Heaton and Randall, 2010; Li et al., 2012; McLean et al., 2012; Blazquez et al., 2013; Jin et al., 2013), the hepacivirus HCV (Sir et al., 2008b; Dreux et al., 2009), and the pestivirus classical swine fever virus (CSFV; Pei et al., 2014). Interestingly, upregulation of the autophagic pathway in flavivirus-infected cells can occur without noticeable changes in the levels of the polyubiquitin-binding protein that interacts with LC3 p62/SQSTM1, whose degradation has been described following autophagy induction under certain conditions (Klionsky et al., 2008). This may indicate the unique features of the autophagic response during infections with at least some of these viruses (Beatman et al., 2012; Blazquez et al., 2013). The roles of the autophagic response in flavivirus-infected cells have been associated with varied functions including lipid metabolism reordering to support strong viral replication (Heaton and Randall, 2010), apoptosis inhibition (McLean et al., 2012), innate immunity evasion (Jin et al., 2013), or adequate platforms provision for viral replication during early steps of infection (Khakpoor et al., 2009; Panyasrivanit et al., 2009). Even more, high activation of autophagy has been associated with low neurovirulence of JEV strains (Li et al., 2012), suggesting a protective role of autophagy *in vivo* as already described for other viruses (Orvedahl and Levine, 2008). However, for other flaviviruses like WNV, the induction of an autophagic response in infected cells still remains controversial (Beatman et al., 2012; Vandergaast and Fredericksen, 2012). Nevertheless it seems clear that exogenous stimulation of autophagy via a pro-autophagic peptide can protect against neuronal cell death induced by WNV infection (Shoji-Kawata et al., 2013), thus supporting again a protective role of autophagy *in vivo*, at least against some members of the *Flavivirus* genus.

An induction or manipulation of the UPR has also been described for a wide variety of members of the *Flaviviridae* (**Figure 2**), although relationships between activation of the UPR, membrane remodeling, and autophagy induction have not been addressed in most cases or remain controversial. For instance, the induction of autophagy and UPR has been shown for HCV, but the mechanistic link between the induction of these two cellular processes remains unclear. Some authors have addressed the relationship between both mechanisms, reporting that down-regulation of a variety of UPR modulators inhibits HCV-induced LC3-phosphatidylethanolamine conjugation, a hallmark of autophagic vesicle accumulation (Sir et al., 2008a; Ke and Chen, 2011), or suggesting that HCV-induced eIF2α phosphorylation via PERK activates autophagy (Dreux and Chisari, 2011). Conversely, rapid autophagy induction after HCV infection with stimulation of the UPR at later stages of the infection has been described, implying that autophagy induction is independent of the UPR (Mohl et al., 2012). Supporting the independence of UPR and autophagy, expression of a subgenomic replicon of the pegivirus GB virus B, led

to an elevated LC3-II level, but did not induce UPR (Mohl et al., 2012). In the case of flaviviruses, a cause–effect relationship between UPR and autophagy is still lacking. There are contradictory evidences for and against a link between these two processes. For instance, it has been reported that WNV triggers UPR while not always upregulates the autophagic pathway (Vandergaast and Fredericksen, 2012), thus supporting that the induction of the UPR by WNV could be independent of an autophagic response. The only flavivirus protein associated with induction of autophagy has been the DENV NS4A (McLean et al., 2012). This protein is responsible for membrane rearrangements and, in WNV, it is also associated with the induction of the UPR. Although this could support a link between these cellular pathways in flavivirus infection, the involvement of WNV NS4A in autophagy induction has not yet been addressed. All these mixed observations show that there is still a need of new studies to direct evaluate the contribution of UPR to autophagy induction in flavivirus-infected cells.

CONCLUSION AND FUTURE PERSPECTIVES

The detailed knowledge of the interaction of flaviviruses with the ER is attractive to refine current antiviral strategies against these viruses and to explore novel therapeutic approaches. The view of the ER as a mere replication platform in flavivirus infection should be changed and more emphasis should be given to its profound remodeling of its architecture and composition induced by the infection, including the activation/rearrangement of cellular pathways related to this organelle which are connected with other relevant pathways as apoptosis and innate immunity. In this way, deciphering the puzzle between autophagy, the UPR, and their potential connections could help to build a more complete picture of flavivirus interactions with host cells. An important challenge will be the analysis of autophagy and UPR during flavivirus infection *in vivo* using animal models, of course, having in mind the complex biology of these pathogens that include infection of different host cells within their infectious cycle, which could complicate the interpretation of these studies. In fact, autophagy and UPR currently represent druggable pathways under evaluation for the treatment of multiple human disorders (Suh et al., 2012; Cao and Kaufman, 2013), and recent studies have revealed that pharmacological activation of autophagy can be protective *in vivo* against flavivirus infection (Shoji-Kawata et al., 2013).

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Coronavirus infection, ER stress, apoptosis and innate immunity

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The replication of coronavirus, a family of important animal and human pathogens, is closely associated with the cellular membrane compartments, especially the endoplasmic reticulum (ER). Coronavirus infection of cultured cells was previously shown to cause ER stress and induce the unfolded protein response (UPR), a process that aims to restore the ER homeostasis by global translation shutdown and increasing the ER folding capacity. However, under prolonged ER stress, UPR can also induce apoptotic cell death. Accumulating evidence from recent studies has shown that induction of ER stress and UPR may constitute a major aspect of coronavirus–host interaction. Activation of the three branches of UPR modulates a wide variety of signaling pathways, such as mitogen-activated protein (MAP) kinase activation, autophagy, apoptosis, and innate immune response. ER stress and UPR activation may therefore contribute significantly to the viral replication and pathogenesis during coronavirus infection. In this review, we summarize the current knowledge on coronavirus-induced ER stress and UPR activation, with emphasis on their cross-talking to apoptotic signaling.

Keywords: coronavirus, ER stress, apoptosis, signal transduction pathways, proinflammatory cytokines, unfolded protein response

INTRODUCTION

Coronaviruses are a family of enveloped viruses with positive sense, non-segmented, single-stranded RNA genomes. Many coronaviruses are important veterinary pathogens. For example, avian infectious bronchitis virus (IBV) reduces the performance of both meat-type and egg-laying chickens and causes severe economic loss to the poultry industry worldwide (Cavanagh, 2007). Certain coronaviruses, such as HCoV-229E and HCoV-OC43, infect humans and account for a significant percentage of adult common colds (Hamre and Procknow, 1966; Kaye et al., 1972). Moreover, in 2003, a highly pathogenic human coronavirus (SARS-CoV) was identified as the causative agent of severe acute respiratory syndrome (SARS) with high mortality rate and led to global panic (Ksiazek et al., 2003). Later, it was found that the SARS-CoV was originated from bat and likely jumped to humans via some intermediate host (palm civets; Li et al., 2005; Wang and Eaton, 2007). Recently, a live SARS-like coronavirus was isolated from fecal samples of Chinese horseshoe bats, which could use the SARS-CoV cellular receptor – human angiotensin converting enzyme II (ACE2) for cell entry (Ge et al., 2013). This indicates that an intermediate host may not be necessary and direct human infection by some bat coronaviruses is possible. Moreover, a novel human coronavirus – the Middle East respiratory syndrome coronavirus (MERS-CoV), emerged in Saudi Arabia in September 2012 (de Groot et al., 2013). Although the risk of sustained human-to-human transmission is considered low, infection of MERS-CoV causes ∼50% mortality in patients with comorbidities (Graham et al., 2013). Initial studies had pointed to bats as the source of MERS-CoV (Annan et al., 2013); however, accumulating evidence strongly suggested the dromedary camels to be the natural reservoirs and animal source of MERS-CoV (Hemida et al., 2013; Alagaili et al., 2014). Thus, coronaviruses can cross the species barrier to become lethal human pathogens, and studies on coronaviruses are both economically and medically important.

Taxonomically, the family *Coronaviridae* is classified into two subfamilies, the *coronavirinae* and the *torovirinae*. The *coronavirinae* is further classified into three genera, namely the Alphacoronavirus, Betacoronavirus, and Gammacoronavirus (Masters, 2006). The classification was originally based on antigenic relationships and later confirmed by sequence comparisons of entire viral genomes (Gorbalenya et al., 2004). Almost all Alphacoronaviruses and Betacoronaviruses have mammalian hosts, including humans. In contrast, Gammacoronaviruses have mainly been isolated from avian hosts.

Morphologically, coronaviruses are spherical or pleomorphic in shape with a mean diameter of 80–120 nm. They are characterized by the large (20 nm) "club-like" projections on the surface, which are the heavily glycosylated trimeric spike (S) proteins (Masters, 2006). Two additional structural proteins are found on the envelope. The abundant membrane (M) proteins give the virion its shape, whereas the small envelope (E) proteins play an essential role during assembly (Sturman et al., 1980; Liu and Inglis, 1991). Inside the envelope, the helical nucleocapsid is formed by binding of the nucleocapsid (N) proteins on the genomic RNA in a beads-on-a-string fashion. The genome, ranging from 27,000 to 32,000 nucleotides in size, is the largest RNA genomes known to date.

Coronavirus infection starts with receptor binding via the S protein (**Figure 1**). The S proteins of most coronaviruses are

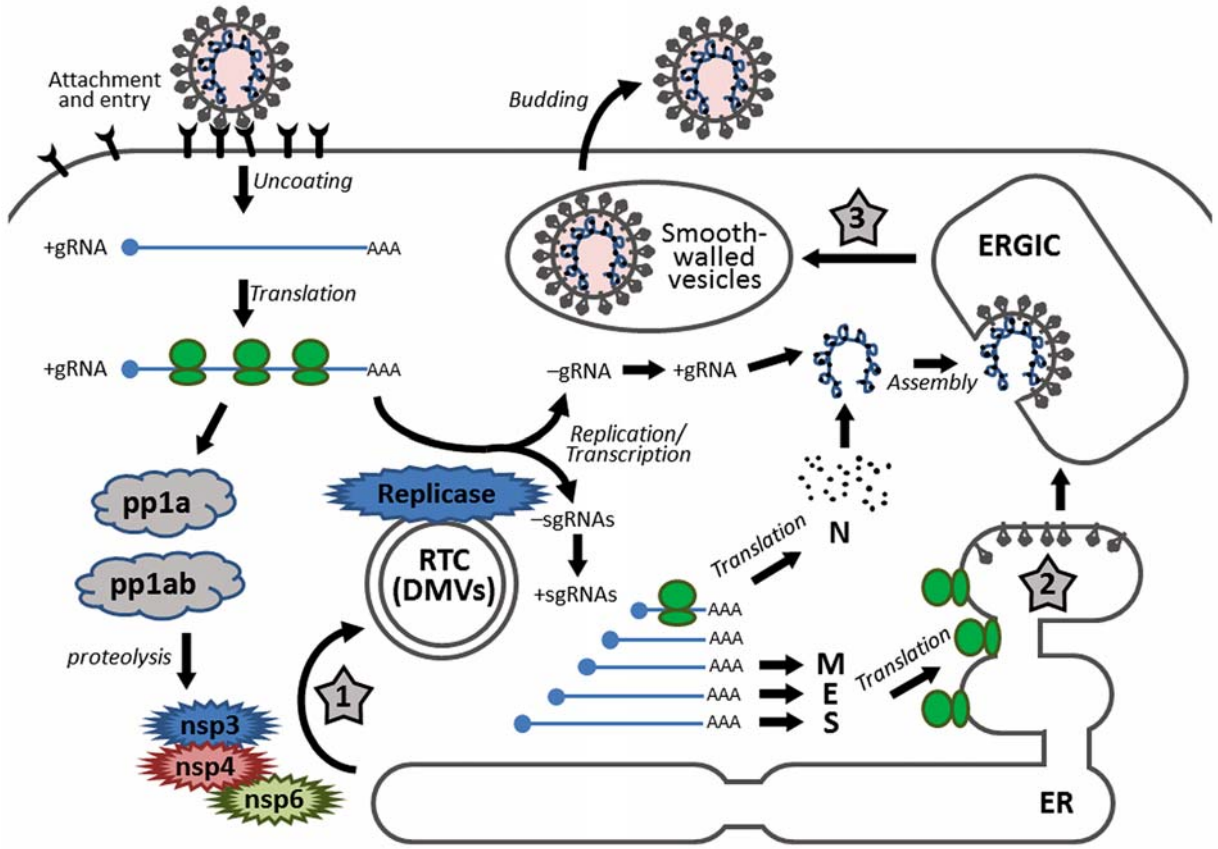

FIGURE 1 | Schematic diagram showing the replication cycle of coronavirus and the stages in which ER stress may be induced during coronavirus infection. Infection starts with receptor binding and entry by membrane fusion. After uncoating, the genomic RNA is used as a template to synthesize progeny genomes and a nested set of subgenomic RNAs. The replication transcription centers are closely associated with DMVs, which are proposed to be adopted from the modified ER, possibly by the combined activities of non-structural proteins nsp3, nsp4, and nsp6. The S, E, and M proteins are synthesized and anchored on the ER, whereas the N protein is translated in the cytosol. Assembly takes place in the ERGIC and mature virions are released via smooth-walled vesicles by exocytosis. The three stages that presumably induce ER stress are highlighted with numbered star signs, namely: (1) formation of DMVs, (2) massive production and modification of structural proteins, and (3) depletion of ER membrane during budding.

cleaved by host protease into two functional subunits: an N-terminal receptor binding domain (S1) and a C-terminal domain (S2) responsible for membrane fusion (Huang et al., 2006; Qiu et al., 2006; Yamada et al., 2009). The interaction between the cell surface receptor and the S1 subunit is the major determinant of the tropism of coronaviruses (Kuo et al., 2000). Upon receptor binding of S1, a conformational change is triggered in the S2 subunit, exposing its hidden fusion peptide for insertion into the cellular membrane. This is followed by the packing of the two heptad repeats in the three monomers into a six-helix bundle fusion core. This close juxtaposition of the viral and cellular membrane enables fusion of the lipid bilayers, and the viral nucleocapsid is thus delivered into the cytoplasm (Masters, 2006).

After uncoating, the genomic RNA first acts as an mRNA for translation of the replicase polyprotein. The replicase gene consists of two open reading frames (ORF1a and ORF1b). Translation of ORF1a produces the polyprotein 1a (pp1a). Meanwhile, a ribosomal frameshifting occurs at the junction of ORF1a and ORF1b, allowing translation to continue onto ORF1b, producing a larger polyprotein 1ab (pp1ab; Brierley et al., 1987). Autoproteolytic cleavage of pp1a produces 11 non-structural proteins (nsp1–nsp11), while cleavage of pp1ab produces 15 non-structural proteins (nsp1–nsp10 and nsp12–nsp16). The functions of these nsps are partially understood. Particularly, the autoproteolytic cleavage relies on nsp3 (a papain-like proteinase) and nsp5 (the main proteinase), whereas the RNA-dependent RNA polymerase (RdRp) is contained within nsp12 (Baker et al., 1993; Lu et al., 1995a).

Using the genomic RNA as a template, the replicase then synthesizes the negative sense genomic RNAs, which are used as templates for synthesizing progeny positive sense RNA genomes. On the other hand, through discontinuous transcription of the genome, the replicase synthesizes a nested set of subgenomic RNAs (sgRNAs; Sawicki et al., 2007). Replication and transcription of the coronavirus genome involve the formation of the replication/transcription complexes (RTCs), which are anchored to the intracellular membranes via the multi-spanning transmembrane

proteins nsp3, nsp4, and nsp6 (Oostra et al., 2007). Also, inside the infected cells, coronaviruses induce modification of the intracellular membrane network and formation of the double membrane vesicles (DMVs; Knoops et al., 2008). Several studies have shown that the DMVs are closely associated with the coronavirus RTCs and the *de novo* synthesized viral RNAs (Gosert et al., 2002; Snijder et al., 2006).

The sgRNAs are translated into structural proteins and accessory proteins. Transmembrane structural proteins (S, M, and E) are synthesized, inserted, and folded in the endoplasmic reticulum (ER) and transported to the ER–Golgi intermediate compartment (ERGIC). The N proteins are translated in the cytoplasm and encapsidate the nascent progeny genomic RNA to form the nucleocapsids. Virion assembly occurs in the ERGIC and is likely to be orchestrated by the M protein through protein–protein interactions (Masters, 2006).

The virions budded into the ERGIC are exported through secretory pathway in smooth-wall vesicles, which ultimately fuse with the plasma membrane and release the mature virus particles (Krijnse-Locker et al., 1994). For some coronaviruses, a portion of the S protein escapes from viral assembly and is secreted to the plasma membrane. These S proteins cause fusion of the infected cell with neighboring uninfected cells, resulting in the formation of a large, multinucleated cell known as a syncytium, which enables the virus to spread without being released into the extracellular space (Masters, 2006).

In eukaryotic cells, ER is the major site for synthesis and folding of secreted and transmembrane proteins. The amount of protein entering the ER can vary substantially under different physiological states and environmental conditions. When protein synthesis surpasses the folding capacity, unfolded proteins accumulate in the ER and lead to ER stress. ER stress can also be activated by excessive lipids or pro-inflammatory cytokines (Kharroubi et al., 2004; Pineau et al., 2009). To maintain homeostasis, cells have evolved signaling pathways that are collectively known as the unfolded protein response (UPR; Ron and Walter, 2007). The UPR signaling starts with the unfolded proteins activating the three ER stress transducers: PKR-like ER protein kinase (PERK), activating transcriptional factor-6 (ATF6), or inositol-requiring protein-1 (IRE1; **Figure 2**). Once activated, these sensors transmit the signal across the ER membrane to the cytosol and the nucleus, and the cell responds by lowering the protein synthesis and increasing the ER folding capacity. If homeostasis cannot be re-established, apoptosis is induced for the benefit of the entire organism (Tabas and Ron, 2011).

In this review, current studies on the involvement of the UPR in coronavirus infection and pathogenesis will be summarized. The role of UPR activation in host response, in particular the induction of apoptosis, will also be reviewed.

CORONAVIRUS INFECTION AND ER STRESS

Global proteomic and microarray analyses have shown that the expression of several genes related to the ER stress, such as glucose-regulated protein 94 (GRP94) and glucose-regulated protein 78 (GRP78, also known as immunoglobulin heavy chain-binding protein, or BiP), is up-regulated in cells infected with SARS-CoV or in cells overexpressing the SARS-CoV S2 subunit (Jiang et al., 2005; Yeung et al., 2008). Using a luciferase reporter system, Chan et al. (2006) found that both GRP94 and GRP78 were induced in SARS-CoV-infected FRhK4 cells. Consistently, the mRNA level of homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1 (HERPUD1), an ER stress marker, was up-regulated in L cells infected with mouse hepatitis virus (MHV) or SARS-CoV (Versteeg et al., 2007). Data from this group have shown a similar induction of ER stress in IBV-infected Vero, H1299, and Huh-7 cells (unpublished observations). Although no parallel studies have been performed on Alphacoronaviruses, it is likely that all three genera of coronaviruses may induce ER stress in the infected cells. Current evidence suggests the following three main mechanisms.

FORMATION OF DOUBLE MEMBRANE VESICLES

It is well-known that the replication of many plus-stranded RNA viruses induces modification of cellular membranes (Miller and Krijnse-Locker, 2008). Among them, coronaviruses have been shown to induce the formation of DMVs in infected cells (David-Ferreira and Manaker, 1965). Based on immunocytochemistry electron microscopy data, the DMVs co-localize with coronavirus major replicase proteins and are presumably the sites where coronavirus RTCs are located (Gosert et al., 2002; Snijder et al., 2006). Indeed, DMVs are induced in HEK293T cells co-expressing the SARS-CoV nsp3, nsp4, and nsp6, which are all multispanning transmembrane non-structural proteins (Angelini et al., 2013). There have been different perspectives regarding the origin of the coronavirus-induced DMVs. The late endosomes, autophagosomes, and the early secretary pathway have all been implicated as the membrane source of DMVs (van der Meer et al., 1999; Prentice et al., 2004; Verheije et al., 2008). Also, co-localization has been observed between SARS-CoV non-structural proteins and protein disulfide isomerase (PDI), an ER marker (Snijder et al., 2006). Using high-resolution electron tomography, Knoops et al. (2008) have shown that infection of SARS-CoV reorganizes the ER into a reticulovesicular network, which consists of convoluted membranes and interconnected DMVs. Recently, Reggiori et al. (2010) have proposed a model in which coronaviruses hijack the EDEMosomes to derive ER membrane for DMV formation. The EDEMosomes are COPII-independent vesicles that export from the ER, which are normally used to fine-tune the level of ER degradation enhancer, mannosidase alpha-like 1 (EDEM1), a regulator of ER-associated degradation (ERAD; Calì et al., 2008). It has been demonstrated that MHV infection causes accumulation of EDEM1 and osteosarcoma amplified 9 (OS-9, another EDEMosome cargo), and that both EDEM1 and OS-9 co-localize with the RTCs of MHV (Reggiori et al., 2010). These results thus add mechanical evidence to support the ER-origin of the coronavirus-induced DMVs.

GLYCOSYLATION OF CORONAVIRAL STRUCTURAL PROTEINS

Except for the N protein, all coronavirus structural proteins are transmembrane proteins synthesized in the ER. The M protein, which is the most abundant component of the virus particle, is known to undergo either O-linked (for most betacoronaviruses)

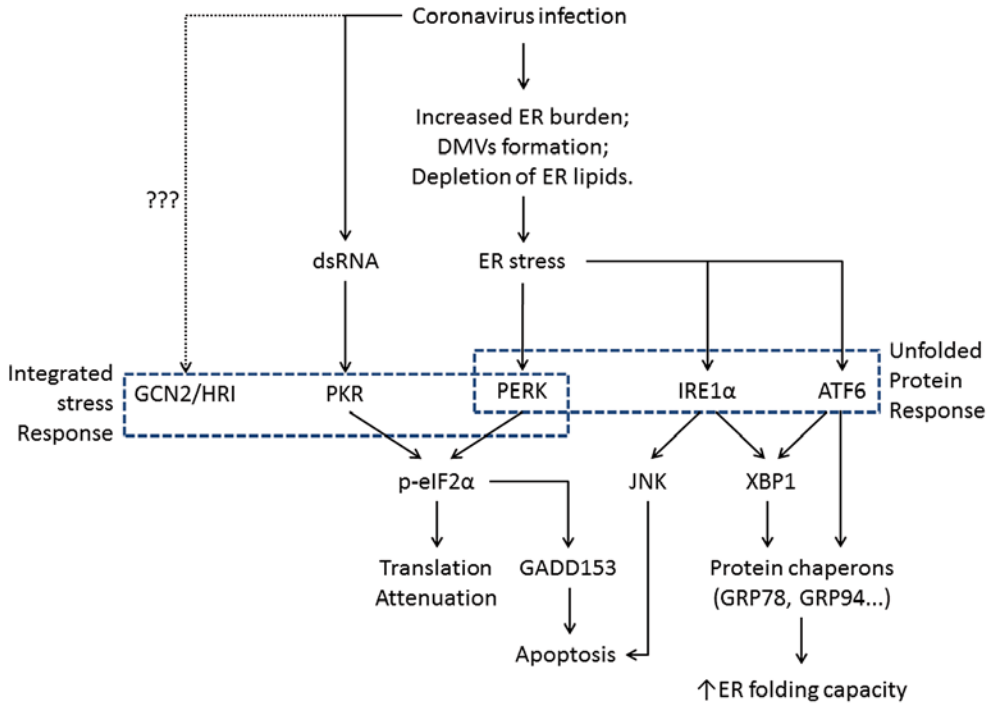

FIGURE 2 | Flowchart showing the induction of ER stress and its physiological outcomes during coronavirus infection. The integrated stress response pathways (including PERK) trigger translation shutdown and modulate apoptosis. The ATF6 pathway enhances the ER folding capacity, and the IRE1 pathway affects both ER folding and apoptosis induction. Pointed arrows indicate activation. The dotted line suggests uncharacterized function of GCN2 and HRI during coronavirus infection.

or N-linked (for all alpha- and gammacoronaviruses) glycosylation in the ER (Jacobs et al., 1986; Cavanagh and Davis, 1988; Nal et al., 2005). The glycosylation of M protein is proposed to play a certain function in alpha interferon (IFN) induction and *in vivo* tissue tropism (Charley and Laude, 1988; Laude et al., 1992; de Haan et al., 2003). The pre-glycosylated S monomers are around 128–160 kDa, whereas sizes can reach 150–200 kDa post-glycosylation (exclusively N-linked), indicating that the S protein is highly glycosylated (Masters, 2006). At least for transmissible gastroenteritis coronavirus (TGEV), glycosylation is presumed to facilitate monomer folding and trimerization (Delmas and Laude, 1990). Moreover, the glycans on SARS-CoV S proteins have been shown to bind C-type lectins DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) and L-SIGN (liver lymph node-specific intercellular adhesion molecule-3-grabbing non-integrin), which can serve as alternative receptors for SARS-CoV independent of the major receptor ACE2 (Han et al., 2007). The folding, maturation, and assembly of the gigantic S trimeric glycoprotein rely heavily on the protein chaperons inside the ER, such as calnexin. In fact, the N-terminal part of the S2 domain of SARS-CoV S protein has been found to interact with calnexin, and knock-down of calnexin decreases the infectivity of pseudotyped lentivirus carrying the SARS-CoV S protein (Fukushi et al., 2012). Also, treatment of α-glucosidase inhibitors, which inhibit the interactions of calnexin with its substrates, dose dependently inhibits the incorporation of S into pseudovirus and suppresses SARS-CoV replication in cell cultures (Fukushi et al., 2012). During coronavirus replication, massive amount of structural proteins is synthesized to assembly progeny virions. The production, folding, and modification of these proteins undoubtedly increase the workload of the ER.

DEPLETION OF ER LIPID DURING THE BUDDING OF VIRIONS

Budding of coronaviruses occurs in the ERGIC, which is a structural and functional continuance of the ER. Thus, the release of mature virions by exocytosis in effect depletes the lipid component of the ER. Taken together, coronavirus infection results in: (1) massive morphological rearrangement of the ER; (2) significant increase ER burden for protein synthesis, folding and modification; and (3) extensive depletion of ER lipid component. These factors together may contribute to the coronavirus-induced ER stress.

In the following sections, the activation of the three individual branches of the UPR by coronavirus infection will be discussed in detail.

THE PERK BRANCH OF UPR

PERK-EIF2α-ATF4 SIGNALING PATHWAY

The PERK branch of the UPR is believed to be activated first in response to ER stress (Szegezdi et al., 2006). Activation of PERK begins with the dissociation from ER chaperon BiP, followed by oligomerization and auto-phosphorylation. Activated PERK then phosphorylates the α-subunit of eukaryotic initiation factor 2

(eIF2α). Phosphorylated eIF2α forms a stable complex with and inhibits the turnover of eIF2B, a guanine nucleotide exchange factor that recycles inactive eIF2-GDP to active eIF2-GTP. This results in a general shutdown of cellular protein synthesis and reduces the protein flux into the ER (Ron and Walter, 2007). Besides PERK, three other kinases are known to phosphorylate eIF2α, namely the protein kinase RNA-activated (PKR), heme-regulated inhibitor kinase (HRI), and general control non-derepressible-2 (GCN2; Ron and Walter, 2007). PKR is induced by IFN and activated by the binding of double-stranded RNA (dsRNA) after virus infection (Clemens and Elia, 1997). HRI is activated in red blood cells and hepatocytes by low levels of heme (McEwen et al., 2005). GCN2 senses amino acid deficiency and is activated via binding to uncharged transfer RNAs (Sood et al., 2000). Due to common outcome (eIF2α phosphorylation and translation suppression), activation of these kinases is collectively known as the integrated stress response (ISR; Ron and Walter, 2007).

Interestingly, the mRNAs of certain genes contain small ORFs in their 5′ UTR and bypass the eIF2α-dependent translation block. One of these is the activating transcription factor 4 (ATF4), which is preferentially translated under ISR. ATF4 in turn transactivates genes involved in amino acid metabolism, redox reactions, and stress response. One of ATF4's target genes is the growth arrest and DNA damage-inducible protein 153 (GADD153, also known as C/EBP homologous protein, or CHOP). GADD153 induces the growth arrest and DNA damage-inducible protein 34 (GADD34), which recruits protein phosphatase 1 (PP1) to dephosphorylate eIF2α and release the translation block. To this end, if ER stress is resolved, normal protein synthesis can be resumed. However, if ER stress persists, GADD153 can induce apoptosis by suppressing the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and inducing the pro-apoptotic proteins such as Bcl-2-interacting mediator of cell death (Bim; Puthalakath et al., 2007). GADD153 also activates ER oxidoreductin-1α (ERO1α), which encodes an ER oxidase. The increase protein influx to a hyper-oxidizing ER aggravates ER stress and induces apoptosis (Marciniak et al., 2004; **Figure 3**).

INVOLVEMENT OF THE PERK PATHWAY DURING VIRAL INFECTIONS

Translation attenuation has been widely observed as a defensive mechanism of the host cells against viral infection. By reducing the translation of viral proteins, virus replication is hampered and the spread of infection is limited, giving enough time for the immune system to initiate effective antiviral responses. Among the four eIF2α kinases, PKR, due to its IFN-inducible nature and specific recognition of viral dsRNAs, plays an especially important role in inducing translation attenuation in virus-infected cells (He, 2006). It is therefore not surprising that viruses have evolved various mechanisms to counteract PKR. For example, the non-structural 5A (NS5A) protein of hepatitis C virus directly interacts with the catalytic site of PKR, whereas the NS1 protein in the influenza A virus binds to dsRNAs and thus blocks PKR activation (Lu et al., 1995b; Gale et al., 1997).

During virus infection, massive production of viral proteins can overload the folding capacities of ER and lead to activation of another eIF2α kinase – PERK. Activation of PERK has been observed in cells infected with various DNA and RNA viruses, such as vesicular stomatitis virus, bovine viral diarrhea virus and herpes simplex virus 1 (HSV1), to name just a few (Jordan et al., 2002; Baltzis et al., 2004; Cheng et al., 2005). However, similar to PKR, viruses have adopted counter measures to inhibit PERK-mediated translation attenuation. For example, the E2 protein of hepatitis C virus (HCV) and the glycoprotein gB of HSV1 bind to PERK and inhibit its kinase activity to rescue translation (Pavio et al., 2003; Mulvey et al., 2007).

ACTIVATION OF PERK PATHWAY DURING CORONAVIRUSES INFECTION AND ITS INVOLVEMENT IN CORONAVIRUS-INDUCED APOPTOSIS

There have been diverging results on the activation of PKR and/or PERK during coronavirus infection. In an early study, it has been found that there is minimal transcriptional activation of PKR and another IFN-stimulated gene, 2′5′-oligoadenylate synthetase (OAS) in cells infected with MHV-1 (Zorzitto et al., 2006). In a separate study, phosphorylation of PKR and eIF2α was also not observed in MHV A59-infected cells (Ye et al., 2007). However, Bechill et al. (2008) have detected significant eIF2α phosphorylation and up-regulation of ATF4 in cells infected with MHV A59, although no induction of GADD153 and GADD34 was observed. It has been suggested that due to the lack of GADD34-mediated eIF2α dephosphorylation, MHV infection induces sustained translation repression of most cellular proteins (Bechill et al., 2008). However, the translation of MHV mRNAs seems to be resistant to eIF2α phosphorylation, and the detailed mechanisms for such evasion are yet to be investigated. As for SARS-CoV, PKR, PERK, and eIF2α phosphorylation are readily detectable in virus-infected cells (Krähling et al., 2009). However, knock-down of PKR using specific morpholino oligomers did not affect SARS-CoV-induced eIF2α phosphorylation but significantly inhibited SARS-CoV-induced apoptosis (Krähling et al., 2009). It is possible that eIF2α is phosphorylated by PERK in SARS-CoV-infected cells, but similar loss-of-function experiments have not been performed, although overexpression of SARS-CoV accessory protein 3a has been shown to activate the PERK pathway (Minakshi et al., 2009).

The discrepancy regarding the activation of PKR/PERK during coronavirus infection may be a result from the different cell culture systems and virus strains used. The interpretation is further complicated by the IFN-inducible nature of PKR. It is generally believed that coronaviruses are poor type I IFN inducers *in vitro* (Garlinghouse et al., 1984; Spiegel et al., 2005; Roth-Cross et al., 2007), although the IFN response may be essential for antiviral activities *in vivo* (Ireland et al., 2008). Moreover, it is known that coronaviruses employ multiple mechanisms to antagonize the IFN response. For example, the nsp16 has been shown to utilize the 2′-*O*-methyltransferase activity to modify coronavirus mRNAs, so as to evade from the cytosolic RNA sensor melanoma differentiation-associated protein 5 (MDA5) and type I IFN induction (Roth-Cross et al., 2008; Züst et al., 2011). Furthermore, the activities of several IFN-induced genes (ISGs) have also been shown to be modulated by coronaviruses during infection. For instance, Zhao et al. (2012) have demonstrated that the MHV accessory protein ns2 cleaves 2′,5′-oligoadenylate, the product of

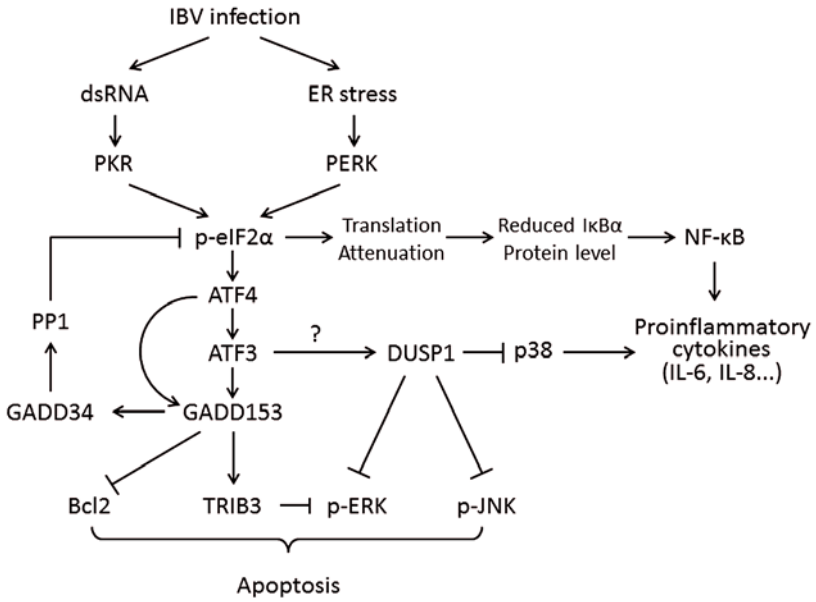

FIGURE 3 | Working model of PKR/PERK-eIF2α-ATF4-GADD153 pathway activation during coronavirus infection, using IBV as an example. Phosphorylation of eIF2α by PERK and PKR induces the expression of ATF4, ATF3, and GADD153. GADD153 exerts its pro-apoptotic activities via suppressing Bcl2 and ERKs by inducing TRIB3. The potential induction of DUSP1 by ATF3 may modulate phosphorylation of p38 and JNK, thus regulating IBV-induced apoptosis and cytokine production. The translation attenuation due to eIF2α activation can also lead to reduced inhibition of IκBα on NF-κB, which in turn promote cytokine production. Pointed arrows indicate activation, and blunt-ended lines indicate inhibition. The question mark indicates hypothetical mechanism.

an ISG called OAS. This results in the suppression of the cellular endoribonuclease RNase L activity and facilitates virus replication *in vitro* and *in vivo* (Zhao et al., 2011, 2012). Thus, similar uncharacterized mechanisms may be used by MHV and other coronaviruses to block the activation and/or downstream signaling of PKR. In this regard, the activation of PERK via ER stress seems to be an alternative pathway to activate eIF2α, although coronaviruses may counteract by directly targeting eIF2α, as described below.

Studies done by this group have shown that, phosphorylation of PKR, PERK, and eIF2α was detectable at early stage of IBV infection (0–8 hpi) but diminished quickly later (Wang et al., 2009; Liao et al., 2013). The rapid de-phosphorylation of eIF2α is likely due to the accumulation of GADD34, which is a component of the PP1 complex and a downstream target gene induced by GADD153 (Wang et al., 2009). Despite of the rapid de-phosphorylation of eIF2α, significant induction of GADD153 was observed at late stage of infection (16–24 h) at both mRNA and protein levels (Liao et al., 2013). The up-regulation of GADD153 was likely mediated by both PKR and PERK, since knock-down of either PKR or PERK by siRNA reduces IBV-induced GADD153 (Liao et al., 2013). The up-regulation of GADD153 promotes apoptosis in IBV-infected cells, possibly via inducing the pro-apoptotic protein tribbles-related protein 3 (TRIB3) and suppressing the pro-survival kinase extracellular signal-related kinase (ERK; Liao et al., 2013). Based on the findings so far obtained, it is safe to conclude that the PERK/PKR-eIF2α-ATF4-GADD153 pathway is activated by some, but not all, coronaviruses. In the infected cells, this pathway is activated at an early stage but quickly modulated by feedback de-phosphorylation. The PERK/PKR-eIF2α-ATF4-GADD153 most likely plays a pro-apoptotic function during coronavirus infection.

INTEGRATED STRESS RESPONSE PATHWAYS AND INNATE IMMUNITY

Several recent studies have demonstrated the critical roles of cellular stress response pathways in modulating the innate immune activation (Cláudio et al., 2013). One of the key regulators that bridge stress and innate immunity is GADD34, a negative regulator of eIF2α activation. It has been shown that when stimulated with polyriboinosinic:polyribocytidylic acid (polyI:C), the integrated stress response pathways were activated in dendritic cells (DCs), leading to up-regulation of ATF4 and GADD34 (Clavarino et al., 2012). Interestingly, GADD34 expression did not significantly affect protein synthesis in DCs, but was shown to be crucial for the production of interferon β (IFN-β) and pro-inflammatory cytokines interleukin-6 (IL-6; Clavarino et al., 2012). In contrast, GADD34 has also been shown to specify PP1 to dephosphorylate the TGF-β-activated kinase 1 (TAK1), thus negatively regulating the toll-like receptor (TLR) signaling and pro-inflammatory cytokines [IL-6 and TNF-α (Tumor necrosis factor alpha)] production in macrophages (Gu et al., 2014). The functional disparities of GADD34 in DCs and macrophages indicate that the integrated stress response may be regulated by some other signaling pathways, resulting in cell-type specific outcomes in the innate immune activation. Since GADD34 induction was readily observed in cells infected with IBV (Wang et al., 2009), it will be intriguing to ask whether GADD34 also contributes to IBV-induced pro-inflammatory

cytokine production, and to determine potential cross-talks between the PERK pathway and innate immune activation during IBV infection.

The massive production of pro-inflammatory cytokines (cytokine storm) has been associated with the immunopathogenesis and high mortality rate of SARS-CoV (Perlman and Dandekar, 2005). The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a master regulator of pro-inflammatory response and innate immunity (Hayden and Ghosh, 2012). It has been well established that NF-κB is required for the induction of pro-inflammatory cytokines (such as IL-6 and IL-8) and the early expression of IFN-β during RNA virus infection (Libermann and Baltimore, 1990; Kunsch and Rosen, 1993; Wang et al., 2010; Balachandran and Beg, 2011; Basagoudanavar et al., 2011). Interestingly, induction of TNF-α, IL-6, and IL-8 has been detected in cells overexpressing the spike protein of SARS-CoV via the NF-κB pathway (Wang et al., 2007; Dosch et al., 2009). Thus, it is intriguing to consider the involvement of ER stress in activating the NF-κB pathway during coronavirus infection. In its inactive form, NF-κB is sequestered in the cytoplasm by inhibitor of NF-κB alpha (IκBα), which masks the nuclear localization signal of NF-κB (Karin and Ben-Neriah, 2000). The basal level of IκBα is maintained by constitutive synthesis and degradation of the protein (Kanarek et al., 2010). Under various stress conditions, phosphorylation of eIF2α leads to global translation repression and a net decrease in IκBα protein level (Jiang et al., 2003). This then results in the activation of NF-κB and induction of pro-inflammatory response (**Figure 3**). Nonetheless, further studies are needed to characterize the actual contributions of ER stress in NF-κB-mediated cytokine induction during coronavirus infection.

Previous study done by this group has shown that infection of IBV induced the production of IL-6 and IL-8, which was dependent on the phosphorylation of MAP kinase p38 (Liao et al., 2011). Interestingly, a protein phosphatase called dual-specificity phosphatase 1 (DUSP1) was also up-regulated in IBV-infected cells and dephosphorylated p38 to modulate pro-inflammatory cytokine production (Liao et al., 2011). Previous studies have shown that the mRNA and protein levels of DUSP1 are modulated by ER stress (Boutros et al., 2008; Li et al., 2011). ER stress-induced DUSP1 up-regulation is likely to be mediated by ATF3 in the PERK pathway, since knock-down of ATF3 significantly reduced DUSP1 induction in cells under ER stress (Gora et al., 2010). Thus, it is possible that IBV infection activates the PERK branch of UPR to induce DUSP1 expression, which in turn dephosphorylates p38 to modulate IBV-induced pro-inflammatory cytokine production (**Figure 3**).

Besides p38, DUSP1 has also been shown to dephosphorylate c-Jun N-terminal kinase (JNK) and ERK (Sun et al., 1993; Franklin and Kraft, 1997). It has been long proposed that ERK phosphorylation promotes cell survival, whereas prolonged JNK and p38 phosphorylation is linked to the induction of apoptosis (Xia et al., 1995). Thus, the induction of DUSP1 by ER stress in coronavirus-infected cells may also contribute to virus-induced apoptosis via modulation of the MAP kinase pathways.

THE IRE1 BRANCH OF UPR

IRE1-XBP1 SIGNALING PATHWAY

The IRE1-XBP1 branch of the UPR is evolutionarily conserved from yeast to humans. In response to unfolded proteins, IRE1 undergoes oligomerization (Bertolotti et al., 2000). This results in trans-autophosphorylation of the kinase domain and the activation of IRE1's RNase domain. So far, the only known substrate for IRE1 RNase activity is the mRNA of the X box binding protein 1 (XBP1) gene (Yoshida et al., 2001a; Calfon et al., 2002). IRE1 cuts the XBP1 mRNA twice, removing a 26-nucleotide intron to form a frameshifted transcript, the spliced XBP1 (XBP1s). Whereas the unspliced XBP1 mRNA (XBP1u) encodes an inhibitor of the UPR, XBP1s encode a potent transcriptional activator, which translocates to the nucleus and enhances the expression of many UPR genes, including those encoding molecular chaperones and proteins contributing to ER-associated degradation (Ng et al., 2000; Lee et al., 2003; **Figure 4**).

Apart from the XBP1 pathway, activated IRE1 has been shown to recruit TNF receptor-associated factor 2 (TRAF2) and induce apoptosis by activating the JNK (Urano et al., 2000). This IRE1-JNK pathway is independent of IRE1's RNase activity, but it requires IRE1's kinase domain and involves TRAF2-dependent activation of caspase-12 (Yoneda et al., 2001). Moreover, one recent study has demonstrated that the IRE1-JNK pathway is required for autophagy activation after pharmacological induction of ER stress. It was found that the kinase domain but not the RNase activity of IRE1 was required, and treatment of a JNK inhibitor (SP600125) abolished autophagosome formation after ER stress (Ogata et al., 2006). Therefore, the IRE1 branch of UPR is closely associated with the JNK pathway and involved in JNK-mediated apoptosis and autophagy signaling.

ACTIVATION OF THE IRE1 PATHWAY DURING CORONAVIRUSES INFECTION

The involvement of IRE1-XBP1 pathway during coronavirus infection has been investigated by several studies, using MHV as a model. Either MHV infection or overexpression of the MHV S protein (but not other structural proteins) induces XBP1 mRNA splicing (Versteeg et al., 2007; Bechill et al., 2008). However, although XBP1 mRNA is efficiently spliced, the protein product of spliced XBP1 cannot be detected in either the whole cell lysate or the nuclear fraction. Moreover, UPR downstream genes known to be activated by XBP1s, such as ER DNA J domain-containing protein 4 (ERdj4), EDEM1, and protein kinase inhibitor of 58 kDa ($p58^{IPK}$), are not significantly induced after infection (Bechill et al., 2008). Using a luciferase reporter system, it is shown that MHV infection does not inhibit transactivation of unfolded protein response element (UPRE) and ER stress response element (ERSE) promoter by XBP1s. Because MHV infection is associated with persistent eIF2α phosphorylation and host translational repression, it is likely that failure to translate the XBP1s protein may be the main reason why activation of the IRE1 branch does not occur even though XBP1 mRNA splicing is observed. On the other hand, although SARS-CoV belongs to the same genera of Betacoronavirus as MHV, neither infection with SARS-CoV nor overexpression of SARS-CoV S protein induces XBP1 mRNA splicing (Versteeg et al., 2007; DeDiego et al., 2011). It is

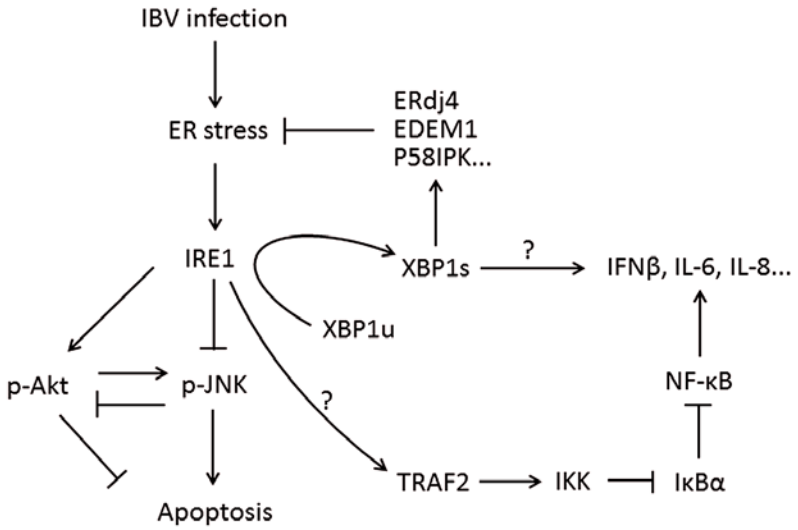

FIGURE 4 | Working model of IRE1-XBP1 signaling pathway during coronavirus infection, using IBV as an example. IRE1 mediates XBP1 splicing, which up-regulates UPR target genes to restore ER stress, and the spliced XBP1 may also modulate the IFN and cytokine secretion. IRE1 activation modulates the phosphorylation of Akt and JNK, thus affecting IBV-induced apoptosis. IRE1 is also responsible for basal activity of IKK, which phosphorylates IκBα to remove its inhibition on NF-κB, thus facilitating the production of type I IFN and pro-inflammatory cytokines. Pointed arrows indicate activation, and blunt-ended lines indicate inhibition. The question mark indicates hypothetical mechanism.

possible that other viral proteins of SARS-CoV (such as the E protein mentioned below), function as an antagonist of IRE1-XBP1 activation.

Result from this group has also shown that the IRE1-XBP1 pathway is activated in cells infected with IBV. In IBV-infected Vero cells, significant splicing of XBP1 mRNA was detected starting from 12 to 16 h post-infection till the late stage of infection. The mRNA levels of XBP1 effector genes (EDEM1, ERdj4, and $p58^{IPK}$) were up-regulated in IBV-infected Vero cells. The activation of IRE1-XBP1 pathway was also detectable, though at a lower level, in other cell lines such as H1299 and Huh-7 cells. Treatment of IRE1 inhibitor effectively blocked IBV-induced XBP1 mRNA splicing and effector genes up-regulation in a dosage-dependent manner. Consistently, knockdown of IRE1 inhibited IBV-induced XBP1 mRNA splicing, whereas overexpression of wild-type IRE1 (but not its kinase dead or RNase domain deleted mutants) enhanced IBV-induced XBP1 mRNA splicing. These results suggest that the IRE1-XBP1 pathway is indeed activated in cells infected with IBV. Interestingly, an earlier onset and more significant apoptosis induction in IRE1-knockdown IBV-infected cells was observed, which is associated with hyper-phosphorylation of pro-apoptotic kinase JNK and hypo-phosphorylation of pro-survival kinase RAC-alpha serine/threonine-protein kinase (Akt). Taken together, IRE1 may modulate IBV-induced apoptosis and serve as a survival factor during coronavirus infection.

Interestingly, a recent report by DeDiego et al. (2011) demonstrates that the coronavirus E protein may modulate the IRE1-XBP1 pathway. Using a recombinant SARS-CoV that lacks the E protein (rSARS-CoV-ΔE), it is found that both XBP1 splicing and induction of UPR genes significantly increase in the absence of E protein. Moreover, E protein also suppresses ER stress induced by RSV and drugs (thapsigargin and tunicamycin; DeDiego et al., 2011). Whether the UPR modulating activity is related to the viroporin property of E protein remains to be investigated, but this study explains, at least in part, why SARS-CoV lacking the E protein is attenuated in animal models (Liao et al., 2004; DeDiego et al., 2007).

IRE1-DEPENDENT DECAY DURING VIRUS INFECTION

Notably, one recent study has demonstrated an alternative function of IRE1. It was found that at the late stage of ER stress, IRE1 mediates non-specific cleavage of membrane-associated mRNA species. This was dubbed IRE1-dependent decay (RIDD) and was proposed to resolve ER stress by reducing the amount of transcripts influx (Hollien et al., 2009). It is intriguing to think of RIDD as a host anti-viral mechanism. During prolonged ER stress induced by infection, non-specific RNase activity of IRE1 may decay the membrane associated viral mRNA. In fact, it has been recently suggested that RIDD is activated during Japanese encephalitis virus (JEV) infection in Neuro2a cells (Bhattacharyya et al., 2014). Interestingly, RIDD specifically degraded known target mRNA transcripts but not JEV RNAs. Also, treatment with IRE1 RNase activity inhibitor suppressed viral replication, indicating that JEV benefits from RIDD activation (Bhattacharyya et al., 2014).

IRE1 PATHWAY AND INNATE IMMUNITY

Similarly to the integrated stress response, the IRE1 pathway has also been implicated in the innate immune response (Cláudio et al., 2013). Martinon et al. (2010) have shown that in murine macrophages, the IRE1-XBP1 pathway is specifically activated by TLR4 and TLR2. Interestingly, the ER stress and TLR activation synergistically activate IRE1 and induce the production of pro-inflammatory cytokines such as IL-1β and IL-6

(Martinon et al., 2010). Consistently, Hu et al. (2011) have demonstrated that the IRE1-XBP1 pathway is also involved in IFN-β and pro-inflammatory cytokines production in murine DCs induced by polyI:C. Significantly, it has been shown that overexpression of the spliced form of XBP1 enhanced IFN-β production in DCs and significantly suppressed vesicular stomatitis virus infection (Hu et al., 2011). Preliminary results from this group have also found that the activation of IRE1-XBP1 pathway is required for IL-8 induction in cells infected with IBV (unpublished data). On the other hand, the kinase but not the RNAse activity of IRE1 has been associated with ER-stress-induced NF-kB activation (Tam et al., 2012). Under ER stress, IRE1 has been shown to phosphorylate TRAF2, which activates the IκB kinase (IKK) and contributes to its basal activity (**Figure 4**). IKK in turn phosphorylates IκBα and promotes its proteasomal degradation, releasing NF-κB to activate downstream genes (Tam et al., 2012). Taken together, these findings suggest that IRE1 may act synergistically with players in innate immunity and serve as a supplementary sensor and/or signaling factors during coronavirus infection.

THE ATF6 BRANCH OF UPR

The ER stress sensor ATF6 has an N-terminal cytoplasmic domain, a single transmembrane segment and an ER luminal domain that sense the presence of unfolded/misfolded proteins. Under ER stress, ATF6 is translocated from the ER to the Golgi apparatus and cleaved by protease S1P and S2P (Haze et al., 1999). The cleavage releases the cytosolic basic leucine zipper (bZIP) domain, which translocates into the nucleus and activates genes harboring the ERSE or ERSE II (Yoshida et al., 2001b). The identified target genes of ATF6 include ER chaperones (such as GRP78, GRP94), PDI, and the UPR transcription factors GADD153 and XBP1 (Szegezdi et al., 2006). Previously, it was proposed the ATF6 pathway is mainly pro-survival, as it enhances the ER protein folding capacity to counteract ER stress (Szegezdi et al., 2006). However, recent studies have demonstrated that, under certain circumstances, ATF6-mediated signals may also contribute to ER-stress-induced apoptosis, possibly via activation of CHOP and/or suppression of myeloid cell leukemia sequence 1 (Mcl-1; Gotoh et al., 2002; Nakanishi et al., 2005; Morishima et al., 2011).

The infection of cells by several viruses has been shown to activate the ATF6 pathway, including the Tick-borne encephalitic virus, African swine fever virus (ASFV), West Nile virus (WNV), and HCV (Ambrose and Mackenzie, 2011; Merquiol et al., 2011; Galindo et al., 2012; Yu et al., 2013). In the case of ASFV, ATF6 activation has been shown to modulate ASFV-induced apoptosis and facilitate viral replication (Galindo et al., 2012). For WNV, it has been shown that ATF6 activation promotes efficient WNV replication by suppressing signal transducer and activator of transcription 1 (STAT1) phosphorylation and late-phase IFN signaling (Ambrose and Mackenzie, 2013). The NS4B protein of HCV has been shown to activate ATF6 signaling in cultured cells (Li et al., 2009). Induction of chronic ER stress and adaptation of infected hepatocyte to UPR have been considered important for HCV persistent infection and pathogenesis *in vivo* (Asselah et al., 2010; Merquiol et al., 2011).

Compared with the PERK and IRE1 pathway, the induction of ATF6 pathway during coronaviruses infection has not been deeply investigated. In MHV-infected cells, significant cleavage of ATF6 could be detected starting from 7 h post-infection (Bechill et al., 2008). However, the levels of both full length and cleaved ATF6 protein diminished at later time points during infection. Moreover, activation of ATF6 target genes was not observed at the mRNA level, as determined by luciferase reporter constructs under the control of ERSE promoters (Bechill et al., 2008). It is also unlikely that MHV infection suppresses downstream signaling of the ATF6 pathway, because the reporter induction by overexpressed ATF6 was not inhibited by MHV infection. The authors thus conclude that global translation shutdown via eIF2α phosphorylation prevents accumulation of ATF6 and activation of ATF6 target genes (Bechill et al., 2008). The involvement of ATF6 pathway during infection of other coronaviruses has not been well characterized.

Although the spike proteins of coronaviruses have been considered as the major contributor in ER stress induction, overexpression of SARS-CoV spike protein fails to activate ATF6 reporter constructs (Chan et al., 2006). On the other hand, the accessory protein 8ab of SARS-CoV has been identified to induce ATF6 activation (Sung et al., 2009). The 8ab protein was found in SARS-CoV isolated from animals and early human isolates. In SARS-CoV isolated from humans during the peak of the epidemic, there is a 29-nt deletion in the middle of ORF8, resulting in the splitting of ORF8 into two smaller ORFs, namely ORF8a and ORF8b, which encode two truncated polypeptides 8a and 8b (Guan et al., 2003). ATF6 cleavage and nuclear translocation was observed in cells transfected with SARS-CoV 8ab (Sung et al., 2009). Physical interaction between 8ab and the luminal domain of ATF6 was also demonstrated by co-immunoprecipitation. However, similar experiments have not been performed for the 8a and 8b proteins. Also, further studies using recombinant SARS-CoV lacking 8a, 8b, or 8ab would be required.

CONCLUSION

Coronaviruses constitute human and animal pathogens that are medically and economically important. Much remains unknown regarding the host–virus interactions during infection. Recent studies have demonstrated that coronaviruse infection induces ER stress in infected cells and activates the UPR. Activation of the PERK pathway (possibly in synergy with PKR and/or other integrated stress response kinases) leads to phosphorylation of eIF2α and a global translation shutdown. At late stage of infection, up-regulation of transcription factor GADD153 likely contributes to coronaviruses induced apoptosis. Activation of the IRE1 pathway induces XBP1 mRNA splicing and expression of downstream UPR genes. Interestingly, IRE1 but not XBP1 is also shown to modulate the JNK and Akt kinase activities, thus protecting infected cells from virus induced apoptosis. The ATF6 pathway is also activated in coronavirus-infected cells, resulting in the up-regulation of chaperon proteins to counteract ER stress.

However, many questions remain to be addressed. First, although the coronaviruses spike proteins are demonstrated to induce ER stress and UPR, detailed mechanisms regarding

molecular interactions between the spike proteins and PERK/IRE1/ATF6 have not been determined. Second, it should be noted that the phenotypes observed in cells overexpressing viral proteins may not essentially reflect their physiological functions in the setting of a real infection. Further experiments using recombinant viruses with deletion of or modification in the target viral proteins should be performed to validate these findings (DeDiego et al., 2011). Last but not the least, the three branches of UPR should not be considered functionally independent, but rather as an integrated regulatory network (Ron and Walter, 2007). For example, besides being spliced by IRE1, XBP1 is also transcriptionally activated by PERK and ATF6 (Yoshida et al., 2001a; Calfon et al., 2002). Also, it is difficult to separate the translation shutdown effect mediated by PERK and the induction of UPR genes by PERK and the other two ER stress sensors, as in the studies with MHV (Bechill et al., 2008).

Nonetheless, there are scientific and clinical significance for studies on ER stress and UPR induction during infection with coronaviruses and other viruses. As an evolutionarily conserved and well-characterized stress response pathway, it serves as a perfect model to study host–virus interactions and pathogenesis. Moreover, besides apoptosis, UPR has been recently demonstrated to crosstalk with other major cellular signaling pathways, including MAP kinases pathways, autophagy, and innate immune responses (Yoneda et al., 2001; Ogata et al., 2006; Martinon et al., 2010; Hu et al., 2011; Clavarino et al., 2012). Thus, further investigations on coronavirus-induced UPR may also help identifying new targets for antiviral agents and developing more effective vaccines against coronaviruses.

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ER stress, autophagy, and RNA viruses

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Endoplasmic reticulum (ER) stress is a general term for representing the pathway by which various stimuli affect ER functions. ER stress induces the evolutionarily conserved signaling pathways, called the unfolded protein response (UPR), which compromises the stimulus and then determines whether the cell survives or dies. In recent years, ongoing research has suggested that these pathways may be linked to the autophagic response, which plays a key role in the cell's response to various stressors. Autophagy performs a self-digestion function, and its activation protects cells against certain pathogens. However, the link between the UPR and autophagy may be more complicated. These two systems may act dependently, or the induction of one system may interfere with the other. Experimental studies have found that different viruses modulate these mechanisms to allow them to escape the host immune response or, worse, to exploit the host's defense to their advantage; thus, this topic is a critical area in antiviral research. In this review, we summarize the current knowledge about how RNA viruses, including influenza virus, poliovirus, coxsackievirus, enterovirus 71, Japanese encephalitis virus, hepatitis C virus, and dengue virus, regulate these processes. We also discuss recent discoveries and how these will produce novel strategies for antiviral treatment.

Keywords: ATF6, eIF2α, enterovirus 71, ER stress, IRE1, unfolded protein response

INTRODUCTION

The endoplasmic reticulum (ER) is a eukaryotic organelle in which an array of cell functions takes place. These include the transportation of cellular materials, provision of increased surface area for cellular reactions, and the production of proteins, steroids, and lipids. The ER may be overloaded with molecular chaperones, folding enzymes, and massive protein products during normal processes, such as in the differentiation of B lymphocytes into antibody-secreting plasma cells (Shaffer et al., 2004; Ma et al., 2010) or in highly specialized cells for secretion (Harding and Ron, 2002). In addition, dysfunction of the ER, known as ER stress, results from pathogenic stress signals, such as hypoxia (Koumenis, 2006), ER–Ca^{2+} depletion, viral infections, or agents that affect Ca^{2+} balance (i.e., thapsigargin), protein glycosylation (i.e., tunicamycin), and ER–Golgi vesicular transport (i.e., brefeldin A), which lead to accumulation of misfolded and unfolded proteins (Kaufman, 1999). To reduce the adverse effects of accumulating misfolded or unfolded proteins, the cell operates an adaptive response known as the unfolded protein response (UPR) to reduce the load of newly synthesized proteins within the ER and eliminate inappropriately folded proteins through upregulation of ER chaperone expression. In addition, proteins that fail to correctly fold are then deployed to the distal secretory pathway from the ER by the ER-associated protein degradation (ERAD) pathway of the UPR (Hampton, 2000; Yoshida et al., 2003).

There are two ERAD models for protein degradation: ubiquitin-proteasome ERAD, designated as ERAD (I), and autophagy-lysosome ERAD, designated as ERAD (II) (Fujita et al., 2007; Korolchuk et al., 2010). Both models depend on retrotranslocation of ERAD substrates from the ER back to the cytoplasm with the help of the Cdc48p–p97 complex. Most soluble misfolded proteins are cleared through the ubiquitin-proteasome system, which involves action of a cascade of three canonical ubiquitin enzymes: E1 ubiquitin-activating enzyme initiates the reaction by using ATP to covalently activate and then conjugate the ubiquitin to an E2 ubiquitin-conjugating enzyme. Ubiquitin is then transferred from the ubiquitin-charged E2 to the lysine residue of a specific target or a growing ubiquitin chain by E3 ubiquitin ligase, which results in a multiubiquitin chain-tagged substrate. Proteins that are ubiquitinated with K48-linked chains are specifically recognized by the 26S proteasome and subjected to degradation (Hershko et al., 1983). In contrast, ERAD (II) degrades both soluble and insoluble misfolded protein aggregates in autolysosome. Autophagy receptors and adaptors, called p62/SQSTM1, NBR1, HDAC6, and ALFY, bind to proteins with K63-specific monoubiquitination or polyubiquitin chains and then guide them to the concave side of developing autophagosomes (Behrends and Fulda, 2012). Notably, p62 also recognizes K48 polyubiquitin-tagged proteins for autophagic clearance upon proteasome dysfunction. In addition to the protective role of UPR, prolonged and/or excess ER stress typically activates caspase-12, an ER-resident caspase, leading to UPR-mediated cell death (Szegezdi et al., 2006).

Basal autophagy plays a key role in maintaining cellular homeostasis through eliminating unwanted proteins and damaged organelles by cellular self-digestion in the lysosome to fulfill the

demand for the building blocks required for cell survival (Levine and Klionsky, 2004; Shintani and Klionsky, 2004). Recently, the study of autophagy regulation has grown in different research areas, including regulation of cancer development and progression (Mahoney et al., 2013a), lipid metabolism (Singh et al., 2009), degenerative diseases (Wang et al., 2006), and the control of viral pathogenesis (Jackson et al., 2005). The first step of autophagy relies on the formation of an isolation membrane at the so-called preautophagosomal site (PAS) where a system of evolutionarily conserved proteins (Atg proteins) comes together. Recent reports have revealed that the ER serves as a subcellular platform for autophagy initiation (Axe et al., 2008). The elongation of the initial autophagic membrane requires continued processing by two ubiquitin-like protein-conjugation systems, the Atg12 and LC3 systems, which modify the autophagy proteins, Atg5 and Atg8/LC3, respectively (Geng and Klionsky, 2008). The autophagosome then fuses with endosomal and/or lysosomal vesicles to create an autolysosome, where digestion of intracellular components occurs (Eskelinen, 2005). In addition, autophagy can be induced by various physiological and pathological conditions such as nutrient deprivation, oxidative stress, and pathogen infections. The live-or-dead signal is modulated by UPR and autophagy and several lines of evidence suggest there is communication between these two pathways (Bernales et al., 2006; Ogata et al., 2006; Yorimitsu et al., 2006; Salazar et al., 2009); thus, it is believed that these two pathways could be a therapeutic target in certain circumstances (**Figure 1**). Herein, we review recent findings, focusing on the regulation of the UPR and autophagy involved in RNA virus infection as a new antiviral strategy.

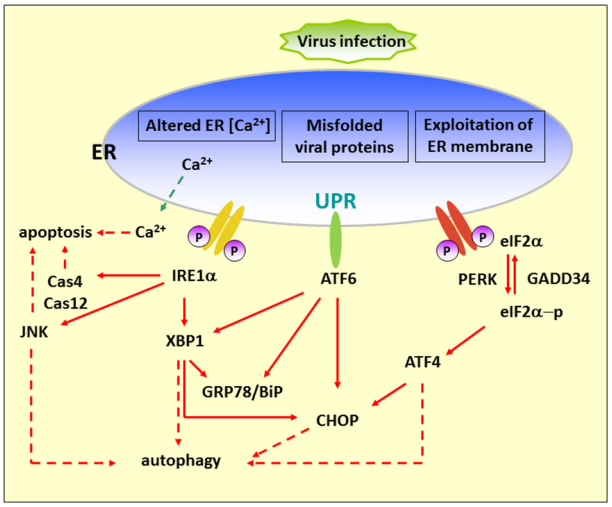

FIGURE 1 | Diagram of the UPR arms and their connection to autophagy. Alteration of ER functions results from stress signals by RNA virus infection, by the exploitation of ER membrane for viral replication, rapid accumulation of viral proteins, imbalance of calcium concentration by viroporin, and the sabotage or depletion of ER membrane for viral release. This leads to the accumulation of misfolded and unfolded proteins, which triggers ER stress. To alleviate this adverse effect, the cells operate an adaptive UPR to reduce the load of the newly synthesized proteins in the ER by activating the PERK–eIF2α branch and eliminating inappropriate protein accumulation by upregulating ER chaperone proteins through IRE1 and ATF6 branches. In addition, the incurable misfolded proteins undergo retrotranslocation from the ER into cytosol for degradation by an ERAD mechanism. ER stress can contribute to autophagy via activation of JNK, XBP1, CHOP, and ATF4. Red dash arrows indicate the final outcome of the activated pathways, such as apoptosis and autophagy, caused by viral infection. Red solid arrows indicate the UPR pathways.

HOW RNA VIRUS INFECTION CAUSES ER STRESS

Viral virulence is determined by successful entrance, replication in the host cell, and release of mature virion. During the life cycle, ER stress may arise from the exploitation of the ER membrane, accumulation of misfolded proteins, imbalance of calcium concentration by viroporin, and the sabotage or depletion of the ER membrane during virion release. Details of viral effects are given as follows.

EXPLOITATION OF ER MEMBRANES

Many positive-strand RNA viruses cause the rearrangement of host intracellular membrane compartments that house replication complexes. ER, *trans*-Golgi, or lysosomes are the likely origin of virally induced membranes (Miller and Krijnse-Locker, 2008; Korolchuk et al., 2010). Upon poliovirus (PV) and coxsackievirus B3 (CVB3) infection, clusters of vesicles have been considered to derive from ER, although other cellular compartment marker proteins also colocalized with viral nonstructural proteins (Schlegel et al., 1996; Van Kuppeveld et al., 1997). Consistent with these findings, our previous study indicates that enterovirus 71 (EV71) nonstructural 2C protein, which participates in viral replication, is associated with the ER membrane through direct interaction with ER membrane protein reticulon 3 (RTN3), which is required and sufficient for immediate early virus replication and translation (Tang et al., 2007). In the RTN3 siRNA knockdown cells, synthesis of the 2C protein was ablated. However, in the RTN3 rescue cell line 2A3, the synthesis of viral protein and RNA was restored. Moreover, the interactions between RTN3 and two EV71 2C homologs of PV and CVA16 have been confirmed (Tang et al., 2007). Immunofluorescence studies reveal that replication of *Flaviviruses* dengue virus (DENV) and hepatitis C virus (HCV) may take place on perinuclear ER membranes (El-Hage and Luo, 2003). DENV2 nonstructural protein 2 (NS2A) is a 22-kDa hydrophobic protein containing five integral transmembrane segments that span the ER membrane. Functional analysis reveals that NS2A involves both DENV RNA synthesis and virion assembly/maturation (Xie et al., 2013). Furthermore, DENV infection induces ROCK-dependent vimentin rearrangement and subsequent ER redistribution (Lei et al., 2013). In addition, the HCV ER integral membrane protein, NS4B, is responsible for rearranging the ER membrane and inducing the formation of new ER-derived membrane structures, and this is possibly negatively regulated by RTN3-NS4B interaction (Lundin et al., 2003; Wu et al., 2014).

INTERFERENCE WITH HOST PROTEIN GLYCOSYLATION BY VIRUSES

The N-glycosylation pathway in the ER modifies a mass of proteins at the asparagine residue of the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except Pro (Kornfeld and Kornfeld, 1985; Gavel and Von Heijne, 1990). The modification influences protein folding and attributes various functional

properties to the protein. Thus, interference with host protein glycosylation by viral proteins competing for the modification process may cause ER stress.

Viruses, including influenza A virus (IAV), hepatitis virus, and Japanese encephalitis virus (JEV), use this host cell process to enhance viral pathogenesis through facilitating folding and trafficking, affecting receptor interaction, and modulating host immune responses (Tatu et al., 1995; Dubuisson and Rice, 1996; Zai et al., 2013). Hemagglutinin (HA) of IAV is a type I transmembrane glycoprotein that determines viral antigenicity. Throughout the glycosylation process, HA rapidly associates with calnexin in a monoglucosylated form. Once folded, the HA monomers dissociate from calnexin and assemble into trimeric structures in the ER or in the intermediate compartment (Tatu et al., 1995). HCV envelope glycoproteins E1 and E2 have been shown to cooperate for the formation of a functional noncovalent heterodimer (Dubuisson et al., 1994; Dubuisson and Rice, 1996). Based on studies of HCV pseudoparticles, coexpression of both envelope glycoproteins has been shown to be necessary to produce infectious pseudoparticles (Bartosch et al., 2003). Glycosylation also occurs in JEV and WNV proteins, namely the precursor of membrane protein (prM), the envelope protein (E), and the nonstructural protein NS1, which affects the efficiency of virus release and infection (Hanna et al., 2005; Zai et al., 2013).

VIROPORINS

Typically, viroporins are composed by integral membrane proteins to form a hydrophilic pore, which targets different cellular compartments and ions, thus affecting various viral functions (Nieva et al., 2012). For example, IAV M2 reduces the acidity of vesicular compartments to trigger virus uncoating. It is also required for viral assembly and release. In the case of ER-targeting viroporins, rotavirus-encoded NSP4 modifies the calcium homeostasis by enhancing the calcium permeability of the ER membrane. This may be associated with virus-induced cell death and subsequent release of NSP4, which in turn causes activation of the phospholipase C-IP3 cascade in neighboring noninfected cells and is responsible for viral pathogenesis (Tian et al., 1995, 1996; Dong et al., 1997). On the other hand, 2B proteins of picornaviruses also participate in the remodeling of membrane structures and the formation of replication complexes (De Jong et al., 2008). Among them, CBV3 2B, PV1, and rhinovirus 2B are present at the membranes of the ER and Golgi complex and are responsible for the release of Ca^{2+} and H^{+} from these organelles.

VIRION BUDDING

Rotavirus studies propose that the double-layered particle (DLP)–VP4–NSP4 complex breaches the ER membrane and penetrates into the ER. The viral capsid protein, VP7, re-envelopes the immature particle (DLP) after removal of the ER membrane and NSP4, and forms the infectious triple-layered particle (Tian et al., 1996; Trask et al., 2012).

REGULATION OF UPR BY VIRUSES

The induction of individual branches or part of the UPR by viruses was reported previously. Viruses have also evolved different means to modulate the arms of the UPR, which consequently expanded both the temporal and spatial superiority for virus replication or completion of the life cycle.

eIF2α PATHWAY

It has been reported that viruses regulate the host translational machinery to promote viral protein synthesis by inhibiting the synthesis of proteins involved in host immune responses. In enteroviruses, 2A and 3C proteases target translation factors such as eIF4GI and poly(A)-binding protein (PABP) to impede host translation (Lloyd, 2006). Moreover, modulation of the integrated stress response (ISR), which is determined by phosphorylation of eIF2α to attenuate cellular translation, is another strategy for promoting virulence (**Figure 2**) (Sonenberg and Hinnebusch, 2009). Four eIF2α kinases have been identified: heme-regulated inhibitor (HRI), which is a response to heme deficiency (Chen, 2007); double-stranded RNA-dependent protein kinase (PKR), which is induced by interferon (IFN) and activated by double-stranded RNA (dsRNA) during viral infection (Meurs et al., 1990); general control nonderepressible-2 (GCN2), which is activated by serum and amino acid deprivation (Harding et al., 2000); and finally, PKR-like ER kinase (PERK or PEK), which is activated by unfolded proteins in the ER (Ron, 2002).

Some researchers consider that eIF2α phosphorylation plays a role in hampering viral protein synthesis. For example, upon VSV infection, the induction of activated PERK only correlates with eIF2α phosphorylation at the later stage of infection. In MEF cells carrying a phosphorylation-insensitive eIF2α S51A variant, viral protein synthesis increased compared with a wild-type control, indicating that eIF2 phosphorylation is inhibitory to viral protein synthesis. As demonstrated by matrix (M) protein mutant virus (rM51RM), a viral protein (M protein) is involved in counteracting the antiviral response of the phosphorylation of eIF2α (Connor and Lyles, 2005). Like VSV infection, Chikungunya

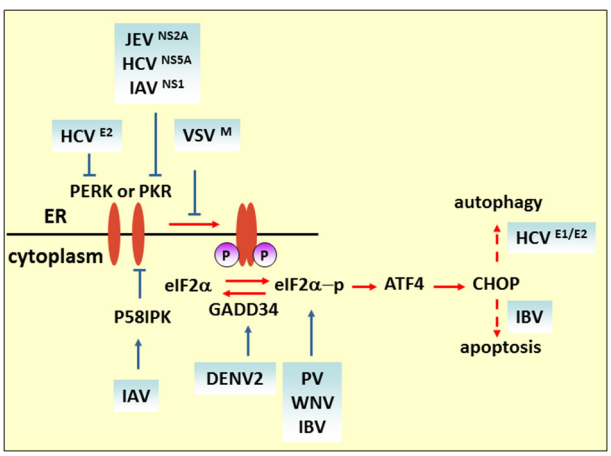

FIGURE 2 | eIF2 pathway under viral infection. The M protein of VSV, the E2 and NS5A proteins of HCV, and NS2A of JEV counteract the phosphorylation of eIF2α for viral replication. Blue solid arrows indicate the direct target of the virus or viral proteins. IAV also targets eIF2α by inducing P58IPK, a cellular inhibitor of PERK and PKR. IBV upregulates eIF2α–ATF4–CHOP-mediated apoptosis to benefit viral replication.

virus (CHIKV) induces PERK activation but delays eIF2α phosphorylation. The expression of CHIKV NSP4, which is the RNA-dependent-RNA polymerase, contributed to suppression of eIF2α phosphorylation, thus ensuring translation of viral proteins (Rathore et al., 2013). Furthermore, viruses containing type I or type II internal ribosomal entry sites (IRESs), such as PV, foot-and-mouth disease virus, mengovirus and EMCV, require many canonical translation initiation factors for initial replication (Beales et al., 2003; Sarnow, 2003). It is reported that PV switches translation mode from an eIF2-dependent to an eIF2-independent one during the course of infection to ensure efficient proliferation. Furthermore, studies have shown that the C terminal of the eIF5B fragment, cleavage by 3C proteases, and proteolytic activity of $2A^{pro}$ can stimulate virus IRES translation of enteroviruses (De Breyne et al., 2008; Redondo et al., 2011). Interestingly, it is reported that phosphorylation of eIF2α is required for activation of IRES during cell differentiation (Gerlitz et al., 2002). Thus, whether the phosphorylation level of eIF2α positively correlates with IRES-dependent viral mRNA translational efficiency remains to be determined. Some viruses regulate the eIF2α pathway by interfering with the activation of eIF2α kinases. HCV NS5A protein, containing an IFN sensitivity-determining region (ISDR), interferes with PKR activity by binding to a PKR dimerization domain (PKR residues 244–296) (Gale et al., 1998), while HCV E2 protein binds to PERK and inhibits downstream eIF2α phosphorylation by acting as a pseudosubstrate (Pavio et al., 2003). Interestingly, it is reported that NS5A stimulates eIF2α phosphorylation in the absence of PKR, implying that NS5A may activate other eIF-2α kinases to regulate eIF2α phosphorylation (Tardif et al., 2002). Overexpression of HCV NS2 induces eIF2α phosphorylation (Von Dem Bussche et al., 2010). Taken together, these studies indicate that HCV proteins modulate eIF2α pathway in a complex way, and the effect of regulation on virus replication cannot be established unequivocally. The N-terminal region of NS2A of JEV contains a sequence that is highly similar to HCV NS5A ISDR and also inhibits PKR-induced eIF2α phosphorylation (Tu et al., 2012). DENV2 infection triggers and then suppresses PERK-mediated eIF2α phosphorylation by elevating the expression of growth arrest and DNA damage-inducible protein-34 (GADD34), which acts together with phosphatase 1 (PP1) to dephosphorylate eIF2α-P (Pena and Harris, 2011). Influenza virus nonstructural protein NS1 interferes with dsRNA binding to PKR, and the infection also induces and activates P58IPK, a cellular inhibitor of PKR and PERK. Both strategies deployed by NS1 and P58IPK prevent PKR dimerization and autophosphorylation, which limits eIF2α phosphorylation (Lee et al., 1992; Lu et al., 1995; Yan et al., 2002).

In some circumstances, such as when the host immune system specifically recognizes foreign viruses and kills them with cytotoxic T lymphocytes, or when cell death is directly induced in virus infected cells to prevent completion of the replication cycle, apoptotic cell death is considered to be a host strategy for fighting against viral infections. ATF4 is a transcriptional activator of the ISR, which is involved in the expression of ISR target genes such as c/EBP homologous protein (CHOP) and GADD34 (Ma and Hendershot, 2003). CHOP was originally identified as a transcriptional factor eliciting ER stress-induced apoptosis. In cells subjected to West Nile virus (WNV) infection, eIF2α phosphorylation and CHOP-mediated apoptosis were induced. Both viral protein expression level and virus titer are increased in CHOP-deficient cells (Medigeshi et al., 2007). On the other hand, a virus may induce apoptosis to facilitate replication or the spread of viral progeny. It is reported that coronavirus infectious bronchitis virus (IBV) upregulates eIF2α–ATF4–CHOP signaling in infected cells and that it relies on PERK or PKR activation. Knockdown of CHOP reduces IBV-induced apoptosis through activation of the extracellular signal-related kinase (ERK). Viral protein expression level is moderately suppressed in CHOP-knockdown cells, which suggests that upregulation of CHOP-mediated apoptosis during IBV infection probably promotes virus replication (Liao et al., 2013).

In addition to regulation of cell death, it is reported that HCV induces the expression of CHOP at mRNA and protein levels and is correlated with autophagy induction; knockdown of CHOP not only increases HCV PAMP-mediated innate immune activation, but also elevates its inhibitory effect on virus replication (Ke and Chen, 2011). However, upstream CHOP induction is a matter of debate. Overexpression of HCV E1 and/or E2 induces the expression of CHOP in a PERK-dependent manner (Chan and Egan, 2005); while upon HCV infection, CHOP protein is upregulated by PERK, activating transcription factor (ATF6), and inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) collectively.

ATF6 PATHWAY

ATF6 is a type 2 transmembrane protein of 670 amino acids and is constitutively expressed as a 90-kDa protein (p90ATF6). Its C-terminal region is located in the ER, whereas the N-terminal region is located on the cytosolic side (**Figure 3**). Upon ER stress, ATF6 is cleaved to an N-terminal 50-kDa protein (p50ATF6) sequentially by the Golgi site-1 and site-2 proteases (S1P and S2P)

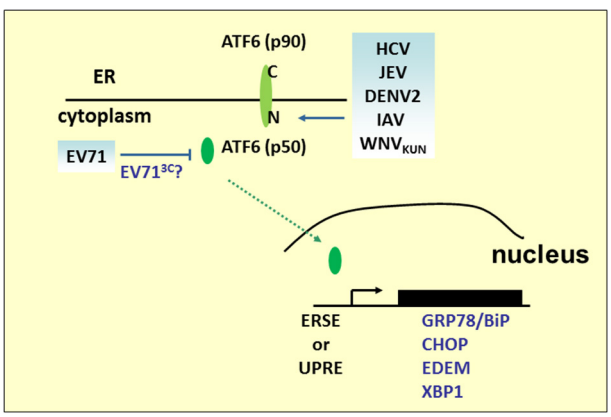

FIGURE 3 | ATF6 pathway under viral infection. Many RNA viruses activate the UPR pathway by cleaving ATF6 to release the p50 fragment. The N-terminal p50 with transcription activity enters the nucleus to activate the expression of ER stress and ERAD genes, such as GRP78/BiP, CHOP, XBP1, or EDEM. However, the p50 fragment was not detected in the EV71 infection.

(Ye et al., 2000). Nuclear translocation of p50ATF6, as a transcription factor, activates expression of ER stress and ERAD genes including ER chaperones, CHOP (*aka* GADD153), EDEM1, and X-box-binding protein 1 (XBP1) by targeting the *cis*-acting ER stress response element (ERSE) (CCAAT-N9-CCACG) and UPR element (UPRE) (GATGACGTG(T/G) NNN(A/T)T), although ATF6 has a much higher affinity for ERSE (Yoshida et al., 1998). In addition to directly regulating gene expression, ATF6 also modulates the innate immune response. Under subtilase cytotoxin (SubAB) treatment, cleavage and degradation of GRP78/BiP leads to activation of the AKT–NF-κB pathway through ATF6 activation (Yamazaki et al., 2009). Based on its pivotal role of connecting the arms of the UPR and converging the UPR and immune response, many viruses preferentially regulate ATF6 pathways to benefit replication. In WNV strain Kunjin (WNV_{KUN})-infected cells, expression of ATF6-target genes increases, but viral production decreases in ATF6 knockout MEF cells. Moreover, in ATF6 knockout MEF cells, phosphorylation of eIF2α, downstream CHOP activity, and Jak–STAT1 phosphorylation induced by IFNα are upregulated upon infection, which implies that virus-induced ATF6 activation is a prosurvival mechanism required for replication and inhibition of the antiviral signaling pathway (Ambrose and Mackenzie, 2013). However, it is still unclear whether WNV_{KUN} NS4A and NS4B, potent inducers of the UPR, inhibit IFNα-induced Jak–STAT signaling in an ATF6-dependent manner (Ambrose and Mackenzie, 2011). Other *Flavivirus* infections, including HCV, JEV, and DENV2, also induce cleavage of ATF6, nuclear translocalization of ATF6 and increases in chaperone proteins expression. In HCV replication, silencing of ATF6 reduces HCV intracellular mRNA levels (Ke and Chen, 2011). However, in JEV-infected cells, knockdown of the ATF6-targeted gene, GRP78, by siRNA did not affect JEV viral RNA replication, although it did impair virus assembly or release. In sucrose gradient, mature JEV viruses that do not cofractionate with GPR78 displayed a significant decrease in viral infectivity, indicating that JEV acts with GPR78 to promote its infectivity (Wu et al., 2011). Notably, DENV2 triggers ATF6 signaling in a cell-type-specific manner. In A549 cells, nuclear-localized ATF6 was observed (Umareddy et al., 2007); however, no activating events can be detected in human fibrosarcoma 2fTGH cells, therefore, GPR78 upregulation may be mediated in an ATF6-independent fashion (Pena and Harris, 2011). This cell-type-specific regulation of ATF6, also observed in IAV infection, p50ATF6, and its target gene ERp57/GRP58 expression (Roberson et al., 2012), has been shown to increase in murine primary tracheal epithelial cells infected with influenza A/PR/8/34, which is known to be involved in influenza virus HA protein folding (Solda et al., 2006). Knockdown of ERp57 abrogates viral progeny production. However, ATF6 activity is not induced in infected human tracheobronchial epithelial (HTBE) cells (Hassan et al., 2012).

Although ATF6-mediated transcriptional activation is an ongoing research field, another role for ATF6 in virus infection has emerged. We have previously demonstrated that EV71 infection results in the decline of p90ATF6, while the GRP78 promoter containing classical ERSE sites responsive to p50ATF6 in EV71-infected cells was not activated (Jheng et al., 2010). Indeed, two potential 3C cleavage sites (glutamine–glycine; QG) located at adjacent amino acids 511–512 and 516–517 near the C terminus of p90ATF6 were computationally predicted. It would be interesting to investigate the role of viral 3C in the regulation of ATF6, for its possible contribution in manipulating virus infection.

IRE1 PATHWAY

IRE1 is an ER-localized type I transmembrane protein containing an ER luminal dimerization domain and cytosolic kinase and RNase domains (Mori et al., 1993; Sidrauski and Walter, 1997). During ER stress, accumulation of unfolded proteins in the ER stimulates IRE1 oligomerization and autophosphorylation (**Figure 1**). Its endoribonuclease activity initiates an unconventional splicing of the XBP1 mRNA, excising a 26-nt sequence and shifts the reading frame to produce a functional isoform XBP1(S), which contains a C-terminal transactivation domain absent from the unspliced form, XBP1(U). XBP1(S) then translocates to the nucleus where it induces expression of target genes containing UPRE or ERSE. These target genes are involved in ERAD, chaperone protein production, and ER membrane biosynthesis (Shamu and Walter, 1996; Friedlander et al., 2000).

Studies of the IRE1 signaling pathway demonstrate its significant role in virus infection (**Figure 4**). HCV glycoprotein E2 is an example of a virus-derived ERAD substrate. HCV infection activates the IRE1–XBP1–EDEM pathway, where EDEM1 and EDEM3, but not EDEM2, interact with HCV E2 to accelerate its degradation. Either knockdown of EDEMs or treating cells with kifunensine (KIF), a potent inhibitor of ER mannosidase, interferes with the binding of EDEMs with SEL1L, a component of ERAD complex, stabilizes E2 expression, and enhances virus replication and viral particle production. However, there is no interaction between EDEM proteins and the JEV envelope protein and abolishing the ERAD pathway by KIF does not affect JEV production (Saeed et al., 2011). The results emphasize the pivotal role of the ERAD pathway in the life cycle of specific viruses. Interestingly, UPRE reporter activity or ERAD of misfolded null Hong Kong α-antitrypsin is reduced in cells carrying HCV replicons, which lack structural proteins, even though upstream XBP1 splicing occurs (Tardif et al., 2004). This implies that HCV structural proteins play a key role in XBP1-mediated UPRE activation, and this is supported by a related study demonstrating that HCV E1 and/or E2 activates the XBP1–ERAD pathway (Chan and Egan, 2005). Furthermore, the IRE1 signaling pathway also participates in viral protein retrotranslocation. Hepatitis E virus (HEV) ORF2 is an N-linked glycoprotein which is cotranslationally translocated into the ER while a significant fraction of it is also observed in the cytoplasm. Based on the results of tunicamycin and KIF treatment, it is believed that glycosylation and ERAD are essential for ORF2 retrotranslocation from the ER to the cytoplasm (Surjit et al., 2007). However, no ubiquitination of ORF2 can be observed, and retrotranslocated ORF2 protein was stable in the cytoplasm when the cells were treated with proteasome inhibitor MG132, which suggests that ERAD is required for ORF2 access to the cytoplasm. Microarray analysis reveals that ORF2 overexpression causes upregulation of Hsp70B, Hsp72, and Hsp40. Hsp72 is an antiapoptotic heat shock protein that directly interacts with ORF2 (John et al., 2011). It is reported that expression of Hsp72 enhances XBP1 mRNA splicing and protects cells from

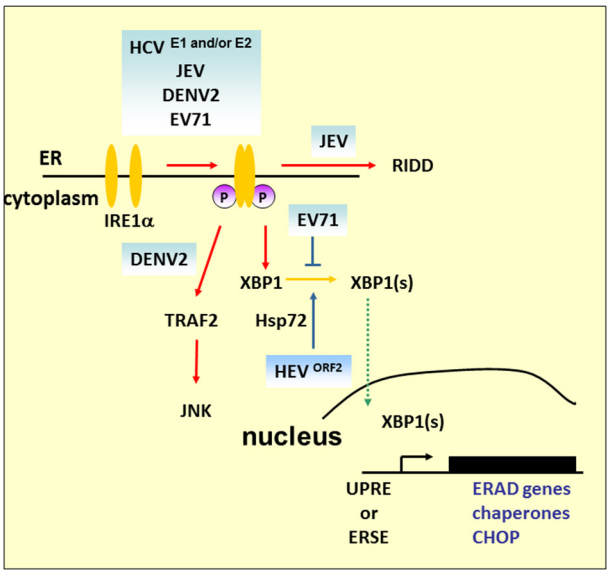

FIGURE 4 | IRE1 pathway under viral infection. In addition to mediating Xbp1 mRNA splicing, studies demonstrated that Ire1 activates RIDD to promote the degradation of mRNAs encoding ER-targeted proteins to reduce the load of ER client proteins during ER stress. The mammalian IRE1–TRAF2–JNK pathway, independent of XBP1 splicing, may lead to the activation of apoptosis after prolonged ER stress. HCV and its structural proteins E1 and E2 play an important role in the activation of the IRE1–XBP1–ERAD pathway. Overexpression of ORF2 of HEV can upregulate antiapoptotic protein Hsp72 to activate XBP1 splicing. However, further study is required to determine whether HEV infection can activate XBP1 via Hsp72. DENV2 infection activates CHOP and GADD34 expression downstream of IRE1–XBP1 signaling. However, apoptosis activation by JNK, but not CHOP, is essential for DENV2 infection.

ER stress-induced apoptosis by association with IRE1 (Gupta et al., 2010). Thus, further investigation is needed to examine the correlations of ORF2, IRE1, and Hsp72 in HEV replication.

Under harsh ER stress, the activation of IRE1–XBP1 can also lead to the induction and expression of CHOP. DENV2 infection induces CHOP, and GADD34 expression is a downstream event of IRE1–XBP1 signaling. Of note is that induction of CHOP does not lead to apoptosis markers such as decreased expression of Bcl-2 or proteolytic cleavage of pro-caspase-9, pro-caspase-3, or PARP, which indicates a role beyond guiding cell death in infected cells (Pena and Harris, 2011). Indeed, it has been reported that CHOP exhibits protective effects against radiation-induced apoptosis or has a role in autophagy induction (Mayerhofer and Kodym, 2003; Ke and Chen, 2011). Another ER stress-induced cell death that relies on the IRE1–TRAF2 pathway is implicated in JNK activation (see **Figure 4**). The role of this pathway is emphasized by DENV2 infection; silencing of IRE1 decreases the virus titer, but the viral progeny output is not affected by silencing of XBP1 (Pena and Harris, 2011). However, JNK pathway inhibitors diminished virus yield significantly, which suggests that activation of JNK is essential for DENV2 infection (Ceballos-Olvera et al., 2010). Our previous findings also demonstrated that EV71 phosphorylates IRE1, but inhibits the expression of XBP1. The overexpression of XBP1 in cells appeared to inhibit viral entry, and therefore reduce viral RNA and viral particle formation (Jheng et al., 2012). As previous studies have reported that picornavirus infections induce JNK activation (Kim et al., 2004; Peng et al., 2014), further detailed studies of the IRE1-JNK activation in EV71 infection would extend our understanding of the contributions of IRE1-JNK in the virus life cycle.

IRE1 has also been linked to the mediation of the selective degradation of a subset of ER-localized mRNAs in a process known as regulated IRE1-dependent degradation (RIDD) (Hollien and Weissman, 2006). Mutation or removal of the signal sequences in targeted mRNAs prevents their decay (Kimmig et al., 2012). However, it has been observed that Drosophila mRNA Smt3, a homolog of a small ubiquitin-like modifier (*aka* SUMO), lacks any ER-targeting sequence, and is a noncanonical RIDD target, which implies that unknown specific features other than ER localization are involved in defining the RIDD substrates (Moore et al., 2013). RIDD has been suggested to play adaptive roles by reducing protein translocation load, such as decrease of proinsulin expression in pancreatic beta-cells faced with chronic high glucose, and protecting liver cells from acetaminophen-induced hepatotoxicity (Lipson et al., 2008; Hur et al., 2012). Alternatively, RIDD has also been suggested to play destructive roles under unmitigated ER stress because continued degradation of mRNAs encoding secretory cargo proteins and proteins involved in ER-resident protein folding occurs.

In addition to IRE1–XBP1 activation, JEV also induces activation of the RIDD cleavage pathway (Bhattacharyya et al., 2014). The addition of STF083010, a specific inhibitor of IRE1 RNase activity, to infected cells decreases the Tg-induced Xbp1 splicing and potential RIDD target transcripts. It also decreases viral protein expression as well as mature progeny formation, but does not affect viral RNA synthesis, which indicates that JEV viral RNA is not a substrate of RIDD, and RIDD activation is beneficial for viral infectivity.

It is not clear whether other viral infections trigger RIDD. To extrapolate from the study of HCV, HCV replicons activate the phosphorylation of IRE1 but impede XBP1 activation (Tardif et al., 2004). Depletion of IRE1 attenuates replicon translation, which implies that RIDD may enhance viral protein synthesis. Thus, the study of HCV replicon may have potential for deciphering the role of RIDD in HCV infection because it could uncouple XBP1 signaling from IRE1 activation.

AUTOPHAGY

Autophagy is a vesicular process that results in the degradation of the sequestered component, which can then be recycled by the cell. In mammalian cells, a complete autophagy includes the following four steps. (1) Induction. Induction is initiated by activation of the Unc-51-like kinase 1 (ULK1) complex. The ULK1 complex contains ULK1, focal adhesion kinase (FAK)-family-interacting protein of 200 kD (FIP200), Atg13 and Atg101 (Mizushima, 2010). ULK1 complex activity would be, at least, modulated by mTORC1, Akt, and AMPK (Inoki et al., 2003; Bach et al., 2011; Egan et al., 2011; Kim et al., 2011). mTORC1 is a serine/threonine kinase complex, which phosphorylates ULK1 and Atg13 and also inhibits autophagy. Akt and AMP-activated protein kinase (AMPK) phosphorylate TSC2 at different residues,

which results in the GTP hydrolysis of Rheb and indirectly antagonizes the mTORC1 signaling pathway. Recently, the combination of bioinformatic and proteomic approaches has identified ULK1 as a direct target of AMPK and as involved in autophagy induction. (2) Vesicle nucleation. The Beclin1–PI3KC3 complex, generating PI3P, is essential for recruitment of PI3P effectors including DFCP1, WIPIs upstream of Atg proteins and lipids recruitment to the PAS, which is required for autophagosome construction (Proikas-Cezanne et al., 2004; Axe et al., 2008). Importantly, the activity of the Beclin1–PI3KC3 complex depends on its subunit composition. Complexes containing Atg14-like protein (ATG14L or Barkor) or ultraviolet irradiation resistance-associated gene (UVRAG) activate autophagy (Itakura et al., 2008); nevertheless, the RUN domain and cysteine-rich domain containing Beclin 1-interacting protein (Rubicon) act as negative regulators of autophagy (Matsunaga et al., 2009). (3) Vesicle expansion and completion. The cytosolic form of LC3 (LC3-I) is cleaved by the cysteine protease Atg4, followed by conjugation with phosphatidylethanolamine (PE) assisted by the Atg12–Atg5–Atg16L complex, which functions as an E3–like enzyme. LC3-PE leads to PAS expansion, and cytosolic cargos are then enclosed into double membrane vesicles called autophagosomes (Geng and Klionsky, 2008). (4) Autophagosome maturation. An autophagosome matures into an autolysosome by sequential fusion with endosomes and with lysosomes, the contents of which are degraded by hydrolases therein. It is reported that autolysosome formation is related to UVRAG and expression of lysosomal-associated membrane protein 2 (Lamp-2) (Liang et al., 2008; Fortunato et al., 2009).

WHY DOES THE RNA VIRUS MODULATE AUTOPHAGY?

Previous studies suggest that autophagy may be an important antiviral defense mechanism (Talloczy et al., 2006; Orvedahl et al., 2010); however, the role of autophagy in virus infection is complicated and may have opposite consequences for the viral pathogenesis. Many viruses manipulate autophagy for their own benefit by the following mechanisms.

FORMING THE MEMBRANE-BOUNDED REPLICATION COMPARTMENTS FOR VIRAL REPLICATION, OR ARRAYING AUTOPHAGIC VESICLES FOR VIRAL PARTICLE ASSEMBLY OR SHEDDING

The exploitation of autophagy has been identified in many RNA viruses including PV, CVB3, JEV, and HCV (Jackson et al., 2005; Wong et al., 2008; Tanida et al., 2009; Ke and Chen, 2011; Li et al., 2012). Increased amounts of autophagosomes, as well as colocalization of the autophagy marker protein LC3 and viral protein, were observed in virus-infected cells. In addition, cells treated with an autophagy inhibitor, or transfected with siRNA specifically obstructed autophagic processes, which reduced virus replication or virus titer. For example, in PV infection, virus yield was correlated with the induction of autophagy. Treating cells with siRNA targeting LC3 or Atg12 to block autophagy leads to reduced virus yield (Jackson et al., 2005). In addition, based on the topology of a double membrane compartment, digestion of the inner membrane under the autolysosome formation would allow efficient fusion of the autophagosomal membrane with the cytoplasm membrane. Thus, an emerging concept is that autophagy may also involve the nonlytic release of cytoplasm under autophagosome maturation, namely autophagic exit without lysis (AWOL), which may participate in the release of PV (Kirkegaard and Jackson, 2005; Taylor et al., 2009).

INCREASED VIRAL INFECTIVITY BY BLOCKING AUTOPHAGIC FLUX

Virus-induced uncompleted autophagy was reported for CVB3-, rotavirus-, and IVA- infected cells (Gannage et al., 2009; Kemball et al., 2010; Alirezaei et al., 2012; Crawford et al., 2012). In CVB3-infected pancreatic acinar cells, an increase in the number of double-membraned autophagy-like vesicles was observed upon infection. However, the accumulation of autophagy substrate p62 and the formation of large autophagy-related structures named megaphagosomes indicate that CVB3 blocks a later stage of the autophagic pathway (Kemball et al., 2010). Further results highlight the impact of autophagy on CVB3 RNA replication and translation (Alirezaei et al., 2012). It was reported that rotavirus NSP4 viroporin initiates autophagy to transport viral proteins to sites of virus replication for assembly of mature particles, which involves an increase of cytoplasmic calcium and subsequent activation of the CaMKK-β–AMPK pathway. Rotavirus also interferes with autophagy maturation; however, the mechanism is still unknown (Crawford et al., 2012). Accumulated studies reveal that M2, HA, and NS1 proteins of IAV are involved in the induction of autophagy, while only M2 has been identified as playing a critical role in impeding fusion of autophagosomes with lysosomes (Gannage et al., 2009; Sun et al., 2012; Zhirnov and Klenk, 2013).

ESCAPING THE HOST IMMUNE RESPONSE

Autophagy-mediated immune responses that benefit virus replication have been reported in VSV, HCV, DENV, and JEV (Jounai et al., 2007; Ke and Chen, 2011; Jin et al., 2013). In VSV infection, the Atg5–Atg12 conjugate targets RIG-I/MDA5–MAVS-dependent type I IFN production by directly interacting with the MAVS and RIG-I, and negatively regulates MAVS-mediated NF-κB and type I IFN promoters, and permits VSV replication. Furthermore, through an unknown mechanism, HCV- or DENV-induced complete autophagy negatively regulates type I IFN production and promotes HCV replication (Ke and Chen, 2011). Recently, research about JEV has shown that in autophagy-impaired cells, virus infection induces aggregates of MAVS and activation of IFN regulatory factor 3 (IRF3), markers for activation of innate immune responses, which suggests that autophagy-mediated immune responses are required for viral replication (Jin et al., 2013).

UPR AND AUTOPHAGY

As ER proliferation, which paradoxically commits the cell to cell death or survival, is observed both in UPR and autophagy, it is reasonable to propose a possible link between UPR pathways and the autophagic response. Indeed, many UPR-related transcription factors manage Atg expression (**Table 1**). As demonstrated previously, yeasts with mutations in the GCN2-signaling pathway are defective in starvation-induced autophagy. GCN4, which undertakes GCN2-dependent transcriptional activation, is essential for autophagy induction (Talloczy et al., 2002). Recently,

Table 1 | Exploitation of autophagy by modulation of UPR transcription factors.

Transcription factor	Target protein	References
ATF4	LC3, p62/SQSTM1, and ULK1	Milani et al., 2009; Rouschop et al., 2010; B'Chir et al., 2013; Pike et al., 2013
CHOP	ATG5, LC3, and p62/SQSTM1	Rouschop et al., 2010; B'Chir et al., 2013; Wang et al., 2014
ATF6	DAPK1	Gade et al., 2012
C/EBPβ	DAPK1, ATG4B, and ULK1	Gade et al., 2008; Ma et al., 2011; Guo et al., 2013
SREBP2	LC3, ATG4B and ATG4D	Seo et al., 2011
XBP1	Beclin1 and Bcl2	Gomez et al., 2007; Margariti et al., 2013

Table 2 | Compounds affecting UPR and autophagy.

Inhibitors/Inducers	Mode-of-action	References
UPR		
GRP78/BiP inducer X (BIX)	GRP78 upregulation	Kudo et al., 2008
Tauroursodeoxycholic acid (TUDCA)	Reduces UPR	Ozcan et al., 2006
Salubrinal	Inhibitor of eIF2α dephosphorylation	Boyce et al., 2005
3,5-dibromosalicylaldehyde	Inhibits the RNase activity of IRE1αs	Volkmann et al., 2011
Sunitinib	Inhibits IRE1α *trans*-autophosphorylation, but promotes oligomerization and activates the RNase domain Inhibitor of PKR	Korennykh et al., 2009; Jha et al., 2011
STF083010	Inhibits the RNase activity of IRE1α	Papandreou et al., 2011
Nelfinavir	Induces UPR autophagy	Mahoney et al., 2013b
Sorafenib	Induces UPR autophagy	Shi et al., 2011
AUTOPHAGY		
Rapamycin	Induces autophagy	Ravikumar et al., 2002
Chloroquine	Inhibits autophagic flux	Yoon et al., 2010
Bafilomycin A1	Inhibits autophagic flux	Van Deurs et al., 1996
Nelfinavir	Induces UPR autophagy	Mahoney et al., 2013b
Sorafenib	Induces UPR autophagy	Shi et al., 2011
Evodiamine	Impairs autophagy	Dai et al., 2012
23-(*S*)-2-Amino-3-phenylpropanoyl-silybin	Impairs autophagy	Dai et al., 2013

results of multiple genetic models showed that the PERK–eIF2α–ATF4 pathway affects cMyc-dependent tumorigenesis by evoking cytoprotective autophagy; while pharmacologic or genetic inhibition of autophagy resulted in enhanced Myc-dependent apoptosis (Hart et al., 2012). Thus, UPR inhibition could provide new targets for the treatment of malignancies, characterized by cMyc overexpression. In addition, IRE1 also mediates autophagy in Huntington's disease under ER stress. Clearance of mutant huntingtin aggregates through autophagic flux was impaired via IRE1–TRAF2 signaling, which results in neuronal cytotoxicity (Lee et al., 2012). Although studies on UPR autophagy mainly focus on the regulation of eIF2α kinase and IRE1, transcriptional regulation of autophagic genes by ATF6 and SREBP2, a membrane-bound transcription factor activated through proteolytic processing upon ER stress, was noticed recently (Ogata et al., 2006; Seo et al., 2011; Gade et al., 2012). Death-associated protein kinase 1 (DAPK1), a positive mediator of IFN-regulated growth suppressor, is principally regulated by transcription factor C/EBP-β, one of the genes that increases expression during ER stress (Chen et al., 2004). DAPK1 promotes autophagy by phosphorylating Beclin 1, and therefore dissociating it from autophagy negative regulator Bcl2. An investigation found that activated ATF6 could directly interact with C/EBP-β carrying an ERK1/2 target site; this heterodimer then coacts to activate the DAPK1 promoter, which in turn induces autophagy. Additionally, XBP1, a downstream target of ATF6, is essential for C/EBP-β expression (Chen et al., 2004). The role of SREBP2 in autophagy was disclosed through gene ontology analysis (Seo et al., 2011). Further study shows that SREBP-2 activates autophagy gene expression, such as LC3B, ATG4B, and ATG4D, accompanied by increased LC3 puncta formation, while SREBP-2 deficiency obtains an opposite result.

In virus infection, HCV is a well-documented model illustrating UPR autophagy regulation. Induction of UPR and incomplete autophagy was observed in cells transfected with HCV JFH1 RNA. Cells treated with siRNA targeting PERK, IRE1, and ATF6 showed a suppression of LC3 conversion and a decrease of HCV RNA replication (Sir et al., 2008). In the HCV infection system, HCV induces complete autophagy and CHOP plays a leading role in UPR autophagy signaling (Ke and Chen, 2011). Further efforts to decipher how HCV activates autophagy revealed that PERK–eIF2α–ATF4 and ATF6 pathways activated CHOP expression in HCV core protein-transfected cells where the core protein had not been demonstrated to induce ER stress previously. Moreover, HCV core protein may promote ATG12 and LC3 protein expression through transcriptional control by ATF4 and CHOP, respectively (Wang et al., 2014).

Recent studies suggest that completed autophagy induced by CHIKV infection is mediated by the independent induction of the endoplasmic reticulum and oxidative stress pathways. Knockdown of IRE1 or treated cells with the ROS inhibitor *N*-acetyl-l-cysteine inhibits formation of autophagosomes as well as the conversion of LC3-I to LC3-II. Moreover, an additive inhibitory effect on autophagosome formation was observed in infected cells silenced for *IRE1*mRNA and treated with *N*-acetyl-l-cysteine (Joubert et al., 2012).

TARGETING UPR OR AUTOPHAGY AS POTENTIAL THERAPY IN VIRUS INFECTION

Because UPR and autophagy play a role in viral pathogenesis, the regulation of UPR and autophagy may be an important strategy for the future development of new therapeutic approaches to combat viruses. For example, we have demonstrated that overexpression of GRP78 to relieve ER stress decreases EV71 replication (Jheng et al., 2010). Thus, agents such as GRP78/BiP inducer X (BIX) (Kudo et al., 2008) or chemical chaperone, tauroursodeoxycholic acid (TUDCA) (Ozcan et al., 2006), will be potentially useful in the treatment of EV71 (**Table 2**).

There are other established strategies to inhibit viruses by modulating UPR target eIF2α phosphorylation or IRE1, e.g., salubrinal is a small molecule that prevents dephosphorylation of eIF2α and 3,5-dibromosalicylaldehyde, an IRE1 inhibitor, may cause restriction of IVA (Boyce et al., 2005; Volkmann et al., 2011).

There is emerging evidence that pharmacological agents that directly activate or deactivate autophagy influence virus replication. Evodiamine and 3-(*S*)-2-amino-3-phenylpropanoyl-silybin have been identified as anti-IVA agents aimed at multiple processes of autophagy (Dai et al., 2012, 2013). Additionally, chloroquine-suppressed HCV replication has been proved (Mizui et al., 2010).

Because UPR and autophagy are closely related, combination treatment may show a synergistic effect of their application, which was demonstrated in cancer research. The combination of nelfinavir (which induces UPR autophagy) and chloroquine enhances cytotoxicity against cancer cells (Mahoney et al., 2013b); therefore, the use of combination treatment with improved efficacy and decreased toxicity represents a promising strategy to fight viruses.

PERSPECTIVES

Although UPR autophagy has been discussed in many research areas, its integrated response to virus infection is only now beginning to emerge. It needs to be experimentally proven whether virus-induced autophagy is associated with UPR. Furthermore, given what we know about the various means that viruses use to modulate UPR or autophagy to advantage their own virulence, the development of specific inducers or inhibitors for these molecules is one of the major challenges in this field.

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Unfolded protein response in hepatitis C virus infection

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Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus of clinical importance. The virus establishes a chronic infection and can progress from chronic hepatitis, steatosis to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). The mechanisms of viral persistence and pathogenesis are poorly understood. Recently the unfolded protein response (UPR), a cellular homeostatic response to endoplasmic reticulum (ER) stress, has emerged to be a major contributing factor in many human diseases. It is also evident that viruses interact with the host UPR in many different ways and the outcome could be pro-viral, anti-viral or pathogenic, depending on the particular type of infection. Here we present evidence for the elicitation of chronic ER stress in HCV infection. We analyze the UPR signaling pathways involved in HCV infection, the various levels of UPR regulation by different viral proteins and finally, we propose several mechanisms by which the virus provokes the UPR.

Keywords: hepatitis C virus, unfolded protein response, endoplasmic reticulum stress, hepacivirus, virus-host interaction

INTRODUCTION

Hepatitis C virus (HCV) infection produces a clinically important disease affecting around 3% of the world population (Thomas, 2013). The disease usually manifests itself as chronic hepatitis, which can progress into fibrosis, cirrhosis and eventually into hepatocellular carcinoma (HCC). How the virus establishes persistence and causes diseases is still far from clear. Understanding how the virus interacts with the host is key to answer these questions. Recently the unfolded protein response (UPR), a host homeostatic response to endoplasmic reticulum (ER) stress, has emerged to be a novel mechanism involved in a number of human diseases including that caused by virus infections (Dimcheff et al., 2003; Favreau et al., 2009; Wang and Kaufman, 2012). UPR has also been frequently manipulated by a number of viruses to aid in infection and to attenuate anti-viral response (Isler et al., 2005; Smith et al., 2006; Yu et al., 2006; Liu et al., 2009; Jheng et al., 2010; Ambrose and Mackenzie, 2011; Pena and Harris, 2011; Burnett et al., 2012; Galindo et al., 2012; Qian et al., 2012; Rathore et al., 2013; Stahl et al., 2013). On the other hand, UPR is recruited by the host anti-viral machinery to help eliminating virus infection (Clavarino et al., 2012). Thus UPR is far from being a homeostatic response in terms of virus infection. For viruses that establish persistent infection it is even more important to be able to adapt to chronic ER stress, otherwise diseases will ensue. It is therefore important to understand how HCV interacts with the host UPR, taking into consideration the genotypes and the various experimental systems used to dissect this virus-host interaction.

HEPATITIS C VIRUS

LIFE CYCLE

HCV is a single-stranded, positive-sense RNA *Hepacivirus* (a genus of the family *Flaviviridae*) with a 9.6 kb genome (**Figure 1**) (Scheel and Rice, 2013; Simmonds, 2013). The virus particles are unusual in that they are associated with low-density lipoproteins (LDLs) and very low-density lipoproteins (VLDLs) to form the lipoviroparticles (Andre et al., 2002). The virus enters host cells (hepatocytes) by initial binding to low-affinity receptors the LDL receptor and glycosaminoglycans on the heparan sulphate proteoglycans, followed by binding to scavenger receptor class B member 1 and stepwise translocation to post-binding co-receptors the tetraspanin CD81 and tight junction proteins claudin 1 and occludin (Pileri et al., 1998; Agnello et al., 1999; Scarselli et al., 2002; Evans et al., 2007; Ploss et al., 2009; Jiang et al., 2012; Lindenbach and Rice, 2013). Interaction between CD81 and claudin 1 facilitates viral uptake by clathrin-mediated endocytosis (Meertens et al., 2006; Farquhar et al., 2012). Exposure to low pH in the endosome triggers membrane fusion and release of the RNA genome into the cytoplasm (Lavillette et al., 2006). Translation of the RNA genome into a single polypeptide is mediated from an internal ribosome entry site element at the 5′ untranslated region (Tsukiyama-Kohara et al., 1992). The polypeptide is then cleaved by the host signal peptidase and signal peptide peptidase and viral autoprotease NS2-3 and serine protease NS3/NS4A co-factor into the structural proteins core, envelopes E1 and E2, and non-structural (NS) proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991b; Grakoui et al., 1993a; Failla et al., 1994; Lin et al., 1994; Santolini et al., 1994; McLauchlan et al., 2002; Schregel et al., 2009; Scheel and Rice, 2013). Replication takes place in a membranous web, which is a re-organized intracellular membrane structure consisting of single, double, and multiple membrane vesicles (Behrens et al., 1996; Romero-Brey et al., 2012; Bartenschlager et al., 2013; Paul et al., 2013). Formation of the membranous web is mainly induced by NS4B and NS5A (Romero-Brey et al., 2012). Replication is catalyzed by an RNA-dependent RNA polymerase, NS5B, via a negative-sense RNA intermediate, and assisted by the helicase activity of NS3 and host factors cyclophilin A and miR-122 (Behrens et al., 1996; Banerjee and Dasgupta, 2001;

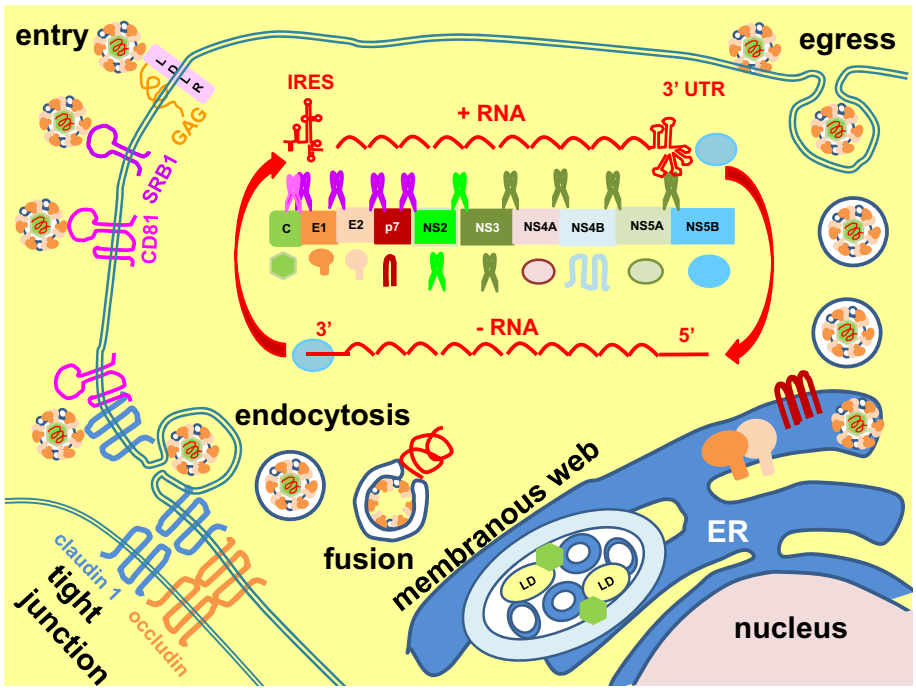

FIGURE 1 | Hepatitis C virus life cycle. Hepatitis C virus enters cells by stepwise binding through host receptors low-density lipoprotein receptor (LDLR), glycosaminoglycans (GAGs), scavenger receptor class B member 1 (SRB1), CD81, and the tight junction proteins claudin 1 and occludin. Interaction between CD81 and claudin 1 facilitates viral uptake by clathrin-mediated endocytosis. Endosomal low pH triggers membrane fusion and release of genome into the cytoplasm. The positive-sense (+), single-stranded RNA is translated by an internal ribosome entry site (IRES) element at its 5′ untranslated region (UTR) into a single polypeptide, which is then cleaved into the core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B by host signal peptidase (purple scissor) and signal peptide peptidase (pink scissor) and viral autoprotease (NS2-3) (fluorescent green scissor) and serine protease (NS3-NS4A) (dark green scissor). Replication is catalyzed by the RNA-dependent RNA polymerase NS5B, assisted by the helicase activity of NS3, via a negative-sense (−) intermediate RNA (red arrows). Replication takes place in the membranous web, which consists of single, double and multiple membrane vesicles. Formation of the membranous web is induced by NS4B and NS5A. Assembly of virion is initiated on core-coated lipid droplets (LD) followed by budding into the endoplasmic reticulum (ER), where it is coated by the ER-resident envelope proteins E1 and E2. Egress follows the secretory pathway to release the virion to extracellular space. The viroporin, p7, forms ion channels to equilibrate pH gradients during trafficking through the secretory pathway to protect the virion.

Jopling et al., 2005; Kaul et al., 2009). Virion assembly is initiated on core-coated lipid droplets followed by budding into the ER, where the two envelope glycoproteins, E1 and E2, form non-covalently-bonded heterodimers and disulphide-bonded aggregates (Dubuisson et al., 1994; Deleersnyder et al., 1997; Lindenbach and Rice, 2013). Virus particles are released via trafficking through the secretory pathway, where the envelope proteins undergo further glycan modifications and structural re-arrangement into higher ordered oligomeric aggregates (Vieyres et al., 2010, 2014). The viroporin, p7, forms ion channels to equilibrate pH gradients during trafficking through the secretory pathway to protect the virion (Wozniak et al., 2010). Assembly, budding and egress are tightly coupled to host lipoprotein synthesis (Lindenbach and Rice, 2013).

GENOTYPES

HCV is classified into 7 genotypes (with >30% sequence variation), sub-divided into sub-types a, b, c, etc. (with 20–25% sequence variation) and then strains/isolates (Simmonds et al., 1994, 2005; Kuiken and Simmonds, 2009). Genotype is a major determining factor in responsiveness to interferon (IFN) treatment and in disease progression in hepatitis C patients (Chayama and Hayes, 2011; Ripoli and Pazienza, 2011). Infection with genotype 1 is more resistant to IFN treatment and presents a more aggressive disease course with the chance of progression into HCC significantly higher. Moreover, HCV in infected patients exists as a population of quasispecies/intrahost variants (Martell et al., 1992; Simmonds et al., 1994, 2005; Holmes, 2010; Domingo et al., 2012). It is anticipated that virus-host interaction is determined at genotypic, sub-genotypic, strain/isolate and even quasispecies/intrahost variants levels. Therefore in this review, we will refer to the genotypes, sub-types and strains/isolates used in various studies.

HCV EXPERIMENTAL SYSTEMS

When HCV was discovered in 1989 as the causative agent of post-transfusional non-A, non-B hepatitis, study on the virus was limited to the use of *in vitro* cell-free systems and cell culture expression systems employing transient transfection or viral vectors (Choo et al., 1989; Hijikata et al., 1991b, 1993; Grakoui et al., 1993b). Nevertheless, much has been known about the genomic structure and viral protein functions. *In vivo* study was made

possible by the successful infection of chimpanzees by intrahepatic inoculation of the RNA transcript (Kolykhalov et al., 1997). However, the use of chimpanzees is limited and restricted (Mailly et al., 2013). Small animal models have become available by the creation of transgenic mice expressing viral proteins in their livers and chimeric mice with humanized livers (Moriya et al., 1998; Mercer et al., 2001; Dorner et al., 2011). It was not until 1999 when a selectable sub-genomic replicon (SGR) of genotype 1b Con1 isolate was successfully established which allowed the study of the intracellular steps of the virus life cycle (**Figure 2A**) (Lohmann et al., 1999). Since then some other SGR and genomic replicons have been established (**Figure 2B**) (Ikeda et al., 2002; Blight et al., 2003; Kato et al., 2003). A pseudotyped virus containing HCV envelope proteins in a retrovirus or lentivirus genomic backbone (HCVpp) was also established to facilitate the study of virus entry (Bartosch et al., 2003). The breakthrough came in 2005 when a cell-cultured infectious system (HCVcc) was established from a wild type genotype 2a JFH1 strain fulminant hepatitis C patient, coupled with derivation of cell lines (Huh7.5, Huh7.5.1) from the parental Huh7 with improved infectivity (**Figure 2C**) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Chimeric viruses were then created by fusing core-NS2 from other genotypes or sub-types to the NS3-5B backbone of JFH1, allowing partial studies of other genotypes (**Figure 2D**) (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008; Li et al., 2011). Currently there has been some success in establishing HCVcc from other genotypes but they all require adaptive mutations, thus do not represent the wild type repertoires (Yi et al., 2006; Date et al., 2012; Li et al., 2012a,b; Ramirez et al., 2014). With the availability of so many systems, therefore in this review, we will refer to the systems and cell lines used in various studies.

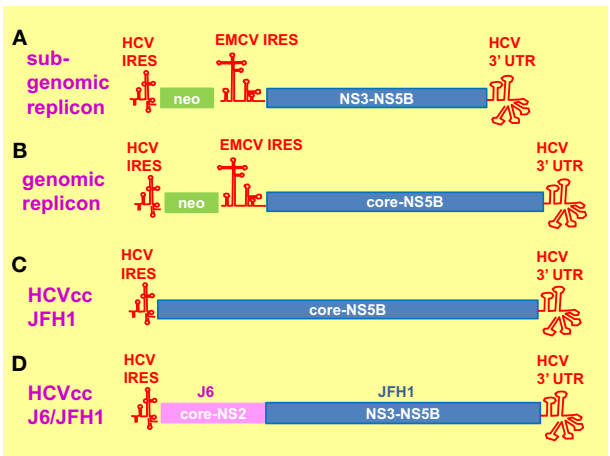

FIGURE 2 | Hepatitis C virus replication systems. (A) Sub-genomic replicon (SGR) consists of a bicistronic mRNA. The 5′ neomycin (neo) mRNA is translated by the hepatitis C virus (HCV) internal ribosome entry site (IRES) element whereas the 3′ mRNA encoding HCV NS3-NS5B plus the 3′ untranslated region (UTR) is translated by the encephalomyocarditis virus (EMCV) IRES element. Cell lines harboring the SGR were established by neomycin selection. **(B)** The genomic replicon is similar to that of SGR, apart from that the 3′ mRNA encodes core-NS5B plus 3′ UTR. **(C)** The HCV cell-cultured infectious system (HCVcc) consists of the entire genomic RNA from JFH1. **(D)** The chimeric J6/JFH1 is created by fusing the core-NS2 from J6 to NS3-NS5B plus 3′ UTR from JFH1.

UNFOLDED PROTEIN RESPONSE

UPR is a cellular adaptive response for restoring ER homeostasis in response to ER stress (**Figure 3**) (Walter and Ron, 2011). UPR transduces into a programme of cellular transcriptional and translational responses culminating in upregulation of the molecular chaperone the immunoglobulin heavy-chain binding protein (BiP) to promote protein folding, global inhibition in protein synthesis to reduce protein load and potentiation of ER-associated degradation (ERAD) to eliminate unfolded/malfolded proteins from the ER (Travers et al., 2000; Walter and Ron, 2011).

BiP has been attributed a pivotal role as the master negative regulator of UPR by binding to and repressing the activities of the three proximal UPR sensors: activating transcription factor (ATF) 6, RNA-dependent protein kinase-like ER-resident kinase (PERK), and inositol-requiring enzyme 1 (IRE1) (Bertolotti et al., 2000; Shen et al., 2002a). Accumulation of unfolded/malfolded proteins " distract" BiP from binding to the UPR sensors. ATF6 de-oligomerizes and migrates to the Golgi where it is cleaved sequentially by site-1 protease and site-2 protease to release an active transcription factor into the nucleus where it transactivates UPR genes harboring an ER-stress element (ERSE) in their promoters e.g., BiP, glucose-regulated protein 94 (GRP94) and $P58^{IPK}$ (Yoshida et al., 1998; Shen et al., 2002a; Nadanaka et al., 2007). $P58^{IPK}$ is an inhibitor of PERK, thus linking the ATF6 pathway to the PERK pathway (Van Huizen et al., 2003).

PERK is an ER stress kinase, activated by dimerisation and autophosphorylation (Harding et al., 1999; Bertolotti et al., 2000). PERK specifically phosphorylates the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) causing global inhibition of protein synthesis but paradoxically enhances translation of the transcription factor ATF4 (Harding et al., 2000). ATF4 transactivates UPR genes with an ATF4 element in their promoters e.g., the CCAAT/enhancer-binding protein-homologous protein (CHOP). CHOP is a pro-apoptotic transcription factor owing to its ability to transactivate a number of apoptotic genes and downregulate the anti-apoptotic Bcl-2 (McCullough et al., 2001; Tabas and Ron, 2011). ATF4 and CHOP co-operate to transactivate downstream effectors e.g., ATF3, growth arrest and DNA damage-inducible protein 34 (GADD34) (Han et al., 2013). GADD34 promotes translational recovery by recruiting protein phosphatase 1 to dephosphorylate eIF2α, thus establishing a negative feedback loop (Connor et al., 2001; Novoa et al., 2001, 2003).

IRE1 is a kinase/endoribonuclease activated by self-oligomerisation and autophosphorylation (Sidrauski and Walter, 1997; Bertolotti et al., 2000; Shen et al., 2002a). The IRE1 pathway is an ancient pathway shared with yeast (Tirasophon et al., 1998; Hollien, 2013). In yeast, there is evidence to suggest that IRE1 is activated by direct binding of unfolded protein ligands to its luminal domain whereas BiP plays a regulatory role by maintaining IRE1 oligomeric equilibrium (Credle et al., 2005; Gardner and Walter, 2011; Gardner et al., 2013). The endoribonuclease activity of IRE1 mediates unconventional splicing of

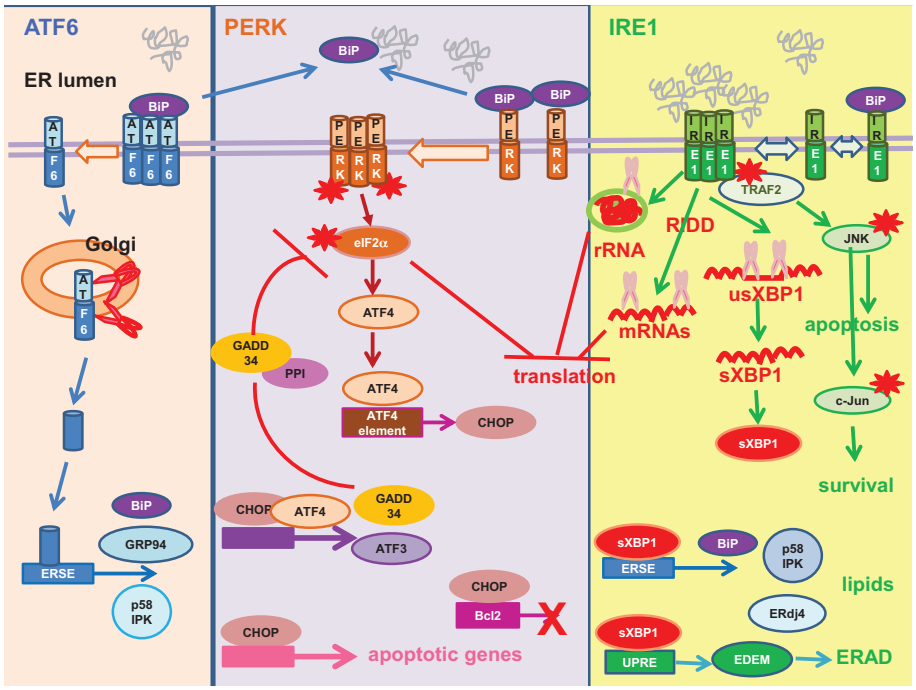

FIGURE 3 | Unfolded protein response. Mammalian unfolded protein response (UPR) is a tripartite response involving three proximal sensors: activating factor (ATF) 6, RNA-dependent protein kinase-like ER-resident kinase (PERK) and inositol-requiring enzyme 1 (IRE1). **Left:** ATF6 is sequestered in an inactive state by the molecular chaperone the immunoglobulin heavy-chain binding protein (BiP). Unfolded/malfolded proteins "distract" BiP from ATF6. ATF6 de-oligomerizes and migrates to the Golgi, where the monomer is cleaved by site-1 and site-2 proteases (red scissor) into an active transcription factor. The truncated N-terminal ATF6 is translocated to the nucleus where it transactivates UPR genes harboring an ERSE in their promoters e.g., BiP, glucose-regulated protein 94 (GRP94), P58^{IPK}. **Middle:** PERK is sequestered in an inactive state by BiP. Unfolded/malfolded proteins "distract" BiP from PERK, allowing its oligomerization and auto-phosphorylation (red asterisk). The activated PERK then phosphorylates its substrate, the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) (red asterisk) to inhibit global protein synthesis. Paradoxically, translation of ATF4 is upregulated to drive transcription of UPR genes with an ATF4 element in their promoters e.g., the CCAAT/enhancer-binding protein-homologous protein (CHOP). CHOP is a pro-apoptotic transcription factor, as it transactivates a number of apoptotic genes and downregulates the anti-apoptotic Bcl-2. ATF4 co-operates with CHOP to transactivate ATF3 and the growth arrest and DNA damage-inducible protein 34 (GADD34). GADD34 is the regulatory subunit of the protein phosphatase 1 (PP1). It recruits PP1 to dephosphorylate eIF2α (red blunt arrow), thus establishing a negative feedback loop. **Right:** Analogous to yeast, it is thought that IRE1 is activated by direct binding of unfolded/malfolded proteins to its luminal domain and BiP plays a regulatory role. IRE1 possesses endoribonuclease and kinase activity. The endoribonuclease activity mediates unconventional splicing of XBP1 (purple scissor) (usXBP1, unspliced XBP1 mRNA; sXBP1, spliced XBP1 mRNA). The sXBP1 mRNA is translated into an active transcription factor sXBP1 to transactivate genes with ERSE or UPRE in their promoters. XBP1 upregulation of UPR genes such as BiP and ERAD genes such as EDEM and ERdj4 provides a link between UPR and ERAD. XBP1 provides a link between the IRE1 and PERK pathways by upregulating P58^{IPK}, an inhibitor of PERK. XBP1 also orchestrates lipogenesis and ER expansion. The other endoribonuclease activity of IRE1 cleaves the ribosomal RNA (rRNA) (purple scissor) and mediates regulated IRE1-dependent decay (RIDD) to cleave a subset of mRNAs (purple scissor) to inhibit protein synthesis. The kinase activity of IRE1 plays a role in cell death/survival. Phosphorylated IRE1 (red asterisk) recruits the adaptor protein tumor necrosis factor receptor-associated factor 2 (TRAF2) to activate a cascade of phosphorylation culminating in pro-apoptotic Jun amino-terminal kinase (JNK) (red asterisk) and pro-survival c-Jun (red asterisk). Red asterisk, activation by phosphorylation.

the X-box binding protein 1 (XBP1) for its productive translation into an active, multi-functional transcription factor, the spliced XBP1 (sXBP1) (Calfon et al., 2002). sXBP1 transactivates ERSE in the promoters of UPR genes and the mammalian UPR element (UPRE) in the promoters of ERAD genes, thus providing a link between UPR and ERAD (Yoshida et al., 2003). Indeed, UPRE-mediated transcriptional induction of the ER degradation-enhancing α-mannosidase-like protein (EDEM) is directly involved in the recognition of malfolded proteins for degradation. Another protein ERdj4 transactivated by sXBP1 also participates in ERAD (Shen et al., 2002b; Lee et al., 2003; Lai et al., 2012). Similar to that of ATF6, XBP1 also links the IRE1 pathway to the PERK pathway by upregulating the inhibitor of PERK, P58^{IPK}, to aid in translational recovery (Yan et al., 2002; Lee et al., 2003; Van Huizen et al., 2003). XBP1 also assumes additional function in the regulation of lipogenesis and ER expansion (Lee et al., 2008; Glimcher and Lee, 2009; Brewer and Jackowski, 2012). The endoribonuclease activity of IRE1 also participates in translational repression by cleavage of the 28S ribosomal RNA and a subset of mRNAs via regulated IRE1-dependent decay (Iwawaki et al., 2001; Hollien et al., 2009). On the other hand, the kinase activity of IRE1 regulates cell death/survival (Urano et al., 2000; Tabas and Ron, 2011). Phosphorylated IRE1 associates with the adaptor protein tumor necrosis factor receptor-associated factor 2 to initiate a cascade of phosphorylation culminating in that of the pro-apoptotic Jun

amino-terminal kinase (JNK) and pro-survival c-Jun (Darling and Cook, 2014).

EVIDENCE OF UPR IN HEPATITIS C

There is as yet no consistent clinical data to support or refute the presence of ER stress in hepatitis C patients (Asselah et al., 2010; McPherson et al., 2011). Comparison between HCV-positive and -negative liver biopsy using real-time RT-PCR did not reveal any significant variation in the mRNA levels of GRP94, sXBP1 and EDEM (McPherson et al., 2011). Immunohistochemistry also did not detect any overall difference in the intensity of BiP between chronic hepatitis C and non-diseased livers, however, the staining was variable and one HCV sample showed a very high level of BiP. This may be explained by HCV being a focal infection, infecting only 7–20% of the liver (Liang et al., 2009; Stiffler et al., 2009). As a result, random sampling may not be able to detect a significant change in the mRNA/protein level in an area of mixed infected- and uninfected-hepatocytes. Indeed, using electron microscopy, dilated and disorganized ER indicative of ER stress was observed in hepatocytes from liver biopsy of mild chronic hepatitis C patients (Asselah et al., 2010). Evidence of ER stress in these liver samples was further confirmed using Western blotting which showed marked elevation in the levels of the proximal sensors ATF6α, ATF6β, sXBP1, and phosphorylated PERK and select subsets of downstream effectors BiP, phospho-eIF2α, ATF4, and EDEM. A study on a cohort of HCV HCC patients also demonstrated increased UPR markers of sXBP1, BiP, and ATF6 in liver biopsy by using immunohistochemistry and Western blotting (Shuda et al., 2003). It is therefore essential that concrete clinical evidence should await the use of more sensitive methods to detect, at single cells level, co-localization of ER stress markers in infected cells as compared to neighboring uninfected cells. Nevertheless, there is overwhelming evidence from *in vivo* and *in vitro* experiments to suggest that the ER stress response plays an important role in the life cycle of HCV (Liberman et al., 1999; Tardif et al., 2002, 2004; Benali-Furet et al., 2005; Chan and Egan, 2005, 2009; Ciccaglione et al., 2005, 2007; Zheng et al., 2005; Tumurbaatar et al., 2007; Sekine-Osajima et al., 2008; Joyce et al., 2009; Li et al., 2009; Mishima et al., 2010; Von Dem Bussche et al., 2010; Funaoka et al., 2011; Merquiol et al., 2011; Shinohara et al., 2013). Importantly, by using immunohistochemistry and confocal microscopy, increased level of the UPR marker, BiP, was found to co-localize with HCV-infected hepatocytes in SCID/Alb/uPA mice (chimeric mice with humanized livers) infected with genotype 1a H77 or intrahepatically inoculated with H77 RNA (Joyce et al., 2009). Infection of humanized mice with another genotype (2a) JFH1 strain also resulted in increased levels of BiP and CHOP in the livers (Mishima et al., 2010). Further *in vivo* evidence of ER stress was obtained in transgenic mice stably expressing the entire open reading frame, the core protein or inducibly expressing C-E1-E2-p7 in the livers (Benali-Furet et al., 2005; Tumurbaatar et al., 2007; Merquiol et al., 2011). Modulation of the UPR was widely observed in tissue-cultured hepatocytes infected with HCV; in cells harboring the HCV genomic replicon and SGR and in cells ectopically expressing individual viral proteins (Liberman et al., 1999; Tardif et al., 2002, 2004; Benali-Furet et al., 2005; Chan and Egan, 2005, 2009; Ciccaglione et al., 2005, 2007; Zheng et al., 2005; Sekine-Osajima et al., 2008; Li et al., 2009; Von Dem Bussche et al., 2010; Funaoka et al., 2011; Shinohara et al., 2013).

UPR SIGNALING IN HEPATITIS C

Some viruses can selectively activate or suppress one or more of the UPR tripartite pathways to facilitate their own replication (Isler et al., 2005; Smith et al., 2006; Yu et al., 2006; Jheng et al., 2010; Ambrose and Mackenzie, 2011; Pena and Harris, 2011; Burnett et al., 2012; Galindo et al., 2012; Qian et al., 2012; Rathore et al., 2013; Stahl et al., 2013). It is apparent that HCV infection activates all three proximal sensors (Ke and Chen, 2011; Merquiol et al., 2011). Infection of the hepatocyte sub-line Huh7.5.1 with JFH1 (2a) induced an acute ER stress peaking at 2–5 days post-infection (dpi), concomitant with phosphorylation of IRE1, eIF2α, and JNK, XBP1 splicing, ATF6 cleavage and upregulation of GADD34, ERdj4, $P58^{IPK}$, ATF3, ATF4, and CHOP (Merquiol et al., 2011). It then subsided into a chronic and milder ER stress response persisting up to 14 dpi, with elevated mRNA levels of CHOP, ATF3, sXBP1, and $P58^{IPK}$ and increased level of phospho-eIF2α. ER stress response is not restricted to the sub-line Huh7.5.1, as infection of the parental lines Huh7 or Huh7.5 with JFH1 (2a) also induced ER stress (Ke and Chen, 2011). Infection of Huh7 with JFH1 provoked an acute ER stress response concomitant with ATF6 cleavage, XBP1 splicing and PERK phosphorylation at 6–9 dpi followed by a chronic and milder ER stress with a diminished CHOP level at 15–22 dpi. Similarly, infection of Huh7.5 with JFH1 (2a) has been shown to transactivate the Bip, CHOP, and ATF6 promoters (Von Dem Bussche et al., 2010).

Currently *in vitro* infection study with wild type genotype is only achievable with the strain JFH1 and yet JFH1 was isolated from a patient with fulminant hepatitis-a rare manifestation of HCV diseases (Wakita et al., 2005; Lohmann and Bartenschlager, 2014). It is therefore important that studies should be extended to other genotypes before it can be generalized that ER stress is a common phenomenon of chronic hepatitis C. Chimeric HCV has been created by fusing the structural proteins from all seven genotypes with the NS proteins of JFH1, which should at least allow us to study the role of genotypic structural proteins in UPR (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008; Li et al., 2011). An intragenotypic chimera J6/JFH1 has already been shown to be capable of eliciting the UPR, as evident by the increased levels of CHOP and sXBP1 at 1–3 dpi (Mohl et al., 2012). Some success has been achieved to establish cell-cultured infectious systems for genotypes 1a (H77 and TN), 1b (NC1), 2a (J6), and 2b (J8, DH8, DH10) but they require a number of adaptive mutations (Yi et al., 2006; Date et al., 2012; Li et al., 2012a,b; Ramirez et al., 2014). At the moment, studies with wild type genotypes other than JFH1 still rely on the use of genomic replicons (Benali-Furet et al., 2005; Shinohara et al., 2013). Similar to that in JFH1-infected Huh7, all three pathways have been activated in Huh7 cells harboring a genomic replicon of genotype 1b O strain, as indicated by the phosphorylation of eIF2α, XBP1 splicing and increased levels of IRE1, phospho-JNK, and phospho-c-Jun (Shinohara et al., 2013).

WHICH VIRAL PROTEINS MEDIATE UPR?

A number of steps in the virus life cycle are potential trigger of ER stress e.g., the maturation of the viral envelope glycoproteins in the ER, formation of replication complex on the ER, virus assembly, and budding of virus particles into the ER (Scheel and Rice, 2013).

ENVELOPE PROTEINS

SGR (devoid of C-E1-E2-p7-NS2) is generally less capable (or incapable) of triggering the UPR than its full-length counterpart, suggesting that the main contributors to ER stress lie within the structural-NS2 region (Von Dem Bussche et al., 2010; Mohl et al., 2012). A genomic replicon devoid of the envelope proteins E1 and E2 failed to elicit UPR in transfected Huh7 cells, implicating a pivotal role of the envelope proteins in the elicitation of ER stress (Mohl et al., 2012). This is consistent with the ER residence of the envelope proteins. Using transient transfection of envelope proteins to physiological levels, we have confirmed that the HCV envelope proteins are capable of inducing the UPR in hepatocytes HepG2 and Huh7 as well as non-hepatocyte HeLa (Chan and Egan, 2005, 2009).

CORE

The core protein, which does not enter the ER lumen but is important in lipid droplet formation and virus assembly and budding, also elicits the UPR (McLauchlan et al., 2002; Benali-Furet et al., 2005; Funaoka et al., 2011; Scheel and Rice, 2013). Evidence of ER stress has been documented in tissue-cultured cells transfected with the HCV-core and in the livers of HCV-core transgenic mice (Benali-Furet et al., 2005). The significant role of the core protein can be illustrated by the considerable effects of mutating the core residues R70Q, R70H, L91M on the UPR in Huh7 cells infected or transfected with JFH1 (Funaoka et al., 2011).

NS2

NS2 does not enter the ER lumen despite being a transmembrane protein (Bartenschlager et al., 2013). Apart from harboring a protease, NS2 is important in organizing the virus assembly complex (Lindenbach and Rice, 2013). One study implicated a major role of NS2 in provoking the UPR based on the detection of increased BiP in Huh-7 cells transfected with core-E1-E2-p7-NS2 compared with that transfected with core-E1-E2-p7, however, expression of NS2 was barely detectable (Von Dem Bussche et al., 2010). Whereas the increases of BiP at the promoter and mRNA levels were very modest, it is not clear why the more pronounced increase of BiP protein level necessitated detection by immunoprecipitation-Western blotting rather than the more straightforward Western blotting. Ectopic expression of NS2 from genotype 1a in Huh7 cells resulted in eIF2α phosphorylation and modest increases of BiP, CHOP, and ATF6 at the promoter and mRNA levels together with a more pronounced increase in BiP protein level (again, detected by immunoprecipitation-Western blotting). In contrast, another hepatocyte cell line Hep3B stably expressing NS2 from genotype 1b (k isolate) failed to induce ATF6 cleavage (Li et al., 2009). Therefore, whether NS2 is the main contributor of ER stress, as claimed, still needs robust testing (Von Dem Bussche et al., 2010).

NS4B

Huh7 cells harboring SGR were capable of inducing ATF6 cleavage and XBP1 splicing but suppressing the downstream activation of UPRE and EDEM by sXBP1 (Tardif et al., 2002, 2004). This suppressive effect could be attributed to NS4B as ectopic expression of NS4B in Huh7 cells displayed a similar pattern of ATF6 cleavage and XBP1 splicing without downstream activation of EDEM (Zheng et al., 2005; Li et al., 2009). NS4B is important in membranous web/replication complex formation (Bartenschlager et al., 2013). Similar to NS2, it also does not enter the ER lumen despite being a transmembrane protein. The role of NS4B in UPR could be modulating. Indeed, ERAD activation, as demonstrated by XBP1 splicing and upregulation of EDEMs mRNAs, clearly exists during infection of Huh7.5.1 with JFH1 (Saeed et al., 2011). EDEM interaction with E1 and E2 resulted in ubiquitination of E2 and decrease in virus particle production. This is not ideal for the virus. Subsequently, NS4B may act to modulate the UPR by suppressing the ERAD to help damping down the inhibitory effect of EDEM in order to regulate and fine-tune virus particle production.

NS5A/5B

NS5A/5B are integral to viral replication (Scheel and Rice, 2013). Infection of humanized mice with NS5A/5B mutants of JFH1 led to increased expression of BiP and CHOP, suggesting a role of the NS5 proteins in ER stress although it is not clear whether they act directly or indirectly (Mishima et al., 2010). It is possible that the enhancing effect of the NS5 mutants on the UPR may be an indirect result of a higher replication rate of these mutants leading to increased production of the responsible proteins i.e., core, E1, E2. Whether the NS5 proteins directly induce the UPR still needs to be shown but Hep3B cells stably expressing NS5B genotype 1b (k isolate) failed to induce ATF6 cleavage (Li et al., 2009).

HOW DO ENVELOPE PROTEINS ELICIT UPR?

Enveloped viruses either bud through the plasma membrane or an intracellular compartment e.g., ER (**Figure 4**) (Garoff et al., 2004; Stertz et al., 2007; Murakami, 2012; Prange, 2012; Vieyres et al., 2014). In either case, the envelope proteins will be first targeted to the ER for post-translational modification and maturation. Many viral envelope proteins are significant inducers of UPR, whether they are ER-resident proteins (for viruses budding into the ER) or are just trafficking through the ER *en route* to the plasma membrane (for viruses budding through the plasma membrane) (Dimcheff et al., 2003, 2004; Wang et al., 2003, 2006; Hsieh et al., 2004; Liu et al., 2004, 2006; Nanua and Yoshimura, 2004; Qiang et al., 2004; Chua et al., 2005; Chan et al., 2006; Yoshimura and Luo, 2007; Yoshimura et al., 2008; Zhao and Yoshimura, 2008; Favreau et al., 2009; Portis et al., 2009; Barry et al., 2010; Dediego et al., 2011; Hung et al., 2011).

MUTANT ENVELOPE PROTEINS AS UPR INDUCERS

Several mutated viral envelope proteins are significant UPR inducers and major determinants of virulence, in analogous to

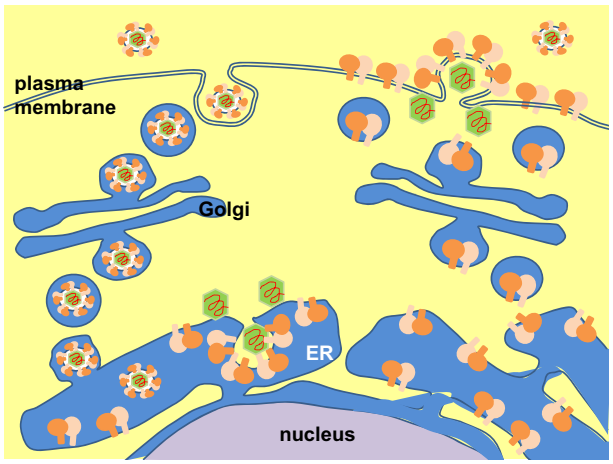

FIGURE 4 | Two modes of virus budding. Left: Virus budding into the endoplasmic reticulum (ER). Virion assembles and buds into the ER, where it is coated by the ER-resident envelope proteins. Egress of virus particle follows the host secretory pathway and released into the extracellular space. **Right:** Virus budding from the plasma membrane. Envelope proteins are targeted to the ER and transported to the cell surface via the host secretory pathway. Virion assembles and buds through the plasma membrane.

many human diseases which are caused by retention of mutated cellular proteins in the ER e.g., the genetic variant null Hong Kong of α1-antitrypsin and the ΔF508 cystic fibrosis transmembrane conductance regulator (Oda et al., 2003; Gnann et al., 2004). Retrovirus buds through the plasma membrane (Murakami, 2012). Virulent strains of retrovirus harbor mutations in the envelope proteins resulting in retention of inefficiently folded envelope proteins in the ER, leading to elicitation of the UPR which is a major determinant of neurovirulence (Dimcheff et al., 2003, 2004; Liu et al., 2004, 2006; Nanua and Yoshimura, 2004; Qiang et al., 2004; Yoshimura and Luo, 2007; Yoshimura et al., 2008; Zhao and Yoshimura, 2008; Portis et al., 2009). Hepatitis B virus buds into the ER-Golgi intermediate or other intracellular compartments, mutations in the large surface protein resulted in ER retention, provoking ER stress which is associated with hepatocarcinogenesis (Wang et al., 2003, 2006; Hsieh et al., 2004; Chua et al., 2005; Hung et al., 2011; Prange, 2012). Coronavirus also buds into the ER-Golgi intermediate compartment (Garoff et al., 1998; Stertz et al., 2007). Its spike protein is an UPR inducer (Chan et al., 2006; Versteeg et al., 2007; Siu et al., 2014). Mutations in the spike protein have been associated with persistence and translational attenuation and these mutations have also been found to enhance UPR, cytotoxicity and cell death and confer neurovirulence (Favreau et al., 2009).

IMMATURE VIRION AS UPR INDUCER

For viruses that bud into the ER it is not clear how the ER-residing envelope proteins will induce UPR. Many of these envelope proteins will undergo further processing and re-organization/conformational changes after incorporation into the immature virion and trafficking through the secretory pathway. In flavivirus, 60 trimeric prM/E assemble as immature virion in the ER (Pierson and Diamond, 2012). During transit through the Golgi E undergoes dramatic re-organization and collapses onto the virion surface whereas a cleavage site on prM is exposed for furin proteolysis. Flaviviruses are prolific inducers of UPR (Jordan et al., 2002; Su et al., 2002; Yu et al., 2006; Medigeshi et al., 2007; Umareddy et al., 2007; Ambrose and Mackenzie, 2011; Klomporn et al., 2011; Paradkar et al., 2011; Pena and Harris, 2011; Wu et al., 2011; Ambrose and Mackenzie, 2013; Blazquez et al., 2013; Yu et al., 2013; Bhattacharyya et al., 2014). UPR has been documented in infections of Dengue virus, West Nile virus, Japanese encephalitis virus, tick-borne encephalitis virus and Usutu virus. The HCV envelope proteins are synthesized as part of a single polypeptide (Grakoui et al., 1993b). After importing into the ER by signal peptides at their respective N-termini, they are cleaved into E1 and E2 by cellular signal peptidase (Hijikata et al., 1991a; Lin et al., 1994). Inside the ER, E1 and E2 form two types of complexes: non-covalently-bonded E1-E2 heterodimer and disulphide-bonded aggregates, neither is the mature form (Dubuisson et al., 1994; Dubuisson and Rice, 1996; Deleersnyder et al., 1997). It is not clear which of these forms of E1E2 is acquired by the virion when the virus buds into the ER as E1E2 undergo further conformational changes into aggregated oligomers when the virus particles transit through the secretory pathway (Vieyres et al., 2010, 2014). There has been evidence to suggest that HCV envelope proteins are major UPR inducers (Mohl et al., 2012). Alphavirus does not bud through the ER and yet its maturation resembles that of flavivirus in that the immature prE2/E1 trimer assembled in the ER undergoes furin cleavage in the Golgi into E3(=pr)/E2/E1 trimer (Garoff et al., 2004; Vaney et al., 2013). UPR has been documented in infections of Chikungunya virus and Sindbis virus (Joubert et al., 2012; Abraham et al., 2013; Rathore et al., 2013). The envelope proteins of Semliki Forest virus have been shown to be responsible for the induction of UPR (Barry et al., 2010). Therefore, we speculate that the immature ER form may be in itself a trigger of the UPR, irrespective of whether the virions bud through the ER or other sites.

HOW DO HCV ENVELOPE PROTEINS ACTIVATE UPR?

E1 and E2 accumulate in the ER, placing them in proximity to interact with BiP (Choukhi et al., 1998). Folding of E1 and E2 into the non-covalently-bonded heterodimer utilizes the canonical chaperone calnexin and calreticulin whereas the E2 aggregates are bound by BiP (**Figure 5**) (Dubuisson and Rice, 1996; Choukhi et al., 1998). This may explain why E2 was able to elicit the UPR (Liberman et al., 1999). However, Bip binds to the E1 aggregates inefficiently or not at all, leading to the possibility that E1 may induce UPR by other means (Choukhi et al., 1998; Liberman et al., 1999; Merola et al., 2001).

One plausible mechanism is that E1 (or even E2) induces UPR by impairing ERAD as ERAD and UPR exist in a regulatory loop (Travers et al., 2000). It is well known that cytosolic proteins such as the polyQ aggregates induce UPR by perturbation of proteasomal degradative function (Friedlander et al., 2000; Travers et al., 2000; Nishitoh et al., 2002). We have shown ERAD engagement in cells transfected with E1 and/or E2 by the demonstration of XBP1 splicing and UPRE induction in these cells (Chan and

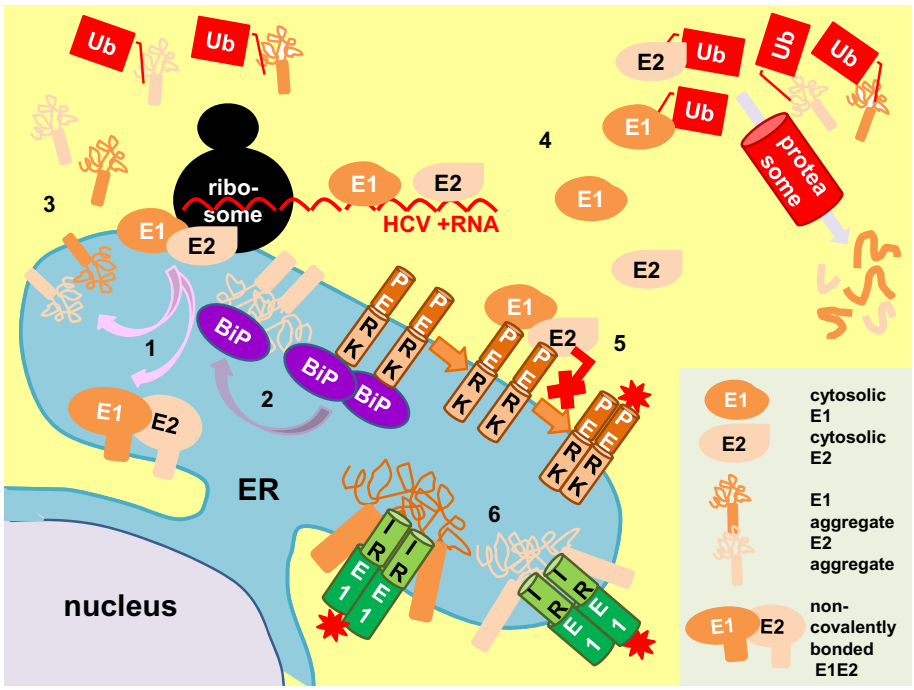

FIGURE 5 | Proposed mechanisms of E1/E2 activation of UPR. E1 and E2 are targeted and mature in the endoplasmic reticulum (ER) to form non-covalently-bonded heterodimers and disulphide-bonded aggregates (pink arrows, 1). The E2 aggregates distract BiP from PERK (purple arrow), allowing PERK oligomerization and activation (brown arrows, 2). Retrotranslocated (3) and cytosolic E1/E2 from surplus protein synthesis (4) can also activate the unfolded protein response (UPR) by perturbation of proteasomal function (Ub, polyubiquitin). Conversely, cytosolic E1/E2 can bind to the cytoplasmic domain of PERK and inhibit its activation (red cross, 5). Direct binding of E1 and E2 aggregates to the luminal domain of IRE1 can also activate UPR (6). Red asterisk, activation by phosphorylation.

Egan, 2005). Although E1/E2 mature in the ER, it is possible that some of them have been directed to the cytoplasm during synthesis or as a result of retro-translocation from the ER. There is *in vivo* evidence of retrograde transport of E1 from the ER to the cytoplasm for proteasome degradation based on the detection of a deglycosylated-deamidated T-epitope from an HCV-infected chimpanzee (Selby et al., 1999). Cytosolic existence of E2 has been demonstrated in vector-expression system although it still yet has to show the cytosolic existence of E2 in infected cells (Pavio et al., 2002). By removing the signal peptides from E1 and E2 we re-directed expression of these proteins to the cytoplasm (Egan et al., 2013). These cytosolic-targeting E1/E2 did not induce UPR. Instead, they repressed tunicamycin-induced UPR possibly as a result of binding to the cytoplasmic domain of PERK and blocking its activation, suggesting that UPR induction by ERAD perturbation is unlikely (Pavio et al., 2003; Egan et al., 2013).

Another possibility is that E1 (or even E2) can trigger UPR by direct binding to one or more of the UPR sensors in the ER lumen. This is especially true when UPR triggered by many virus infections is often skewed suggesting a canonical tripartite-responsive BiP derepression mechanism may not be sufficient to explain these skewed UPR in cases of virus infections. Studies with yeast have shown that UPR can be triggered by direct binding of unfolded proteins to the luminal domain of IRE1 (Credle et al., 2005; Gardner and Walter, 2011; Gardner et al., 2013). The luminal domain of PERK bears secondary structure homology with that of IRE1, by extrapolation, direct binding of unfolded proteins to PERK can also be feasible (Gardner et al., 2013). Direct binding between the herpes simplex virus glycoprotein B and the luminal domain of PERK has been documented but in this case, binding results in repression rather than elicitation of the UPR (Mulvey et al., 2007). Toxic lipids are directly sensed by the transmembrane domains of IRE1 and PERK to provoke the UPR, further supporting the idea that mechanisms other than BiP derepression is possible (Volmer et al., 2013).

HOW DO CORE AND NS PROTEINS ELICIT UPR?

During polyprotein processing, the signal peptide at the C terminus of the core protein directs the translocation of E1 into the ER, after that the signal peptidase will cleave at the C-terminal end of the core protein at amino acid (aa) residue 191 (Santolini et al., 1994). This intermediate core protein is anchored onto the cytosolic side of the ER membrane by a membrane anchor. Maturation of the core protein involves another intramembrane cleavage event at aa173–182 by signal peptide peptidase in the ER membrane (Okamoto et al., 2008a; Pene et al., 2009). The exact C terminus has not been determined but a minimum of 177 aa residues seems to be required for productive virus production (Kopp et al., 2010). The mature core protein is then released from the ER to traffic to lipid droplets to orchestrate virus assembly (McLauchlan et al., 2002). It appears that the core protein is never directed inside the ER to be able to interact with BiP to trigger

the canonical UPR signaling. This is also true for the NS proteins which do not appear to enter the ER lumen even though NS2 and NS4B are transmembrane proteins (Romero-Brey et al., 2012). The question remains how then can their cytosolic presence elicit the UPR?

PROTEASOMAL PERTURBATION

Perturbation of proteasomal activity is one possibility (Friedlander et al., 2000; Travers et al., 2000; Nishitoh et al., 2002). The core, NS2 and NS5B proteins have been shown to interact with the proteasomal pathways (**Figure 6**) (Gao et al., 2003; Moriishi et al., 2003; Franck et al., 2005; Shirakura et al., 2007; Suzuki et al., 2009). Moreover, interaction of the core protein with the proteasome activator PA28γ is responsible for the pathogenesis of steatosis, HCC and other liver pathology in core-transgenic mice and virus propagation in JFH1-infected Huh7 cells (Moriishi et al., 2007, 2010; Tripathi et al., 2012).

PERTURBATION OF MEMBRANOUS WEB PROTEIN CHAPERONE ACTIVITY

The cytosolic chaperone heat shock protein 90 (HSP90) promotes HCV replication by facilitating host and viral protein folding in the replication complex of the membranous web (**Figure 7**) (Taguwa et al., 2009). Inhibition of HSP90 activity reduces protein folding, accelerates proteasome degradation and induces the UPR. HSP90 is recruited into the replication complex by means of interaction between its co-chaperones FK506-binding protein 8 (FKBP8) and the human butyrate-induced transcript 1 (hB-ind1) and NS5A (Okamoto et al., 2006; Taguwa et al., 2008). It is therefore possible that any changes in HSP90-FKBP8/hB-ind1-NS5A interaction can disrupt the chaperone activity of HSP90 leading to UPR. Indeed, interaction of NS5A with FKBP8 has already been implicated in pathogenesis via activation of mammalian target of rapamycin anti-apoptotic function (Peng et al., 2010). In contrast, a JFH1 NS5A/5B multiple mutants exhibited a higher replication rate and yet provoking a stronger UPR in humanized mice (Mishima et al., 2010). However, the sample size is small (only one mouse from each of the test and control groups was dissected for the UPR markers). Moreover, the mutations are not likely to be involved in co-chaperone binding, suggesting another mechanism of UPR regulation. Previously, it has been found that mutation of a single amino acid V/I121A in NS5A is sufficient to abolish its interaction with FKBP8 impairing virus replication (Okamoto et al., 2008b). It would be interesting to see whether this mutation will provoke a stronger UPR.

DIRECT BINDING TO CYTOSOLIC DOMAINS OF UPR SENSORS

Another possibility for cytosolic proteins to trigger UPR is by direct binding to the cytoplasmic domain of the UPR sensors. Currently there is no evidence for interaction of the core protein with any of the cytosolic domains of the UPR sensors. NS4B, however, interacts with the bZIP motif of ATF6β via a predicted

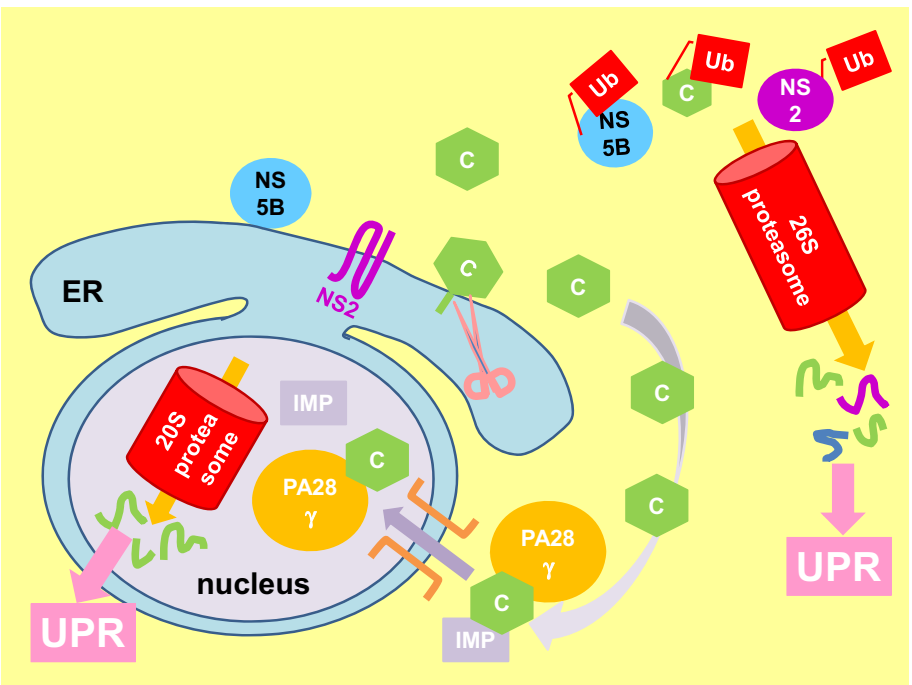

FIGURE 6 | Viral proteins perturb proteasomal function to elicit UPR. Immature core protein (C) attaches to the cytosolic side of the endoplasmic reticulum (ER) membrane by a membrane anchor. Cleavage of the membrane anchor (pink scissor) releases mature core to the cytoplasm where it can be polyubiquitinated (Ub) and degraded by the cytosolic 26S proteasome. The mature core protein can also be imported into the nucleus by association with the proteasome activator PA28γ and importin (IMP) (purple arrow), where it is degraded by the 20S proteasome independent of ubiquitin. Also shown is the polyubiquitination and degradation of non-structural protein 2 (NS2) and NS5B by the 26S proteasome. Perturbation of proteasomal function elicits the unfolded protein response (UPR).

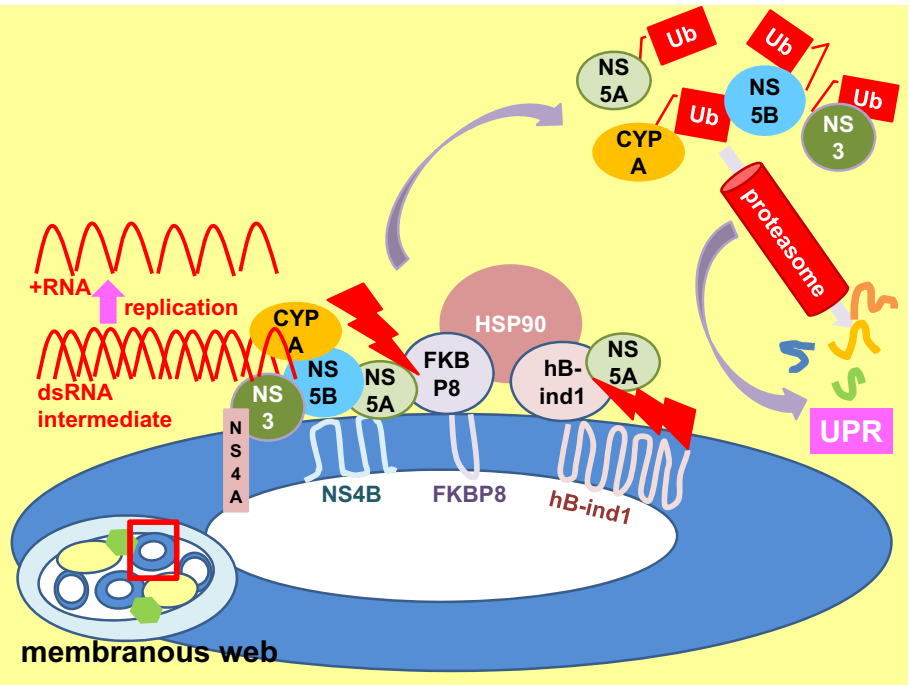

FIGURE 7 | Perturbation of replicase chaperone activity results in UPR. An enlarged view of a membrane vesicle (enclosed by a red square) within the membranous web. Heat shock protein 90 (HSP90) chaperones folding of viral non-structural (NS) 3-NS5B proteins and host protein cyclophilin A (CYPA) etc. in the replication complex to facilitate viral replication. HSP90 forms a complex with NS5A via interactions with its co-chaperone FK506-binding protein 8 (FKBP8) and the human butyrate-induced transcript 1 (hB-ind1). Disruption of co-chaperone-NS5A interaction (red serrated arrows) interrupts HSP90 chaperone activity, resulting in increased degradation of polyubiquitinated (Ub) proteins and perturbation of proteasomal function, leading to the unfolded protein response (UPR) (purple arrows).

bZIP motif in its N-terminal cytoplasmic domain although there is as yet no functional analysis of whether this interaction leads to induction or suppression of ATF6β activity (**Figure 8**) (Tong et al., 2002; Welsch et al., 2007). On the other hand, NS4B also interacts with ATF6α, despite to a lesser extent, and it is plausible that this interaction signals ATF6α cleavage as observed in hepatocytes and non-hepatocytes expressing NS4B (Tardif et al., 2002; Tong et al., 2002; Zheng et al., 2005; Li et al., 2009). However, expression of NS4B alone also induced XBP1 splicing, suggesting that at least one other mechanism is operating to induce the UPR (Li et al., 2009).

LIPID PERTURBATION

UPR regulates lipogenesis and ER membrane expansion (Lee et al., 2008; Glimcher and Lee, 2009; Brewer and Jackowski, 2012). On the contrary, lipid perturbation is one of the triggers of UPR (Volmer et al., 2013). HCV virus particle formation is intimately coupled to the host lipogenesis (**Figure 9**). The core protein targets to the lipid droplets which is the initial site of virus assembly (McLauchlan et al., 2002; Lindenbach and Rice, 2013). Virus infection also induces massive intracellular membrane re-organization to form the membranous web as the site of virus replication (Behrens et al., 1996; Romero-Brey et al., 2012; Bartenschlager et al., 2013; Paul et al., 2013). Budding of virion into the ER and egress through the secretory is tightly linked to

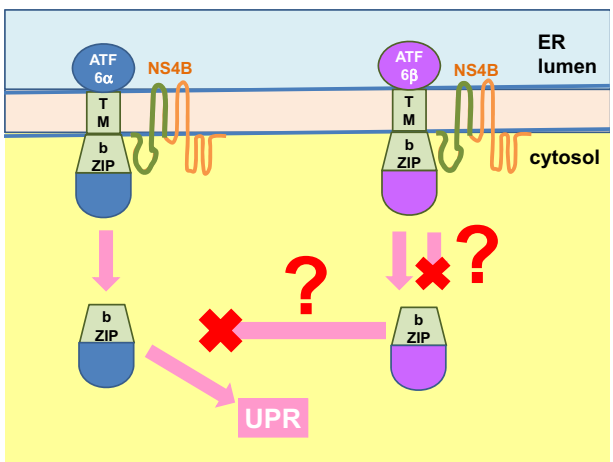

FIGURE 8 | NS4B interacts with ATF6 to modulate UPR. The hepatitis C virus (HCV) non-structural (NS) 4B protein binds to the b-ZIP and transmembrane (TM) domains of the activating factor 6 (ATF6) α and β (binding domains shown in green for both NS4B and ATF6). Binding of ATF6α likely triggers its Golgi translocation and cleavage into an active transcription factor to mediate the unfolded protein response (UPR). The fate of NS4B binding to ATF6β is unknown. It can either trigger or inhibit ATF6β Golgi translocation and activation. The end result could be modulation of the UPR but whether ATF6β is an inhibitor of ATF6α is controversial. ER, endoplasmic reticulum.

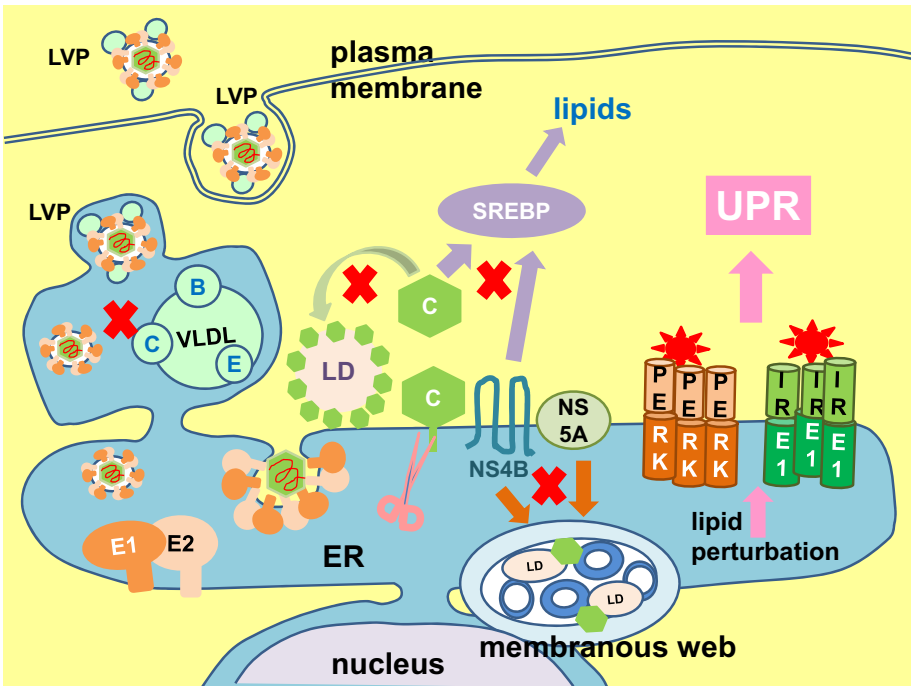

FIGURE 9 | Lipid perturbation elicits unfolded protein response. The hepatitis C virus (HCV) core protein (C) plays a part in lipid droplet (LD) formation (green arrow), and also coats the lipid droplet to initiate virion assembly and budding into the envelope proteins E1- and E2-coated endoplasmic reticulum (ER). Maturation and egress of the virion is tightly coupled to host very low density lipoprotein (VLDL) synthesis (A, apolipoprotein A; C, apolipoprotein C; E, apolipoprotein E), resulting in the formation of a lipoviroparticle (LVP). The non-structural (NS) 4B and NS5A proteins induce formation of the membranous web (brown arrow). The core and NS4B proteins have been shown to transactivate the sterol regulatory element binding protein (SREBP), the master regulator of lipogenesis (purple arrow). Perturbation in any of these lipid synthesis pathways will easily signal to elicit the unfolded protein response (UPR) (pink arrow). Putative lipid metabolic steps vulnerable to perturbation are marked with red crosses.

lipoprotein synthesis (Lindenbach and Rice, 2013). Finally, the virus particle associates with lipoproteins to form lipoviroparticle which is essential for virus infectivity (Andre et al., 2002; Felmlee et al., 2013). Therefore, throughout the life cycle of the virus, there is a constant need for lipids and lipoproteins. Transactivation of the sterol regulatory element binding proteins, the master regulator of lipogenesis, has been observed in Huh7 cells infected with JFH1 (2a) or harboring a SGR and in cells ectopically expressing the core protein or NS4B (Waris et al., 2007; Rahman et al., 2009). It is not difficult to imagine that this can easily lead to perturbation of lipid homeostasis and trigger the UPR. Indeed, hepatitis C patients exhibit many lipid and lipoprotein metabolism disorders such as hepatic steatosis (fatty liver), hypobetalipoproteinaemia, and hypocholesterolemia (Serfaty et al., 2001; Colloredo et al., 2004; Felmlee et al., 2013). Experimentally, both exogenous and endogenous sources of fatty acids were capable of inducing ER stress in Huh7 cells infected with JFH1 or harboring a SGR (Rahman et al., 2009; Gunduz et al., 2012).

CONCLUDING REMARKS

Despite overwhelming evidence from *in vivo* (transgenic and humanized mice) and *in vitro* studies to indicate that HCV infection causes ER stress and induces the UPR, we still need to confirm the presence of ER stress in hepatitis C patients by conducting clinical studies at single cells level (Liberman et al., 1999; Tardif et al., 2002, 2004; Benali-Furet et al., 2005; Chan and Egan, 2005, 2009; Ciccaglione et al., 2005, 2007; Zheng et al., 2005; Tumurbaatar et al., 2007; Sekine-Osajima et al., 2008; Joyce et al., 2009; Li et al., 2009; Mishima et al., 2010; Von Dem Bussche et al., 2010; Funaoka et al., 2011; Merquiol et al., 2011; Shinohara et al., 2013). Clinical data will need to be further corroborated and elaborated using well-controlled experiments. Genotype is a major determinant of IFN responsiveness and disease progression, therefore, it is important that studies should be extended to other genotypes before it can be generalized that ER stress is a common phenomenon of chronic hepatitis C (Chayama and Hayes, 2011; Ripoli and Pazienza, 2011).

A number of steps in the virus life cycle are potential trigger of ER stress e.g., the maturation of the viral envelope glycoproteins in the ER, formation of replication complex on the ER and virus assembly and budding of virus particles into the ER (Scheel and Rice, 2013). Current evidence suggests a major role of the structural proteins, with the NS proteins playing a modulating role (Tardif et al., 2002, 2004; Mishima et al., 2010; Von Dem Bussche et al., 2010; Funaoka et al., 2011; Mohl et al., 2012). More work still needs to be done to decipher the mechanisms of UPR induction and the answer will lead to a better understanding of virus-host interaction and may uncover novel mechanisms of UPR sensing in general.

AUTHOR CONTRIBUTIONS

Shiu-Wan Chan conceived, designed, and wrote the review article.

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Modulation of the unfolded protein response by the human hepatitis B virus

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During productive viral infection the host cell is confronted with synthesis of a vast amount of viral proteins which must be folded, quality controlled, assembled and secreted, perturbing the normal function of the endoplasmic reticulum (ER). To counteract the ER stress, cells activate specific signaling pathways, designated as the unfolded proteins response (UPR), which essentially increase their folding capacity, arrest protein translation, and degrade the excess of misfolded proteins. This cellular defense mechanism may, in turn, affect significantly the virus life-cycle. This review highlights the current understanding of the mechanisms of the ER stress activation by Human Hepatitis B virus (HBV), a deadly pathogen affecting more than 350 million people worldwide. Further discussion addresses the latest discoveries regarding the adaptive strategies developed by HBV to manipulate the UPR for its own benefits, the controversies in the field and future perspectives.

Keywords: hepatic viruses, ER stress, degradation, autophagy

INTRODUCTION

An important amount of experimental data accumulated in the last decade have not only demonstrated that viruses can induce endoplasmic reticulum (ER) stress in mammalian cells, but importantly, that the cellular response to this stress may play a crucial part in the evolution of the disease (Netherton et al., 2004; Tardif et al., 2005). This is not surprising, since an infected cell must manage a vast quantity of viral proteins that are synthesized over a short period of time during productive infection, often leading to perturbation of the ER homeostasis, protein misfolding and aggregation.

Discovered more than 40 years ago, with an efficient vaccine developed against it, the Human Hepatitis B virus (HBV) infection is still a frequent viral disease and a major cause of chronic liver pathogenesis. About 350 million people are currently HBV carriers worldwide and at high risk to develop hepatocellular carcinoma (HCC), the third leading cause of cancer death in humans. Despite decades of intensive research and comprehensive investigations at large scale, such as by using proteomics and transcriptomics technologies, the HBV interactions with the host cells and the molecular mechanisms underlying the viral pathogenesis and progression are not clearly understood. The lack of an efficient and robust *in vitro* infectivity model has been a major drawback, preventing in depth studies in a natural infection system (Gripon et al., 2002). However, HBV is one of the few viruses demonstrated to induce ER stress, with strong support by *in vivo* data.

HBV is the representative of the *Hepadnaviridae* family of DNA viruses. Its genome consists of a partially double-stranded DNA molecule of 3.2 kb encoding the envelope (surface) proteins, a core protein, the reverse transcriptase and the regulatory X protein (HBx), within four overlapping open reading frames (ORF) (Schadler and Hildt, 2009). The surface proteins, namely the large (L), middle (M), and small (S) are synthesized from three different initiation codons within the same ORF and share the tetra-spanning transmembrane S domain. In addition to S, the M and L proteins contain the preS2 and the preS1-preS2 regions, respectively at their N-terminal end (**Figure 1A**). The surface proteins are co-translationally inserted into the ER membrane, fold and oligomerize by extensive disulfide bonding, before being transported to the budding sites for assembly into virions and non-infectious subviral particles (SVP) (Chai et al., 2008). The preS1 domain adopts a dual topology at the ER membrane enabling the interaction of the L protein with both, the core protein in the cytoplasm and the viral receptors at the plasma membrane (Lambert and Prange, 2001). Due to this intriguing feature, the L protein is indispensable to HBV assembly and entry into hepatocytes, but not to SVP formation. Interestingly, when expressed alone, the L protein assembles into particles that are retained within the ER lumen. Secretion of the L protein is rescued when either M and/or S proteins are co-expressed and incorporated into these particles (Bruss and Ganem, 1991). Similarly, excessive production of L, over M and S, results in retention of all three proteins and oligomers as well as virions, demonstrating that the ratio between the surface proteins plays a crucial role in the HBV life-cycle (Chisari et al., 1986). Therefore, it is not surprising that expression of the surface proteins is tightly regulated

Abbreviations: Akt, protein kinase B; Atg, autophagy-related; BiP, immunoglobulin heavy chain binding protein; GGH, ground glass hepatocytes; GRP78, glucose-regulated protein of 78 kDa; GRP94, glucose-regulated protein of 94 kDa; LC3, microtubule-associated light chain 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor kB; PI3KC3, class III phosphatidylinositol 3-kinase; V-ATPase, vacuolar ATPase; VEGF-A, vascular endothelial growth factor A.

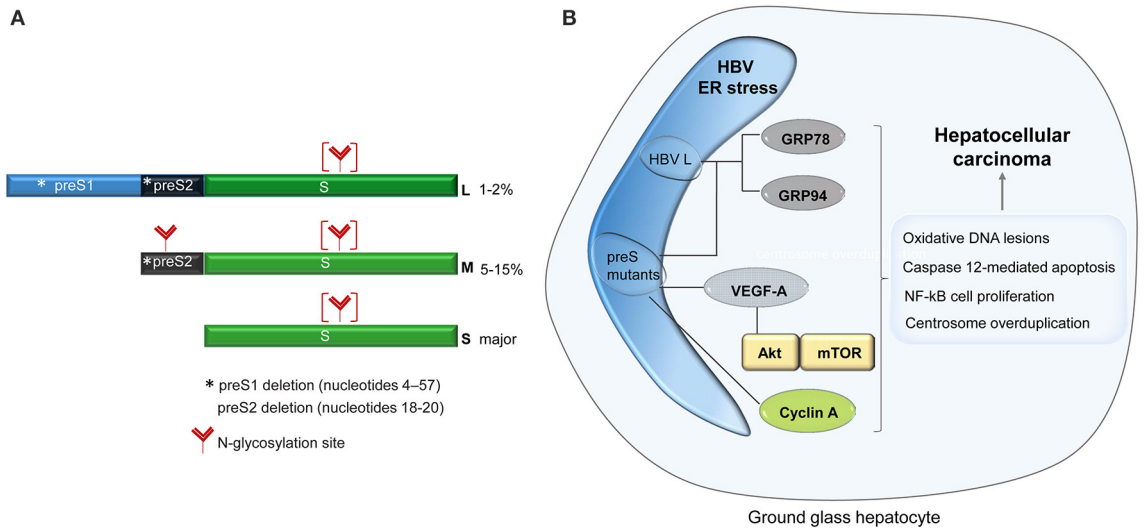

FIGURE 1 | Schematic representation of the HBV surface proteins. The asterisk shows the location of the major mutations identified in patients with ground glass hepatocytes **(A)**. Signaling pathways activated in ground glass hepatocytes, with potential role in carcinogenesis **(B)**.

by different promoters, namely, the preS1 promoter, which controls the L transcript and the S promoter regulating transcription of the M and S mRNAs. This control results in differential expression of the surface proteins, S being the most abundant of them, while M and L are expressed at much lower levels of about 5–15% and 1–2%, respectively (Yokosuka and Arai, 2006).

HBV INDUCES ER STRESS

The first indication of the ER stress and the intracellular morphological modification induced by HBV infection came more than 40 years ago, from studies of cirrhotic and carcinomas liver biopsies, showing a strong hypertrophy of the ER within hepatocytes. Initially associated with a disregulated glycogen metabolism, the presence of the altered subcellular structures was later clearly related to the expression of HBV surface proteins (Hadziyannis et al., 1973). Due to their microscopic appearance, these cells were termed "ground glass hepatocyte" (GGH) and their detection became an important parameter in the diagnostic of HBV-related pathogenesis (Pópper, 1975). The phenotype was classified in type I, characterized by a random distribution of the GGH in the hepatic tissue, and type II, where larger regions of clustered GGH are observed, occurring usually at later stages of infection (Fan et al., 2000, 2001). A potential mechanism of this unusual intracellular accumulation of the secretory proteins was suggested with the discovery that mutations in the pre-S1 domain of the L protein inhibited secretion of the surface proteins, inducing the GGH phenotype (Xu and Yen, 1996). Moreover, a deletion naturally occurring in the pre-S2 region of the same protein produced similar effects, suggesting that mutations in the entire pre-S region are responsible for the accumulation of the viral proteins and the development of the GGH morphology (Fan et al., 2000, 2001). The level of this accumulation appears to play a crucial role in triggering the ER stress since natural pre-S2 deletion leading to a moderate retention of the HBV envelope proteins were not able to induce cellular toxicity, at least *in vitro* (Tai et al., 2002).

The ability of secretion-incompetent pre-S mutants to specifically activate ER stress signaling pathways in host cells was further demonstrated by the up-regulation of well-established stress sensors such as GRP78 (BiP), GRP94, and ER-resident kinases (Wang et al., 2003). These stress signals could also induce oxidative DNA lesions and mutagenesis (Chen et al., 2006; Hsieh et al., 2007), caspase 12-mediated apoptosis, or NF-kB-mediated cell proliferation with an important role in cancer development (Qu et al., 2004). Interestingly, the Akt/mTOR pathway and the VEGF-A synthesis were found significantly activated in GGHs (Yang et al., 2009); it is therefore tempting to speculate that the ER stress induced by the pre-S mutants plays a key role in progression of HBV pathogenesis, leading to neoplastic lesions of the liver in chronically infected patients. The observation that the cell-cycle progression is directly affected by the ER stress provides additional data in support of this hypothesis. The ER accumulation of a pre-S2 mutant protein promotes cyclin A cleavage by the calcium-dependent protease μ-calpain, followed by its translocation from the nucleus into the cytoplasm and centrosome overduplication (Wang et al., 2012). The cytoplasmic distribution of cyclin A reported in transgenic mouse livers expressing pre-S2 mutants appears to favor this mechanism (Wang et al., 2005) (**Figure 1B**). However, these results must be interpreted with care, as overexpression of the wild-type L protein in transgenic mice was shown to be sufficient to induce aberrant changes in the hepatocyte, "ground-glass" appearance, cell death and nodular hyperplasia of the liver (Chisari et al., 1987).

Interestingly, a point L77R mutation occurring naturally in the cytosolic loop of the S protein results in retention of the S protein in the ER-Golgi compartment, probably due to impaired folding (Chua et al., 2005). Despite of this significant accumulation, no ER stress was detected in cells expressing this mutant variant, raising the question whether the pre-S-independent accumulation of the envelope proteins is able to induce UPR signaling.

REGULATORY MECHANISMS ACTIVATED IN HBV-INFECTED CELLS TO REDUCE THE ER STRESS

To alleviate the ER stress, cells initiate a complex signaling cascade termed the unfolded protein response (UPR), which relies on activation of three complex signaling pathways at the ER level and continued in the cell nucleus. The sensors of the ER stress and the key regulators of these pathways are the transmembrane proteins inositol-requiring protein 1 (IRE1) α and β, the activating transcription factor 6 (ATF6) α and β and the protein kinase RNA-like ER kinase (PERK), all regulated by BiP. Binding of these sensors to BiP maintains them in an inactive state; this process is competed by the accumulation of unfolded proteins within the ER, which also require interaction with BiP to prevent terminal misfolding and aggregation. Eventually BiP is released from the interaction with IRE1, ATF6, and PERK (Kimata et al., 2004) which promotes their activation by dimerization and autophosphorylation (PERK and IRE1) (Liu et al., 2000; Su et al., 2008) or by trafficking to the Golgi apparatus and further proteolytic processing (ATF6) (Ye et al., 2000; Chen et al., 2002). Activated IRE1 directs splicing of the full-length (unspliced) XBP1 mRNA (XBP1u) generating a shorter variant. This encodes a highly active transcription factor (XBP1s) which is able to induce transcription of selected genes by binding the ER stress response elements (ERSE) containing the consensus sequence CCAAT-N9-CCACG (Yoshida et al., 1998, 2001; Lee et al., 2002). Activated PERK phosphorylates the α subunit of the eukaryotic initiation factor-2 α (eIF2α), blocking the assembly of the ribosomal complex and thus protein translation (Ma et al., 2002). ATF6 cleavage within the Golgi releases its N-terminal domain which translocates to the nucleus and functions as a transcription factor on target genes, including that encoding for XBP1 (Haze et al., 1999). Collectively, these complex transcriptional activities and cross-talks between the signaling pathways result in: (a) attenuated protein translation (b) increased transcription of ER chaperone genes; (c) up-regulation of key molecules of the ER degradation machinery.

Interestingly, a first regulatory mechanism involved in alleviation of the ER stress produced by HBV infection was first described at viral level. It was observed that overexpression of the L protein increased the transcriptional activity of the S promoter by up to 10 folds, while neither M, S, or a secreted variant of L had a similar effect (Xu et al., 1997). Moreover, L accumulation also activated the promoters of the genes encoding for GRP78 and GRP94, the two chaperone proteins up-regulated during ER stress (Ramakrishnan et al., 1995; Roy and Lee, 1995). Activation of these promoters by the L protein was shown to depend on the transcription nuclear factor NF-Y and its binding to the CCAAT element, also present in the S promoter. Two *cis*-acting elements in the S promoter, namely Z1 and Z2 flanking the CCAAT region were also required for its activation by the ER stress, through a, yet unknown, transcription factor called "Z." The activation of the S promoter was later shown to be independent of the PERK pathways (Huang et al., 2005); rather, expression of the "Z" factor was induced by XBP1(s) the transcription factor resulted following IRE1 activation (Calfon et al., 2002). Since XBP1(s) and ATF6-alpha interact with each other to form heterodimers and bind to similar DNA sequences (Lee et al., 2002), a potential involvement of the ATF6-alpha pathway in this regulation, although not directly demonstrated, could not be excluded in this study.

An interesting hypothesis emerging from this investigation was based on the observation that induction of the "Z" factor by the ER stress was restricted to certain cell types, including liver and kidney cells, but not fibroblasts. Given the ubiquity of the UPR signaling pathways, this result is rather unusual and may indicate that the cells have also evolved specific mechanisms in response to ER stress stimuli that are characteristic to a specific tissue.

HBx and S proteins were also shown to activate the IRE1/XBP1 branch of the UPR, in independent studies. The HBx protein expressed transiently in Hep3B and HepG2 cells resulted in a significant increase of the XBP1 promoter activity, by up to 7 folds, as demonstrated using a luciferase expression reporter (Li et al., 2007). Moreover, splicing of the Xbp-1 mRNA occurred efficiently in these cells, and the presence of XBP1(s) was clearly evidenced in the same study. Splicing of the Xbp-1 mRNA was also shown in HepG2.2.2.15 cells, which contain two copies of the HBV genome and support HBV replication, assembly and secretion of SVPs and of fully infectious virions. The process depended on the HBx expression level, further supporting the involvement of this protein in UPR activation (Li et al., 2007). Similarly, Huh7 hepatoma cells overexpressing the S protein contained both, the precursor and the spliced form of the Xbp-1 mRNA, suggesting that the envelope protein is also able to trigger UPR via the IRE1/XBP1 pathway (Li et al., 2011). However, whether or not XBP1-specific target genes were actually activated in these systems had not been investigated in either study.

ACTIVATION OF THE ER-ASSOCIATED DEGRADATION (ERAD) BY HBV

The first UPR target demonstrated to depend entirely on the IRE1-XBP1 pathway was the gene encoding for a member of the ER degradation-enhancing, mannosidase-like family of proteins (EDEM1) (Yoshida et al., 2003). EDEM1 and its two homologs EDEM2 (Mast et al., 2005) and EDEM3 (Hirao et al., 2006) belong to the glycoside hydrolase 47 family and are believed to play an important role in alleviating the ER stress during UPR, by targeting misfolded glycoproteins to ERAD (Olivari and Molinari, 2007).

Our investigation on the ERAD function in the HBV life-cycle revealed strong evidence that HBV and the IRE1-XBP1 branch of the UPR are in tight, mutual relationship. Synthesis of the transcripts encoding for the three members of the EDEM family was significantly increased in HepG2.2.215 cells hosting stable HBV replication, or Huh7 cells replicating the virus in a transient manner (Lazar et al., 2012). Moreover, while HepG2 cells express only trace amounts of EDEM1, expression of this protein became clearly detectable in HepG2.2.215 cells. It is important to note that activation of EDEM synthesis was not related to HBV replication and nucleocapsids accumulation within cells, as it occurred with similar efficiency in the presence of lamivudine, a strong DNA replication inhibitor (Doong et al., 1991). Rather, overexpression of the surface proteins appeared sufficient to induce EDEM up-regulation, which is in agreement with the ability of these proteins to activate the IRE1/XBP1 branch of the UPR (Li

et al., 2011). EDEM1 overproduction induced a significant degradation of the wild-type S, M, and L proteins, when expressed either independently, or in the context of a full replication-cycle. This observation was surprising, since EDEM has been traditionally involved in disposal of misfolded proteins and therefore, it called for a deeper investigation.

The incapacity of the ER folding machinery to deal with an excess of viral protein synthesis could be a possible explanation for this result. However, silencing of the endogenous EDEM1 resulted in intracellular accumulation of the HBV surface proteins, accompanied by a significant increase of their secretion. This strongly suggests that an important fraction of folding-, assembly-, and secretion-competent envelope proteins is delivered to degradation during HBV protein synthesis. Interestingly, the proteins rescued from degradation in EDEM1 knocked-down cells were recruited for nucleocapsid envelopment, increasing the amount of secreted virions. Thus, despite the availability of correctly folded surface proteins, their trafficking to the corresponding virus particles and SVP budding sites appear to be competing, rather than successive processes.

In search for a mechanism of action underlying the effects of EDEM1 modulation on the HBV life-cycle, a direct interaction between the endogenous protein and the viral polypeptides was evidenced by co-immunoprecipitation. EDEM1 co-precipitated both, the glycosylated and non-glycosylated isoforms of the envelope proteins. Moreover, investigation of the surface proteins oligomerization in EDEM1-depleted cells showed that EDEM1 acts early during surface protein synthesis, most probably before their assembly into oligomers. Collectively, these experiments point to an N-glycan-independent mechanism of EDEM1 binding to the viral substrates, possibly involving the common S-domain. These data are in support of a more recent model of EDEM1-substrate recognition, implying a glycan-independent binding of the ERAD targets (Cormier et al., 2009). The evidence of EDEM1 interaction with wild-type proteins and the observation that glycoprotein substrates are still targeted to ERAD in the absence of mannose trimming, when EDEM1 is up-regulated (Ron et al., 2011), have added new angles to this model. It was proposed that the significant induction of EDEM1 expression in HBV-replicating cells promotes extraction of conformation-viable viral polypeptides from the ER-resident protein quality control cycle and their premature delivery to degradation (Lazar et al., 2012). This has important consequences on the HBV life-cycle (summarized in **Figure 2**), resulting in reduction of SVP and virion production.

The concept that wild-type proteins can also be degraded during UPR is supported by data obtained from studies on Human Hepatitis C virus (HCV), showing a direct involvement of EDEM1 in regulating the production of wild-type envelope proteins at post-translational level (Saeed et al., 2011). EDEM1 overexpression promotes degradation of the E1 and E2 surface proteins, which results in down-regulation of HCV particles production. Conversely, the E2 protein shows greater stability in EDEM1 knocked-down cells, which also produce an increased amount of infectious HCV, with no effect on viral replication (Saeed et al., 2011).

An important question arising from these investigations regards the validity of this concept for the UPR induced during conditions other than viral infections. Most of the pioneering works leading to characterization of the UPR signaling pathways have used model proteins undergoing severe conformational changes, due to genetic mutations or other environmental factors, often leading to pathogenesis. These conditions are known as "folding diseases," of which some neurodegenerative disorders,

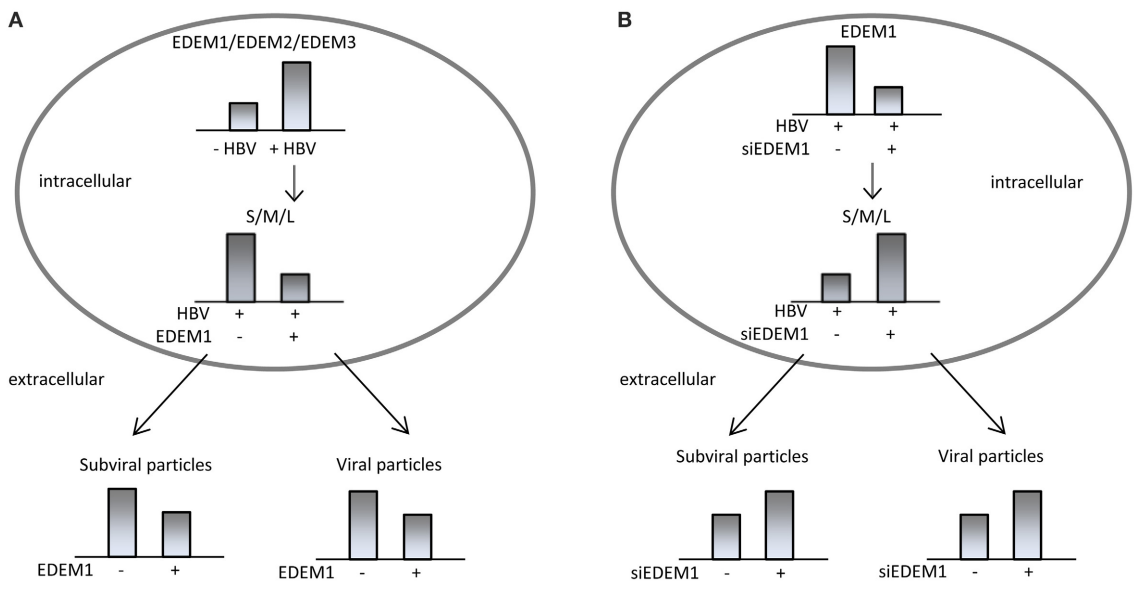

FIGURE 2 | HBV interaction with the ER-associated degradation pathway. HBV activates expression of EDEMs, which promote degradation of wild-type, assembly-competent HBV surface proteins, reducing the production of subviral and viral particles **(A)**. Silencing of endogenous EDEM1 in HBV replicating cells induces accumulation of folding-competent envelope proteins and increases secretion of subviral and viral particles **(B)**.

diabetes, obstructive pulmonary disease are best documented (Yoshida, 2007). It would be interesting to extend this research to other wild-type cellular proteins to understand the subtleties of the molecular discrimination between correctly folded and misfolded proteins and their degradation and identify the cellular factors involved in this recognition.

Unlike the case of "folding diseases," the amount of data regarding the UPR in viral infections is relatively limited and controversial; in addition to HBV and HCV discussed above, only a few other viruses, such as Borna Disease Virus (Williams and Lipkin, 2006), murine leukemia virus (Dimcheff et al., 2006), rotaviruses (Trujillo-Alonso et al., 2011), or the West Nile virus (Ambrose and Mackenzie, 2011) have been reported to induce ER stress. Most of these viruses have adapted mechanisms to use the UPR signaling pathways to their own benefit, whether this is assistance for proper protein folding or replication. In the case of the HBV/HCV, it is tempting to speculate that activation of the ERAD pathway would limit the amount of surface proteins available for nucleocapsid envelopment, on one hand, and avoid extensive damage of the ER due to viral protein accumulation, on the other hand, thus enabling the evolution of infection toward chronicity.

HBV INDUCES AUTOPHAGY

It has been established that prolonged UPR eventually leads to cells death by apoptosis. However, before making the ultimate choice, cells under severe ER stress are able to recruit survival pathways, such as autophagy. Autophagy is a catabolic process, highly conserved during evolution, involving degradation of long-lived macromolecules and defective organelles within double membrane compartments, called autophagosomes (He and Klionsky, 2009). Interestingly, several viruses, including HBV, were shown to induce autophagy and further exploit it in productive or unproductive infections (Pratt and Sugden, 2012). Activation of autophagy by HBV has been clearly demonstrated by two independent groups (Sir et al., 2010; Li et al., 2011), leading to one of the most interesting, recent controversy in the HBV field (**Figure 3**). Both groups have observed conversion of the autophagic marker LC3 from the cytosolic to the lipidated, autophagosome-associated form in HBV transfected cells (Sir et al., 2010; Li et al., 2011) and livers of HBV-infected patients or transgenic mice (Sir et al., 2010). Autophagosomes formation in the presence of HBV was also convincingly confirmed by electron and confocal microscopy; notably, this was not accompanied by protein degradation, suggesting a strong interference of HBV with the autophagy signaling pathway preventing further clearance of the engulfed macromolecules (Sir et al., 2010; Li et al., 2011). However the two studies diverge in their proposed mechanisms of HBV-induced autophagy and the interpretation of the role played by this process at different steps of the viral life-cycle. A first indication of a viral factor involvement in autophagy induction was reported by Tang and collaborators (Tang et al., 2009). It was shown that Beclin-1 expression in hepatic and hepatoma cells was modulated by HBx, which acts at transcriptional level, activating

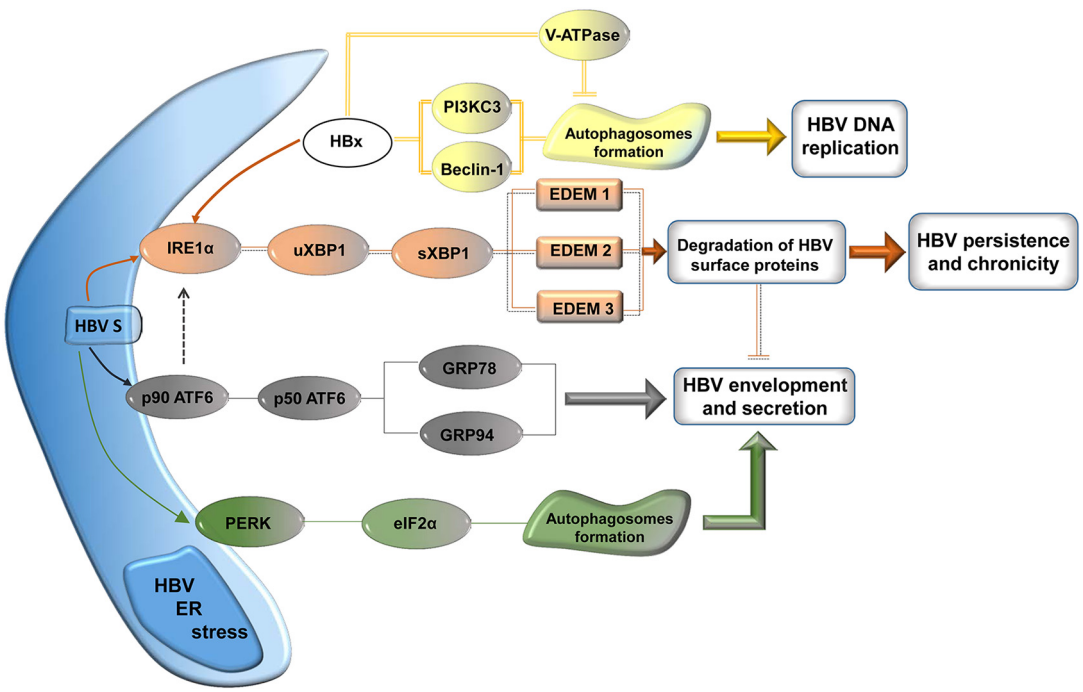

FIGURE 3 | Signaling pathways modulated by HBV for its own benefit. The surface proteins activate the UPR leading to autophagy, involved in nucleocapsid envelopment and virus particle secretion. Activation of the IRE1 pathways results in up-regulation of the EDEM family of proteins, which reduces the load of viral surface proteins, possibly contributing to HBV persistence and chronicity. HBx induces autophagosome formation via PI3KC3/Beclin-1 activation, which is involved in HBV DNA replication. Transport of the V-ATPase to autophagosomes is possibly impaired by HBx, resulting in elevated vacuolar pH and lack of protein degradation.

its promoter. This induces autophagy under nutrient starvation conditions, which could be inhibited by Beclin-1 silencing with specific siRNA. Intriguingly, Beclin-1 up-regulation was not confirmed in a subsequent study, despite HBx being also indicated as the major factor triggering autophagy (Sir et al., 2010). Rather, the process appeared to be mediated by activation of PI3KC3, an enzyme playing a critical role autophagy initiation, following a direct interaction with HBx.

Very recently, an independent study provided additional evidence in support of the HBx role in the formation of autophagosomes and a potential mechanism for the absence of protein degradation observed, despite the activated autophagy (Liu et al., 2014). It was shown that lysosomal activity was significantly perturbed in HBx-expressing cells, possibly due to mistrafficking of the V-ATPase involved in lysosomes acidification. This results in accumulation of defective lysosomes containing immature hydrolases, such as Cathepsin D, which are unable to degrade the cargo proteins (Liu et al., 2014).

However, another series of experiments have implied that S protein expression is sufficient to activate autophagy by a mechanism involving cellular stress, excluding a potential contribution of HBx (Li et al., 2011). High amounts of S protein expressed either alone or in the context of the full viral replication resulted in phosphorylation of PERK and eIF2α, its direct effector. Similarly, XBP1 mRNA splicing was induced by S, while only the unspliced form could be detected in control cells, in the absence of the viral protein. ATF6 activation was also investigated in these cells; although the cleavage products could not be directly evidenced, up-regulation of ATF6 downstream effectors, such as GRP94 (Eletto et al., 2010) was unambiguously demonstrated. Collectively, these data suggest that accumulation of the S protein activates the ER stress pathways regulated by PERK, ATF6, and IRE1. Moreover, knock-down of either signaling route efficiently prevented LC3 lipidation and autophagosome formation induced by the HBV surface protein, further supporting the hypothesis above. Finally, an important fraction of the S protein associated with the autophagosome membrane and a direct interaction with LC3 was demonstrated, providing a potential mechanism for the effects of the HBV-induced autophagy on the viral life-cycle (Li et al., 2011).

These effects have raised additional controversy between the two groups. One group suggested that autophagy is critical for the viral DNA replication, while little effects were observed on the RNA synthesis or its packaging into nucleocapsids (Sir et al., 2010). Based on several lines of evidence, including: (a) the partial co-localization of the HBV core and surface proteins with LC3, (b) cell treatment with 3-MA, a PI3KC3 inhibitor, (c) specific down-regulation of Vps34, the catalytic subunit of PI3KC3 and of Atg7, an enzyme involved in autophagosomes formation, it was proposed that early autophagic vacuoles may function as platforms for viral DNA replication and assembly. Alternatively, an indirect role of the autophagosomes in HBV replication, by hosting signaling molecules involved in regulation of the core protein phosphorylation is also likely. Important evidence for a role of autophagy in the production HBV particles *in vivo* was provided by the experiments using HBV transgenic mice with liver-specific knockout of another autophagy initiating factor, Atg5 (Tian et al., 2011). The impaired autophagy in this system resulted in a significant reduction of both, HBV DNA and SVP secretion in the mice sera.

In contrast, the second group suggested that the UPR- induced autophagy is required for efficient envelopment of the HBV nucleocapsids, while the DNA replication is only moderately affected. This conclusion was based on (a) the decreased secretion of enveloped virions from cells treated with 3-MA, (b) the increased amount of extracellular enveloped virions in the presence of autophagy inducers, such as rapamycin and starvation.

The lines of evidence provided by the two groups in support of their conclusions appear compelling and difficult to reconcile at a first glance. However, it is very likely that in fact, the two mechanisms operate together, one or the other prevailing according to the amount of viral protein expressed, or the hepatoma cell line used, which may activate different levels of ER stress. It is important to note, for instance, that the first study focused on DNA replication in autophagy-inhibited cells, while the efficiency of virion envelopment and secretion were not assessed (Sir et al., 2010). Similarly, a moderate effect of the UPR-induced autophagy on DNA replication was also observed in the second study, which analyzed in more detail virion assembly and secretion (Li et al., 2011). It would be interesting for future investigations to address the relationship between the HBx and the envelope proteins in the HBV-induced autophagy, in a more systematic manner and in the context of a complete viral life-cycle, ideally in a natural infection system.

Activation of the UPR and autophagy signaling during mild ER stress clearly favors the recovery of the cellular homeostasis. As mentioned above, prolonged ER stress may trigger apoptosis to remove the irreversibly damaged cells. This is a complex process which relies on activation of transcription factors (e.g., the C/EPB homologous protein -CHOP), phosphatases and kinases (e.g., the protein phosphatase 1—PP1, the apoptosis signal-regulating kinase 1—ASK1, and JNK) (Nishitoh, 2012), which, in turn, regulate downstream pro-apoptotic factors (Wei et al., 2001). The ability of HBV to induce apoptosis is a matter of intense debate, which is far from being concluded. One set of experimental data indicates the absence of apoptosis in HBV replicating cells (Schulze-Bergkamen et al., 2003) or inhibition of apoptosis by HBV, by several mechanisms (Huo et al., 2001; Marusawa et al., 2003; Liu et al., 2013). In addition, it was recently shown that artificially-induced apoptosis is detrimental to the HBV life-cycle (Arzberger et al., 2010). In contrast, other reports suggest that overexpression of the HBx protein can trigger apoptosis of liver cells, in a p53-dependent (Wang et al., 2008) or -independent manner (Terradillos et al., 1998). However, it remains to be established whether expression of the HBx protein can reach this critical levels in a natural infection and how the stage and progression of infection may influence the pro- or anti-apoptotic responses observed in different HBV experimental systems.

CONCLUSION REMARKS

While the HBV-induced ER stress and UPR signaling have been clearly established, the consequences of this activation on both, the host cell and the virus life-cycle, are far from being elucidated. There are many interesting issues that deserve deeper scrutiny,

such as the relationship between the ER stress and carcinogenesis and the UPR modulation by HBV for own benefits, whether this is protein folding, genome replication, virion assembly, or establishment of chronic infection (**Figure 3**). It is important to note that studies regarding the UPR at early stages of HBV infection have not even been addressed. This may be explained by the difficulty to investigate HBV infection *in vitro* using the infectivity models available. It is expected that the recent discoveries of cellular factors facilitating HBV entry and the development of new, improved cellular systems, permissive for HBV infection (Yang et al., 2014), will help expand this research by approaching the current controversies in the context of the whole viral infection.

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Varicella-zoster virus glycoprotein expression differentially induces the unfolded protein response in infected cells

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Varicella-zoster virus (VZV) is a human herpesvirus that spreads to children as varicella or chicken pox. The virus then establishes latency in the nervous system and re-emerges, typically decades later, as zoster or shingles. We have reported previously that VZV induces autophagy in infected cells as well as exhibiting evidence of the Unfolded Protein Response (UPR): XBP1 splicing, a greatly expanded Endoplasmic Reticulum (ER) and CHOP expression. Herein we report the results of a UPR specific PCR array that measures the levels of mRNA of 84 different components of the UPR in VZV infected cells as compared to tunicamycin treated cells as a positive control and uninfected, untreated cells as a negative control. Tunicamycin is a mixture of chemicals that inhibits N-linked glycosylation in the ER with resultant protein misfolding and the UPR. We found that VZV differentially induces the UPR when compared to tunicamycin treatment. For example, tunicamycin treatment moderately increased (8-fold) roughly half of the array elements while downregulating only three (one ERAD and two FOLD components). VZV infection on the other hand upregulated 33 components including a little described stress sensor *CREB-H* (64-fold) as well as ER membrane components *INSIG* and *gp78,* which modulate cholesterol synthesis while downregulating over 20 components mostly associated with ERAD and FOLD. We hypothesize that this expression pattern is associated with an expanding ER with downregulation of active degradation by ERAD and apoptosis as the cell attempts to handle abundant viral glycoprotein synthesis.

Keywords: herpesvirus, unfolded protein response, autophagy, tunicamycin, ERAD, CREBH, gp78, INSIG

INTRODUCTION

VZV is a human pathogen that spreads to children as varicella or chicken pox and re-emerges later as zoster or shingles (Ross, 1962; Grose, 1981). VZV is one of nine human herpesviruses (Davison, 2010). The virus is supremely adapted to its human host and infects most people in a given community (Hope-Simpson, 1965; Choo et al., 1995). It is endemic throughout the world but largely controlled in some countries by vaccination with a live attenuated virus (Seward et al., 2008; Marin et al., 2011).

Varicella infection, within its natural human host, spreads from the nasopharynx via infection of a limited number of T cells that home to the skin epidermis (Arvin et al., 2010). Once there the infection is passed to the basal keratinocytes making up the innermost layer of the epidermis (Ku et al., 2004). The virus progressively infects other cells in its proximity until reaching the surface of the skin in the form of characteristic VZV vesicles. Within the area of the vesicle, polykaryocytes or multi-nucleated cells are found due to VZV-induced cell to cell fusion (Weigle and Grose, 1984). As the number of viral particles increase within the vesicle, some particles travel retrograde along sensory neurons in the skin to the sensory ganglia emanating from the spinal cord (Gilden et al., 2003). In the ganglia, the virus becomes latent or quiescent until much later (years or decades) in the life of the host. Under conditions of immunosuppression or aging, VZV can reactivate within the ganglia and spread back anterograde to the skin to cause zoster or shingles (Arvin, 1987). Typically, this event only happens from a single ganglion within one dermatome (Hope-Simpson, 1965).

VZV is an alphaherpesvirus that exists as a multilayered structure approximately 200 nm in diameter (Grose et al., 1983). In the virus particle, the genome (dsDNA) is surrounded by a protein capsid structure that is covered by an amorphous layer of tegument proteins. These two structures are surrounded by a lipid envelope that contains viral glycoproteins. The VZV genome is the smallest of the human herpesviruses and encodes at least 71 unique proteins (ORF0–ORF68) with three more opening reading frames (ORF69–ORF71) that duplicate earlier open reading frames (ORF64–62, respectively) (Davison and Scott, 1986). Only a fraction of the encoded proteins form the structure of the virus particle (Kinchington et al., 1992). Among those proteins are nine glycoproteins: ORF5 (gK), ORF9A (gN), ORF14 (gC), ORF31 (gB), ORF37 (gH), ORF50 (gM), ORF60 (gL), ORF67 (gI), and ORF68 (gE). Abundant biosynthesis of viral glycoproteins increases to the point of excluding cellular glycoprotein expression under conditions of infection in cultured cells (Grose, 1980).

Of importance, VZV induces autophagy in infected cells as well as exhibiting evidence of the Unfolded Protein Response (UPR): XBP1 splicing and a greatly expanded ER (Takahashi et al., 2009; Carpenter et al., 2011). More recently, we

found that inhibition of autophagy by either 3-methyl adenine (3-MA) treatment or siRNA knockdown of ATG-5, a necessary autophagy protein, reduced glycoprotein expression and altered post-translational modifications of VZV gE and gI and ultimately VZV infectivity in culture. (Buckingham et al., 2014). These results highlight the role of VZV glycoprotein expression in inducing ER stress and associated autophagy. Our observations of enlarged ER and spliced XBP-1 in VZV infected cells led us to consider what other elements of the UPR are being activated. We decided to use a commercial PCR array that measures the levels of transcripts of 84 different components of the UPR. Herein we report the results of comparing VZV infected cells vs. tunicamycin treated cells with this UPR PCR array.

METHODS

VIRUSES AND CELLS

VZV-32 is a low passage laboratory strain; its genome has been completely sequenced and falls within European clade 1 of VZV genotypes (Peters et al., 2006). MRC-5 human fibroblast cells or HeLa cells were grown in six well tissue culture plates with and without 12 mm round or 22 mm square coverslips in Minimum Essential Medium (MEM; Gibco, Life Technologies) supplemented with 7% fetal bovine serum (FBS), L-glutamine, non-essential amino acids, and penicillin/streptomycin. When monolayers were nearly confluent, MRC-5 cells were inoculated with VZV-infected cells at a ratio of one infected cell to eight uninfected cells by previously described methods (Grose and Brunel, 1978).

TRANSFECTION

HeLa cells were transfected with plasmids containing VZV gE (pTargeT_gE) or VZV ORF62 (pCMV_IE62) under the CMV promoter as described previously (Carpenter et al., 2011). The plasmids were transfected into HeLa cells using ExtremeGene HP (Roche) transfection reagent (Jacobsen et al., 2004) at 10 μl/ml and plasmid DNA at a concentration of 1.0 μg/ml. After 6 h, the culture medium was replaced with plasmid/transfection reagent free medium. At 24 h post-transfection, RNA was extracted from all wells in a culture plate and cells incubated on coverslips were fixed and processed for microscopy.

REAL-TIME RT-PCR

Total RNA was extracted from uninfected, tunicamycin treated and VZV infected fibroblast cells in six well plates at the given time points using the RNEasy mini kit (Qiagen). RNA quality and quantity was assayed by UV spectroscopy using a NanoDrop spectrometer. Both A260/A280 and A260/A230 ratios were within 20% of 2.0 and infected cells from a six well plate well (6.5 sq cm) yielded approximately 3 μg of RNA in 60 μl. Further, the RNA was electrophoresed in an Agilent Bioanalyzer 2100 (Agilent) and yielded RIN values within 20% of 10. Polyadenylated RNA was converted to cDNA using anchored Oligo(dT) primers and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) to yield approximately 20 ng of cDNA. The entire cDNA sample from one well of cells was mixed into 1 ml of 1× diluted Power SYBR Green Master Mix (ABI) and split into all wells of a SA Biosciences UPR PCR array (Life Technologies) with a multichannel pipettor (25 μl per well). The measurements were carried out in triplicate using cDNA from three of the original six wells in the plate for all types of samples using a Model 7000 real time PCR instrument (ABI). The resulting PCR results were processed using the SDS 1.2.3 software (Applied Biosystems). C_T values of each measurement were normalized to an average of 16.0 for housekeeping genes (wells H1–H5 of the UPR array) to form ΔC_T values which were then used to calculate averages and standard deviations between triplicate measurements. Subsequent $\Delta\Delta C_T$ values were calculated by differences between averages of VZV infected ΔC_T values or tunicamycin treated ΔC_T values with the average uninfected ΔC_T values. Uncertainties correspond to propagation of errors using standard deviations between the uninfected and infected or tunicamycin averages.

RT-PCR PRIMERS

To confirm measurements from the UPR specific PCR array, several RT-PCR measurements were carried out using the following primers: BiP: forward 5′-CCC CAA CTG GTG AAG AGG AT-3′ and reverse 5′-GCA GTA AAC AGC CGC TTA GG-3′; DNAJB9/ERDj4: forward 5′-ACA TCT GTG ACT TGC GTT GC-3′ and reverse 5′-TGG GCA ATA AAA CCA TTT CC-3′; CREBH: forward 5′-GGG AGA CGA GCT GTG AGC-3′ and reverse 5′-TGT CTG AGT GTC GGT TCC TG-3′; PERK: forward 5′-GCC TAA GGA GGT AGC AGC AA-3′ and reverse 5′-GGG ACA AAA ATG GAG TCA GC-3′.

ANTIBODIES

Murine MAb antibodies to VZV gE (3B3) and IE62 (5C6) produced in our laboratory were used in addition to a rabbit polyclonal antibody to LC3B (Santa Cruz Biotech sc-28266).

IMAGING PROTOCOLS

Samples of infected and uninfected cells were prepared for confocal microscopy by methods described previously (Carpenter et al., 2008). Briefly, the samples were fixed with paraformaldehyde and permabilized with 0.05% Triton-X-100 in PBS and then blocked in 5% non-fat milk with 2.5% normal goat serum for 2 h at RT. The primary antibody (1:2000) was added for 2 h at RT and overnight at 4°C. After washing (3 × 5 min with PBS) the samples were incubated with the secondary antibody (1:1250) and the Hoechst 33342 dsDNA stain (1:500) for 2 h at RT then washed before mounting on slides for viewing. Following preparation, the samples were viewed on a Zeiss 710 confocal fluorescent microscope (Duus et al., 1995).

TUNICAMYCIN PROTOCOL

Conditions for treatment of cultured cells with tunicamycin (2.5 μg/ml; Calbiochem, #654380) have been described in earlier papers in which we were investigating VZV glycoprotein biosynthesis (Montalvo et al., 1985; Carpenter et al., 2010). For experiments in uninfected cells, tunicamycin (2.5 μg/ml) was added 24 h after subculturing and the monolayer was fixed after another 24 h.

ER LABELING BY DICARBOCYANINE DYE

$DiOC_6$ (3-3-dihexyloxa-carbocyanine iodide) was obtained in powder form from Molecular Probes (D-273) and dissolved (0.7 mg/ml) in ethanol (Sabnis et al., 1997). An aliquot of the $DiOC_6$ stock (2.8 μl/ml yielded a final concentration of $DiOC_6$ of 2 μg/ml) was added to warm cell culture medium; this medium was applied to live cells for 30 min, then rinsed 2× with PBS and processed for fluorescent microscopy as described above.

RESULTS

VZV INFECTED CELLS EXHIBITED ABUNDANT GLYCOPROTEIN EXPRESSION WITH AN ENLARGED ER AND INCREASED AUTOPHAGY

Within cell culture, VZV is entirely cell associated with no release of cell-free virus (Grose and Brunel, 1978; Weller, 1983). Monolayers are inoculated with VZV-infected cells. The susceptibly of cells to VZV determines how long it takes to infect the whole monolayer but spread typically requires 3–5 days. Within infected cell monolayers, we observe a range of fused cells. For example, VZV induces massive syncytia involving hundreds of nuclei in melanoma cells while VZV infection of less fusogenic cells such as lung fibroblasts or skin keratinocytes induces syncytia involving tens of nuclei.

Recently, we observed that VZV induces increased LC3-positive puncta formation indicative of autophagosomes within cultured cells as well as from cells removed from varicella and zoster vesicles (e.g., **Figures 1A1,A2**) (Takahashi et al., 2009). Unlike the closely related herpes simplex virus, VZV does not encode any known inhibitors of autophagy, such as ICP 34.5. Later we observed that VZV infected cells also exhibited signs of ER stress, namely *XBP-1* splicing and a greatly enlarged ER (see, e.g., **Figures 1B1,B2**). The latter results led to the hypothesis that VZV glycoprotein synthesis induces ER stress that is partially relieved by an enlarging ER and increased autophagy (Carpenter et al., 2011).

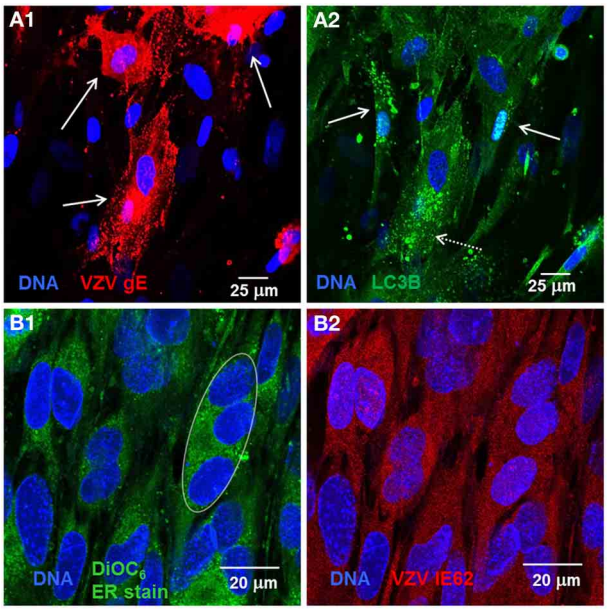

FIGURE 1 | VZV infected cells exhibited abundant glycoprotein expression, increased autophagosomes, and an enlarged ER. Human fibroblast cells (MRC-5) were grown on glass coverslips in tissue culture plates until 60% confluent and then infected with VZV-32 infected MRC-5 cells at a ratio of 1:8. At 48 hpi, the cultures were fixed, permeabilized, blocked and immunolabeled for VZV gE, IE62, or cellular LC3B. The cells were then imaged at 400× with a Zeiss 710 confocal fluorescence microscope. Other cultures grown and infected similarly were incubated in medium containing DiOC6, a polar dye, prior to fixation. **(A1)** VZV glycoprotein gE expression is abundant in three cells (solid arrows) in the image. **(A2)** LC3 puncta indicative of autophagosomes are apparent in cells expressing gE (arrows, dashed line) and nearby cells that are newly infected (arrows, solid line). **(B1)** $DiOC_6$ staining in 19 cells at 630×. A representative white ellipse indicates the enlarged ER in an infected syncytium. **(B2)** VZV IE62 staining indicates that all 19 cells are infected and several are in syncytia.

UPR GENE TRANSCRIPTION WAS DIFFERENT IN VZV INFECTED CELLS VS. TUNICAMYCIN TREATED CELLS

Based on the observations in the previous section, we sought to further document the induction of the UPR within VZV infected cells via a UPR-specific PCR array manufactured by SA Biosciences (now part of Qiagen). This 96 well plate consists of 84 wells containing primers to the 3′ Untranslated Region (UTR) of transcripts associated with the UPR and the remaining 12 wells containing primers to housekeeping genes and PCR and cDNA quality control wells. **Table 1** lists the UPR specific primers or wells where the wells are grouped by association with a given UPR function: ANTI or PRO (anti or pro-apoptotic), ERAD (ER associated degradation), FOLD (primarily folding chaperones), LIPID (transcripts associated with lipid synthesis and metabolism), SENSOR (transcripts associated with ER membrane resident proteins known to "sense" and signal ER stress conditions), TF (other transcription factors like C/EBPβ) and finally TRANS for two components associated with protein translation. Each group will be described more fully in the next sections.

Gene transcripts were measured in uninfected human fibroblasts, tunicamycin (TM) treated fibroblasts and VZV infected fibroblasts. Each measurement was done in triplicate. The measured C_T values were normalized so that in each case the housekeeping gene transcripts measured C_T average was 16 and then the triplicate measurements were averaged and standard deviations computed to generate ΔC_T. Differences between the uninfected ΔC_T and those associated with TM treated and VZV infected cell transcript measurements were then calculated to form the final measurements $\Delta\Delta C_T$ listed in **Table 1**. Graphs of the resulting values (**Figure 2**) showed that tunicamycin treatment, a classical ER stressor by inhibition of N glycosylation, upregulated 66 of the 84 UPR genes, with known folding chaperones, e.g., BiP (in blue), particularly upregulated. Also upregulated is the pro-apoptotic factor *CHOP* (pink). By contrast, only 43 of the UPR genes are upregulated in VZV infected cells. In particular, those genes most upregulated such as *CREB3L3/CREBH* (light blue) are more upregulated than after TM treatment. VZV infected cells also upregulated the LIPID transcripts *AMFR/gp78* and *INSIG* (green) while downregulating a number of ERAD components such as *UBXN4/erasin* and *EDEM3* (red). These differences will be considered by group in the subsequent sections.

Table 1 | UPR qPCR results for tunicamycin treated and VZV infected cells.

Gene	Function	Group	TM treated		VZV infected	
			$\Delta\Delta C_T$	STD	$\Delta\Delta C_T$	STD
ARMET/MANF	ERSE-II regulated; reduces cell proliferation and UPR initiated apoptosis	ANTI	5.0	0.2	6.1	0.5
EDEM3	ER degradation enhancer, mannosidase alpha-like 3	ERAD	−1.5	0.9	−4.7	0.9
PPIA	Peptidylpropyl isomerase A (cyclophilin A)	ERAD	−1.4	0.2	−0.7	0.5
UBE2G2	E2 ubiquitin conjugating enzyme G2	ERAD	−0.9	0.2	0.4	0.5
NPLOC4 (NPL4)	Regulates poly Ub on cytosolic side of ER membrane with VCP	ERAD	−0.7	0.2	1.9	0.5
UBXN4/erasin	UBX domain protein 4—adaptor protein to VCP	ERAD	−0.5	0.3	−6.1	0.5
USP14	Ubiquitin specific peptidase 14 in cytosol	ERAD	−0.4	0.3	−0.3	0.5
SEC62	ERAD translocation pore formation	ERAD	−0.2	0.3	−4.3	0.4
UFD1L	Regulates poly Ub on cytosolic side of ER membrane with VCP	ERAD	0.1	0.1	0.9	0.4
UBE2J2	E2 ubiquitin conjugating enzyme J2	ERAD	0.2	0.1	−0.4	0.4
ATXN3	Ataxin 3—deubiquiting enzyme	ERAD	0.8	0.2	3.8	0.5
FBX06	E3 ubiquition ligase of glycoproteins in ER lumen	ERAD	0.9	0.1	−2.1	0.4
RNF5	Ring Finger protein 5—E3 Ubiquitin ligase in ER membrane	ERAD	1.1	0.1	1.1	0.4
DERL1	Derlin family member E3 Ubiquitin ligase in ER membrane	ERAD	1.2	0.2	2.0	0.5
VCP(p97)	Regulates poly Ub of translocated ER substrates with NPL4 and UFD1L	ERAD	1.3	0.2	−0.4	0.5
EDEM1	ER degradation enhancer, mannosidase alpha-like 1—trims mannose	ERAD	1.4	0.2	1.5	0.4
SEL1L	Adaptor protein of Derlin-3/HRD1 in ER membrane	ERAD	2.0	0.3	−2.6	0.4
OS9	Glycoprotein protein quality control	ERAD	2.0	0.2	−0.7	0.5
DERL2	Derlin family member, E3 Ubiquitin ligase in ER membrane	ERAD	2.4	0.2	−1.3	0.5
SYVN1 (DER3/HRD1)	Synoviolin, Derlin family member E3 Ubiquitin ligase in ER membrane	ERAD	2.7	0.1	1.9	0.4
SELS	Selenoprotein S—oxidoreductase (oxidative stress)	ERAD	3.0	0.2	1.6	0.5
HERPUD1 (HERP)	Mediates degradation of ER Ca channels	ERAD	4.3	0.3	−1.9	0.5
HSPA2	HSP70 protein 2	FOLD	−1.5	0.3	1.2	0.6
HSPA4	HSP70 protein 4	FOLD	−0.8	0.3	1.4	0.5
HSPA1B	HSP70 protein 1B	FOLD	−0.5	0.2	0.0	0.5
GANAB (Glu II)	Glucosidase that trims N-linked glycans	FOLD	−0.5	0.3	−1.6	0.5
PRKCSH	Protein kinase C substrate 80K-H (subunit of glucosidase II)	FOLD	−0.4	0.2	1.7	0.5
GANC	Glycosal hydoloysis	FOLD	−0.2	0.2	2.3	0.4
TCP1	Component of Chaperonin	FOLD	−0.2	0.1	−0.7	0.4
HSPA1L	HSP70 protein 1 like	FOLD	−0.1	0.2	−3.0	0.4
HSPA4L	HSP70 protein 4 like	FOLD	0.0	0.3	−1.7	0.5
CCT4	Chaperonin containing TCP1, subunit 4 (delta)	FOLD	0.3	0.1	−1.3	0.4
CCT7	Chaperonin containing TCP1, subunit 7 (eta)	FOLD	0.4	0.2	0.4	0.5
HSPH1	HSP105 protein 1	FOLD	0.6	0.1	1.6	0.4
PFDN5	Prefoldin subunit 5; co-chaperone of Chaperonin complex	FOLD	0.7	0.2	2.2	0.5
PFDN2	Prefoldin subunit 2	FOLD	1.0	0.2	1.5	0.5
TOR1A	Torsion A—ATPase	FOLD	1.0	0.2	−1.0	0.5
UGCGL2 (UGT2)	UDP-glucose ceramide glucosyltransferase-like 2	FOLD	1.0	0.2	1.6	0.5
UGCGL1 (UGT1)	UDP-glucose ceramide glucosyltransferase-like 1	FOLD	1.1	0.2	0.9	0.5
ERP44	Thiol chaperone	FOLD	1.5	0.3	−1.8	0.5
CALR	Calreticulin; glycoprotein folding chaperone	FOLD	1.5	0.3	−1.1	0.5
RPN1	Ribophorin 1—substrate specific facilitator of N-glycosylation	FOLD	1.6	0.2	−1.6	0.5
ERO1L	Thiol oxidase governs redox state of ER (with Ca2+)	FOLD	1.6	0.3	0.8	0.5
DNAJC10 (ERdj5)	DNAJ (HSP40 homolog), subfamily C, member 10	FOLD	1.7	0.3	−1.0	0.5
DNAJB2	DNAJ (HSP40 homolog), subfamily B, member 2	FOLD	1.8	0.2	2.0	0.5
SEC63	Regulates ER import of membrane proteins	FOLD	2.1	0.2	0.3	0.4
CANX	Calnexin; glycoprotein folding chaperone; binds Ca2+	FOLD	2.2	0.3	1.2	0.5
SIL1(BAP)	Nucleotide exchange factor; binds BiP	FOLD	2.4	0.1	2.0	0.4
PDIA3 (ERP57)	Protein disulfide isomerase family A, member 3	FOLD	2.5	0.3	1.9	0.5
DNAJC3	DNAJ (HSP40 homolog), subfamily C, member 3	FOLD	2.6	0.1	2.4	0.4

(Continued)

Table 1 | Continued

Gene	Function	Group	TM treated		VZV infected	
			$\Delta\Delta C_T$	STD	$\Delta\Delta C_T$	STD
DNAJC4	DNAJ (HSP40 homolog), subfamily C, member 4	FOLD	2.9	0.2	2.9	0.5
ERO1LB	Thiol oxidase governs redox state of ER (with Ca2+)	FOLD	3.8	0.2	1.5	0.4
DNAJB9 (ERdj4)	DNAJ (HSP40 homolog), subfamily B, member 9	FOLD	5.5	0.2	−2.9	0.5
HSPA5	HSP70 protein 5 GRP78 (BIP)	FOLD	6.3	0.1	4.7	0.4
SREBF2	Sterol regulatory element binding TF 2	LIPID	0.9	0.1	−1.9	0.4
RNF139 (TRC8)	E3 Ubiquition ligase associated with INSIG	LIPID	−0.3	0.6	−1.2	0.6
INSIG2	Insulin induced protein isoform 2; regulation of cholesterol synthesis	LIPID	0.3	0.3	4.1	0.4
INSIG1	Insulin induced protein isoform 1; regulation of cholesterol synthesis	LIPID	0.5	0.3	5.3	0.4
AMFR (gp78)	Autocrine motility factor receptor; E3 Ub ligase; regulation of cholesterol	LIPID	0.6	0.3	6.2	0.5
SREBF1	Sterol regulatory element binding TF 1	LIPID	1.1	0.2	0.3	0.5
SCAP	Activates SREBF by cleaving it	LIPID	1.6	0.2	−0.3	0.5
SERP1 (RAMP4)	Stress induced ER protein 1; ER salt channel regulation	LIPID	2.7	0.3	2.9	0.6
MAPK8 (JNK1)	Map kinase K8 aka JNK1; pro-apoptotic in response to TNFα	PRO	−0.8	0.1	2.2	0.4
BAX	BCL2-associated X protein; induces release of COX-2 from mitochondria	PRO	0.5	0.3	−1.0	0.5
MAPK9 (JNK2)	Mitogen-activated protein kinase 9	PRO	0.5	0.2	1.0	0.4
HTRA2	HTRA serine peptidase 4	PRO	0.6	0.2	−0.2	0.5
MAPK10 (JNK3)	Map kinase K10 aka JNK3; pro-apoptotic in neurons	PRO	1.0	0.4	2.3	0.6
HTRA4	HTRA serine peptidase 2	PRO	1.4	0.4	4.0	0.6
CHOP	Aka DDIT3/GADD153; ER stress associated apoptotic protein	PRO	5.3	0.2	0.1	0.4
MBTPS2/S2P	Membrane bound TF peptidase, site 2 (active in Golgi)	SENSOR	−0.2	0.3	1.0	0.4
MBTPS1/S1P	Membrane bound TP peptidase, site 1 (active in Golgi; cleaves ATF6)	SENSOR	−0.1	0.2	0.1	0.4
ERN1 (IRE1α)	IRE1α is an endonuclease that splices XBP1 upon activation	SENSOR	0.0	0.2	1.6	0.4
ATF6B	ATF6 beta	SENSOR	0.5	0.3	−3.0	0.5
CREB3 (LUMAN)	OASIS (B-zip TF) family member; cell proliferation	SENSOR	1.4	0.2	1.0	0.4
ATF6	Activating transcription factor 6	SENSOR	1.5	0.2	0.3	0.5
EIF2AK3 (PERK)	ER stress sensor; PKR-like kinase	SENSOR	1.6	0.2	−4.2	0.5
ERN2 (IRE1β)	ER to nucleus signaling protein 2	SENSOR	1.8	0.5	3.1	0.6
NUCB1	Nucleobindin 1; negative regulation of ATF6	SENSOR	3.1	0.2	4.1	0.5
CREB3L3 (CREBH)	TF regulating lipogenesis and secretory pathway	SENSOR	4.4	1.6	9.3	0.9
XBP1	X box binding protein 1; splicing by IRE1 activates XBP1	TF	0.7	0.1	−0.4	0.4
CEBPB (C/EBPβ)	Bzip TF with wide impact on cell cycle and proliferation	TF	1.3	0.3	−2.9	0.6
ATF4	Activates stress response (including CHOP)	TF	1.7	0.2	1.5	0.5
EIF2A	Eukaryotic translation initiation factor 2A	TRANS	0.0	0.5	0.1	0.4
PPP1R15A	Protein phosphatase 1, subunit 15A	TRANS	1.8	0.2	1.1	0.4

Human fibroblast cells (MRC-5) were grown on glass coverslips in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was extracted from the VZV-32 infected cultures. For the TM treated cultures, RNA was extracted at 24 h post-treatment. RNA from the VZV-32 infected, TM treated and uninfected cell cultures was then converted to cDNA which was applied to UPR specific PCR arrays (SA Biosciences) and real time PCR was carried out on an ABI 7000 PCR instrument The resulting CT values were then normalized (ΔC_T) by housekeeping genes in the plate and then differences ($\Delta\Delta C_T$) between the uninfected and infected or tunicamycin treated values were computed and averaged. Abbreviations: anti-apoptotic (ANTI), ER associated degradation (ERAD), protein folding chaperones (FOLD), lipid and fat metabolism (LIPID), pro-apoptotic (PRO), ER stress sensor proteins (SENSOR), other transcription factors (TF) and protein translation associated proteins (TRANS). Error estimates correspond to standard deviation (STD).

VZV INFECTION SIGNIFICANTLY UPREGULATED THE TRANSCRIPTION FACTOR CREBH

The SENSOR grouping includes the best known ER stress sensors: *PERK*, *IRE1α* and *ATF6* but also two CREB proteins (*CREB3/LUMAN* and *CREBH*) as well as primers to the Golgi resident proteases *S1P* and *S2P* that activate AT6 and the CREB proteins by cleavage (Ye et al., 2000; Asada et al., 2011). Included in the group are lesser known transcripts including *IRE1β*, *ATF6β*, and *NUCB1*.

CREBH, the cAMP responsive element binding protein H, is an ER anchored transcription factor implicated in nutrient metabolism and the proinflammatory response. VZV infected cells displayed more transcripts of *CREBH* and fewer of *ATF6β* and *PERK* (all with $p < 0.001$) than in TM treated cells (**Figure 3A**). TM treatment generally upregulated all ER sensor transcripts with *CREBH* the most upregulated. *CREBH* transcription has previously been described as upregulated in hepatocytes and has been associated with lipid synthesis and acute phase

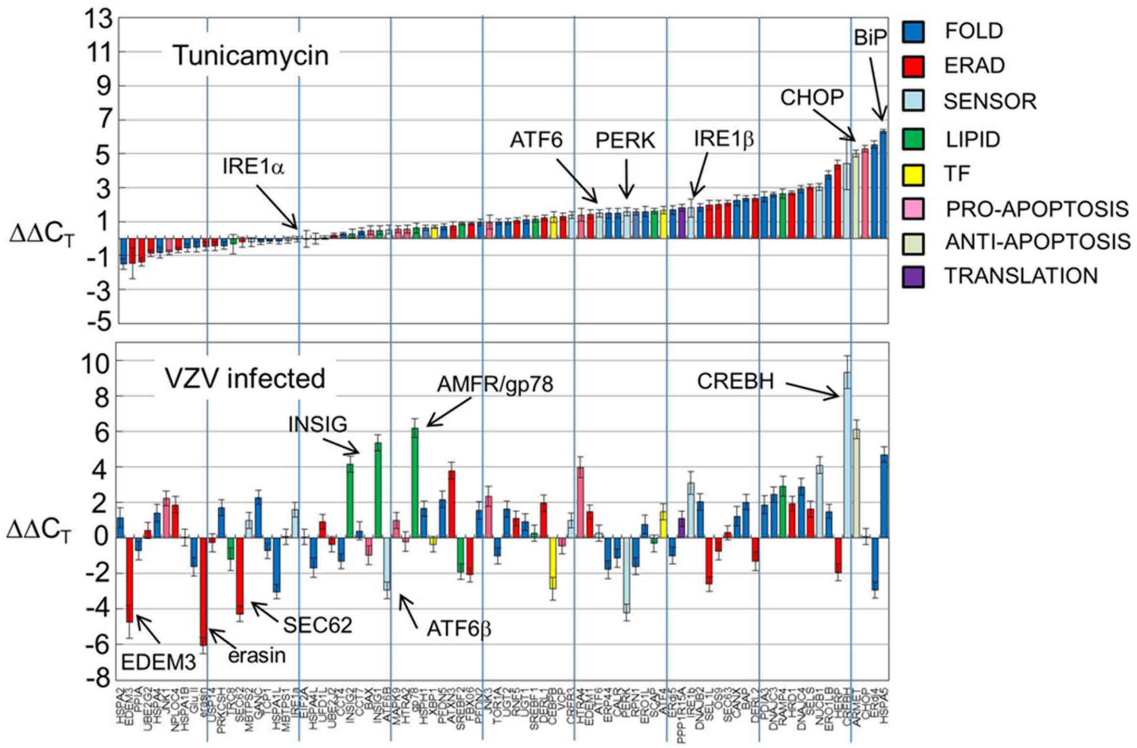

FIGURE 2 | UPR gene transcription was significantly different in VZV infected cells vs. either uninfected cells or tunicamycin treated cells. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was extracted from the VZV-32 infected cultures. For the TM treated cultures, RNA was extracted at 24 h post-treatment. RNA from the VZV-32 infected, TM treated and uninfected cell cultures was then converted to cDNA, which was applied to UPR specific PCR arrays (SA Biosciences); real time PCR was carried out on an ABI 7000 PCR instrument. The resulting C_T values were then normalized (ΔC_T) by the housekeeping genes of the plate and differences ($\Delta\Delta C_T$) between the uninfected and infected or tunicamycin treated values were computed and averaged. Graphs of the resulting values show that tunicamycin treatment, a classical ER stressor, resulted in upregulation of 66 of the 84 UPR genes with known folding chaperones such as *BiP* (in blue). Also upregulated was the pro-apoptotic factor *CHOP*. By contrast, only 43 of the UPR genes were upregulated in VZV infected cells although several, such as *CREBH*, were more upregulated than in tunicamycin treated samples. Error bars correspond to standard deviation when averaging.

transcription in T-cells (Zhang et al., 2006, 2012). More recently, CREBH as a transcription factor has been described as increasing the capacity of the secretory pathway (Barbosa et al., 2013). *ATF6β* and *ATF6α* share similar structures but differ in function. In particular, *ATF6β* has been reported to inhibit transcription of *ATF6α* one of the primary ER stress sensors (Thuerauf et al., 2007).

In order to confirm the results of the UPR specific PCR array, we carried out qPCR measurements using primers to *CREBH* and *PERK* (**Figure 3B**) in TM treated cells and at several timepoints in VZV infected cells. Those measurements confirm the upregulation of *CREBH* by TM treatment but particularly in VZV infected cells ($p < 0.01$). However, the downregulation of *PERK* in VZV infected cells was not confirmed.

VZV INFECTED CELLS EXHIBITED UNEVEN FOLD GENE TRANSCRIPTION

Within the FOLD group, the largest, there are 32 wells with primers to eight HSP-70 homologs including *HSPA5/BiP* and *SIL1/BaP*; five DNAJ HSP-40 homologs including *DNAJB9/ERdj4* and *DNAJC10/ERdj5*; twelve wells contain primers to transcripts encoding ER lumen folding components including *CALR* and *CANX*; three components of the folding chaperonin complex and finally four components in the ER membrane including *RPN1* and *SEC63*. Many of these transcripts encode proteins which assist secretory protein folding but also sense misfolded proteins in the ER (Schroder, 2008). For example, DNAJC10/ERdj5 is a disulfide reductase that associates with ERAD component EDEM (Hagiwara et al., 2011).

VZV infected cells exhibited very uneven transcription of folding chaperones (FOLD) while TM treatment robustly upregulated transcription of these chaperones particularly *BiP* (**Figure 4A**). Measurements with the UPR specific PCR array showed VZV infected cells upregulated *BiP* while downregulating *DNAJB9/ERdj4* and *HSPA1L*. In order to reassess these observations, we carried out qPCR measurements of *BiP* and *DNAJB9/ERDj4* using primers specific to those transcripts (**Figure 4B**) and found that neither the upregulation of *BiP* nor downregulation of *DNAJB9/ERDj4* was confirmed. Rather the qPCR results found *BiP* to be moderately downregulated as the infection progressed to more cells ($p < 0.05$). However, we also reassessed the regulation of the ER-co-chaperone

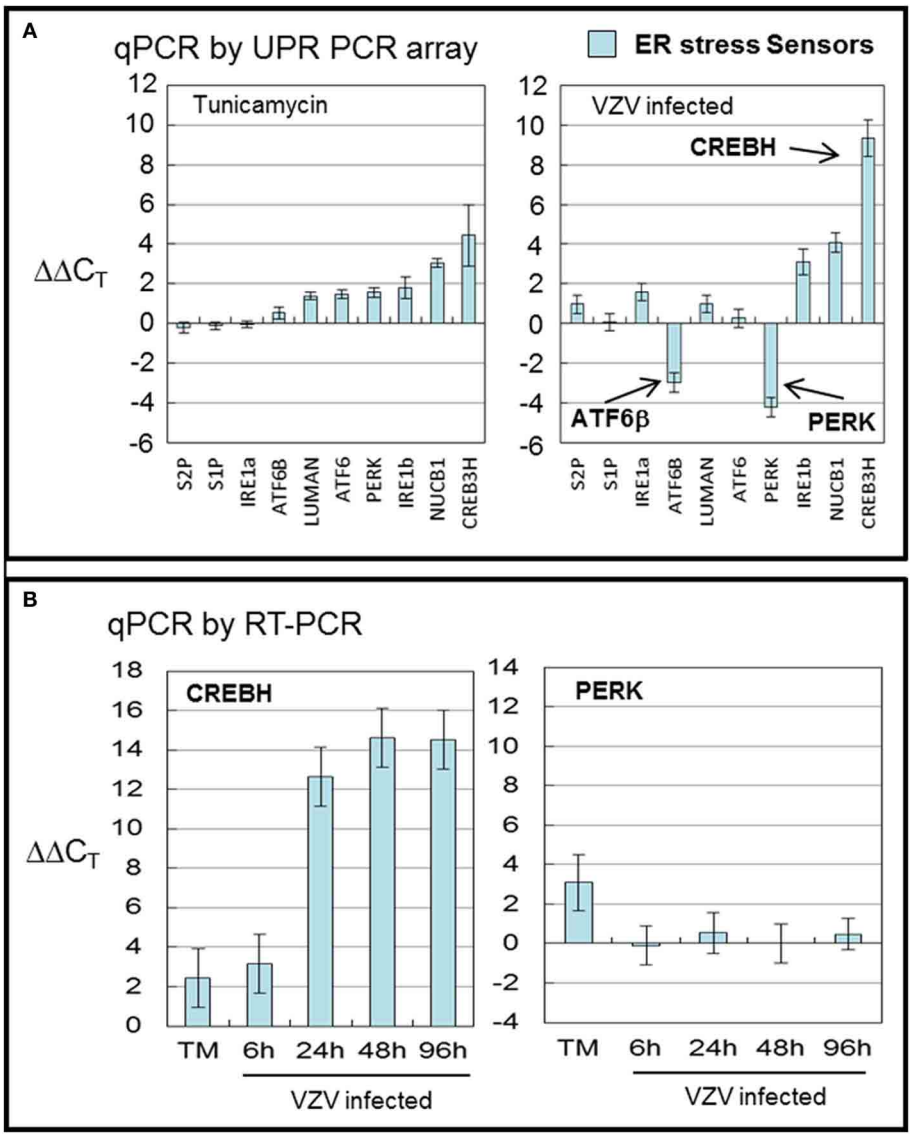

FIGURE 3 | VZV infection significantly upregulated the transcription factor *CREBH*. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was processed as described in legend to **Figure 2**. All gene transcription measurements were graphed for tunicamycin treated and VZV infected cell samples. **(A)** By measurements using the UPR specific PCR array, VZV infected cells showed significant upregulation of *CREBH* with downregulation of *PERK* and *ATF6β*. Tunicamycin treatment upregulated to a lesser extent all stress SENSORs. **(B)** To assess some of the measurements by the UPR array, cDNA from VZV infected and tunicamycin treated cells was submitted for real-time (RT-) PCR using primers specific to *CREBH* and *PERK* (see Methods section for primer information). Error bars correspond to standard deviation when averaging.

DNAJC10/ERdj5 and found a correlation with the UPR-specific array (data not shown).

VZV INFECTED CELLS SIGNIFICANTLY DOWNREGULATED ERAD GENE TRANSCRIPTION

There are 21 ERAD associated wells that amplify a number of known transcripts that code for proteins that are involved in the degradation of misfolded proteins in the ER through a number of steps: recognition of misfolding (*OS9, PPIA* and *SELS* along with a number of FOLD transcripts), trimming of mannose residues prior to recognition by E3 ubiquitin ligases (*EDEM1* and *EDEM3*), recognition of misfolded proteins by E3 ubiquition ligases (*DERL3/HRD1, DERL2, DERL1, HERP*, *RNF5,* and associated factors *SEL1L* and *FBX06*), exportation to the cytosolic side of the ER membrane (*SEC62*) where the VCP/p97 complex poly-ubiquitinates protein substrates before extracting/clipping the protein from the membrane to be ultimately degraded in the cytosol by the proteasome (Schroder, 2008; Merulla et al., 2013). The *VCP/p97* complex includes its cofactors *UFD1L* and *NPLOC4* and regulators *ATAXIN3* and *ARMET/erasin* as well as the E2 ubiquitin-conjugators *UBE2J2* and *UBE2G2* (Ballar et al., 2011).

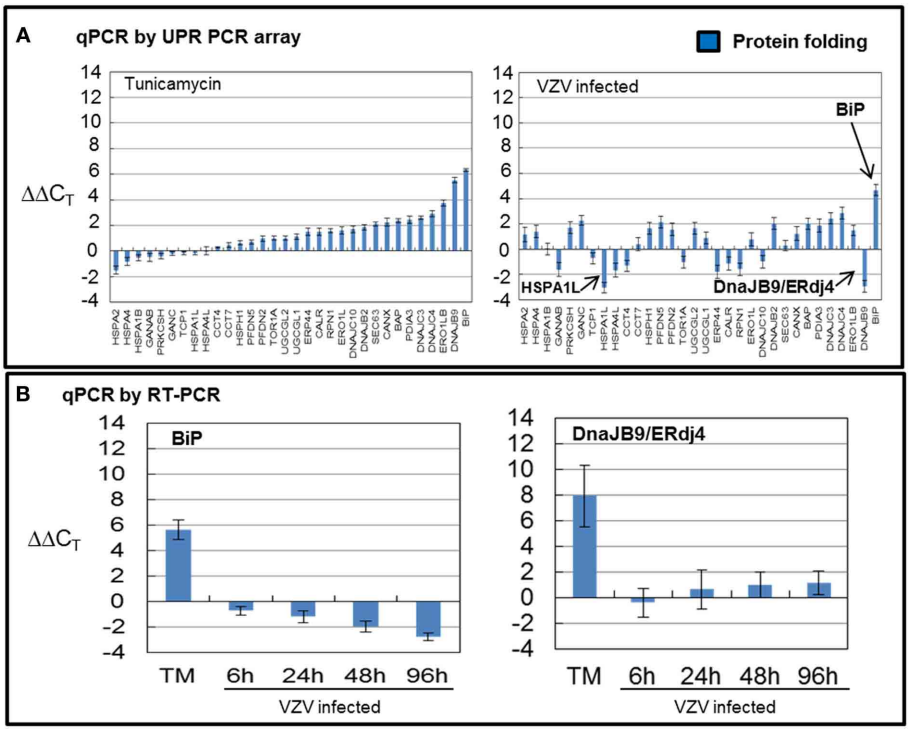

FIGURE 4 | VZV infected cells exhibited uneven transcription of protein folding genes. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was processed as described in legend to **Figure 2**. All gene transcription measurements were graphed for tunicamycin treated and VZV infected cell samples. **(A)** Using the UPR specific PCR array, tunicamycin treated cells exhibited significant upregulation of transcripts of FOLD chaperones while VZV infected cells exhibited a much more uneven pattern of up or down regulation of FOLD transcripts. In particular, *ERdj4/DNAJB9* and *HSPA1L* were downregulated with only *BiP* showing upregulation. **(B)** To assess some of the measurements by the UPR array, cDNA from VZV infected and tunicamycin treated cells was submitted for real-time (RT-) PCR, using primers specific to *BiP* and *ERdj4/DNAJB9* (see Methods section for primer information). Error bars correspond to standard deviation when averaging.

Finally, there is a cytosolic protease, *USP14*, included in this grouping.

While TM treatment showed almost complete upregulation of ERAD transcripts particularly *HERP* (**Figure 5A**), VZV infection showed considerable downregulation of ERAD transcript (**Figure 5A**) where *EDEM3*, *UBXN4/erasin* and *SEC62* were downregulated. However, *ATAXIN3* was upregulated in VZV infected cells. Both erasin and ATAXIN3 are regulators, positive and negative, respectively, of VCP/p97 (Lim et al., 2009; Liu and Ye, 2012). As noted above, VCP/p97 forms the protein complex in the ER membrane on the cytosolic side that poly-ubiqininates ERAD substrates that are then released into the cytosol to be degraded by the proteasome (Ballar et al., 2011). Downregulation of VCP/p97 via its regulators appeared to reduce ERAD in VZV infected cells. All observed differences were significant with $p < 0.001$. Again, in order to reassess two of the more striking observations from the UPR specific PCR array, we carried out qPCR measurements using primers to *UBXN4/erasin* and *ATAXIN-3* (**Figure 5B**). These measurements showed *UBXN4/erasin* to be modestly downregulated in VZV infected cells while ATAXIN-3 was essentially unchanged.

VZV INFECTED CELLS UPREGULATED TRANSCRIPTION OF CHOLESTEROL SYNTHESIS REGULATOR INSIG

Transcripts associated with lipid synthesis and metabolism such as *RAMP4* showed similar transcription in both VZV infected and TM treated cells but VZV infected cells, in particular, showed increased transcription of cholesterol synthesis regulators *AMFR/gp78* and *INSIG* (**Figure 6A**). *AMFR/gp78* is an E3 ubiquitin ligase and INSIG is an insulin signaling factor (Flury et al., 2005; Chen et al., 2012). Both are localized to the ER membrane and when activated function together to degrade HMG COA reductase, a cholesterol synthesis enzyme (Jo et al., 2011; Tsai et al., 2012).

In order to reassess the upregulation of *AMFR/gp78* and *INSIG*, we carried out qPCR measurements using primers to each transcript (**Figure 6B**). Of note, *INSIG* was upregulated in VZV infected cells at early timepoints in agreement with the UPR specific array, while *AMFR/gp78* was not increased. Of note, greater transcription of *INSIG* may lead to reduced cholesterol synthesis in VZV infected cells with the consequence of a more fluid ER in those cells. Cholesterol acts as a stabilizing agent in lipid membranes by supporting adjacent lipid head groups and reducing disorder of the lipid

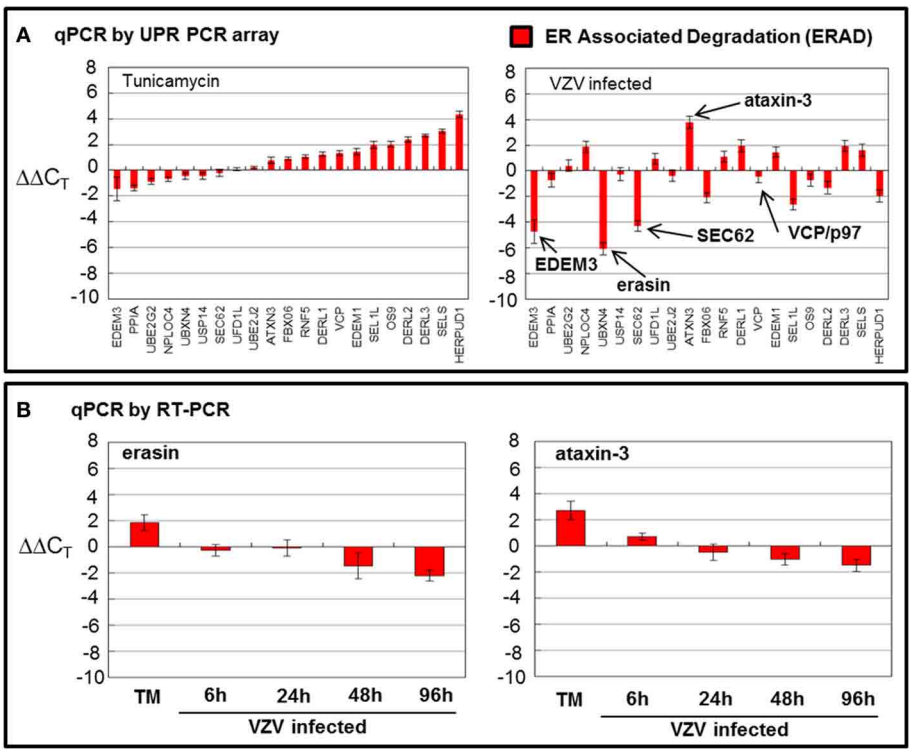

FIGURE 5 | VZV infection moderately downregulated ER associated degradation genes. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was processed as described in the legend to **Figure 2**. All gene transcription measurements were graphed for tunicamycin treated and VZV infected cell samples. **(A)** By measurements using the UPR specific PCR array, VZV infected cells showed significant downregulation of several elements of the ERAD pathway: *EDEM3, ERASIN,* and *SEC62*. Tunicamycin treatment, in contrast, upregulated most of the ERAD transcripts. **(B)** In order to assess two of the ERAD transcript measurements by the UPR specific PCR array, RT-PCR was carried out on cDNA from uninfected, tunicamycin treated and VZV infected cells, using primers to *UBXN4/erasin* and *ataxin-3*.

hydrocarbon chains internal to the bilayer (Mouritsen and Zuckermann, 2004). All observed differences were significant with $p < 0.001$.

VZV INFECTED CELLS DOWNREGULATED THE TRANSCRIPTION FACTOR *C/EBPb* AND DISPLAYED DIFFERENTIAL TRANSCRIPTION OF APOPTOTIC TRANSCRIPTS

Transcription of cellular transcription factor *C/EBPβ* was significantly downregulated ($p < 0.001$) in VZV infected cells as compared to the value in TM treated cells (**Figure 7A**) C/EBPβ is a transcription factor with a large effect on cellular proliferation (Tang and Lane, 2000). Downregulation of this factor may put VZV infected cells into a non-proliferative state. Transcription of apoptotic genes differed considerably between VZV infected cells and TM treated cells. TM treated cells exhibited much higher *CHOP* transcription than VZV infected cells while VZV infected cells showed a greater number of transcripts associated with cellular apoptosis such as *HTRA4* and the MAP kinases *JNK1* and *JNK3* (**Figure 7B**). Finally there was no difference between the levels of two protein translation associated transcripts in VZV infected cells vs. TM treated cells (**Figure 7C**).

TRANSFECTION OF VZV gE UPREGULATED TRANSCRIPTION OF *CREBH* AND *BiP* WHILE TRANSFECTION OF VZV IE62 DID NOT

In 2011, we found that transfecting cells with VZV glycoprotein genes led to increased autophagosome production and inflation of the ER. Transfection with VZV IE62 led to neither increased autophagosomes nor a larger ER. Therefore, we measured by qPCR whether CREBH and BiP transcription was increased by transfection of a glycoprotein vs. a non-glycoprotein that is also the major transactivator encoded by VZV. Transfection with a plasmid encoding VZV gE under the CMV immediate early promoter led to approximately 10% of transfected cells (**Figure 8A1**) while transfection with VZV IE62 also under the CMV immediate early promoter led to a larger number, approximately 40%, of transfected cells (**Figure 8A2**). Even though a low fraction of cells were transfected with VZV gE, increased transcription of *CREBH* and *BiP* were observed in those cells (**Figures 8B1,B2**) whereas not in cells transfected with VZV IE62 even though many more cells were transfected in those samples ($p < 0.01$). We therefore conclude that expression of a single VZV glycoprotein gene in cells is sufficient to activate the CREBH arm of the UPR.

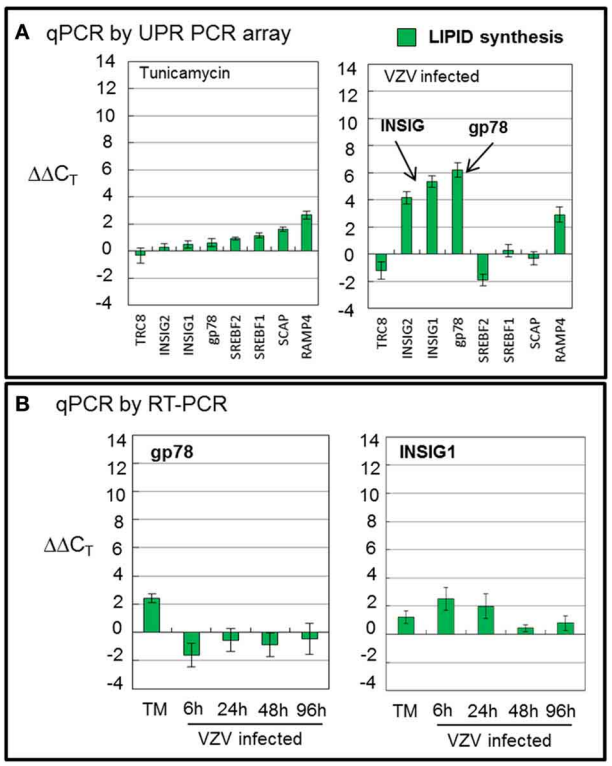

FIGURE 6 | VZV infection significantly upregulated the cholesterol synthesis associated transcript *INSIG*. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was processed as described in the legend to **Figure 2**. **(A)** Transcripts associated with lipid synthesis and metabolism where both VZV infected cells and tunicamycin cells showed similar transcription of *RAMP4;* VZV infected cells in particular showed more transcription of cholesterol synthesis regulator *INSIG*. **(B)** In order to assess two of the lipid transcript measurements by the UPR specific PCR array, RT-PCR was carried out on cDNA from uninfected, tunicamycin treated and VZV infected cells using primers to *AMFR/gp78* and *INSIG1*.

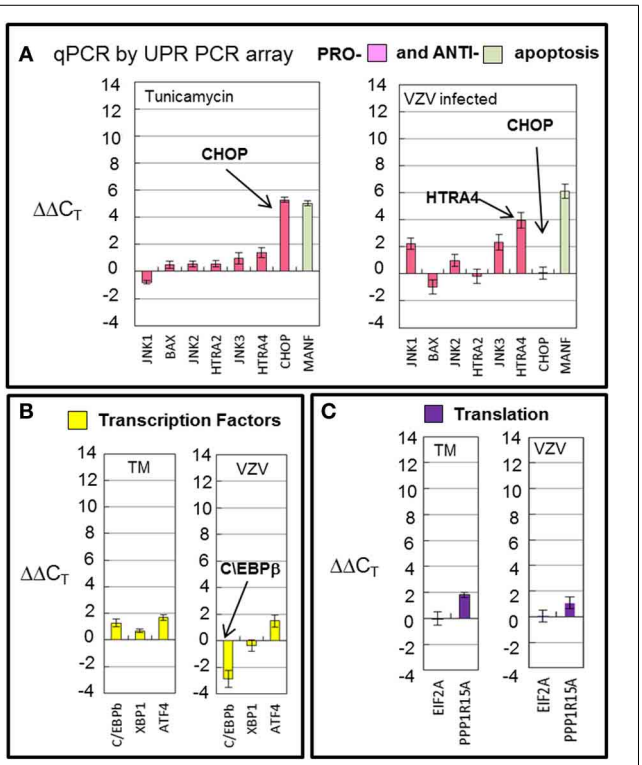

FIGURE 7 | VZV infection downregulated the transcription factor *C/EBPb* and displayed differential transcription of apoptotic transcripts. Human fibroblast cells (MRC-5) were grown in tissue culture plates then infected with VZV-32 infected MRC-5 cells or treated with tunicamycin (TM), a N-glycosylation inhibitor. At 72 hpi, RNA was processed as described in the legend to **Figure 2**. **(A)** Transcription of apoptotic genes differed considerately between VZV infected cells and tunicamycin treated cells. VZV infected cell transcripts showed very fewer *CHOP* transcripts when compared to TM treatment; infected cells had more transcripts associated with cellular apoptosis such as *HTRA4* and MAP kinases *JNK1* and *JNK3*. **(B)** Transcription of cellular transcription factor *C/EBPβ* was significantly downregulated in VZV infected cells as compared to the value in TM treated cells **(C)**. There was no difference between VZV vs. TM treatment for two protein translation associated transcripts. Error bars correspond to standard deviation when averaging.

DISCUSSION

We have previously documented that VZV infection induces an autophagic response in infected cells. The basic observation of this report is that VZV infected cells differentially activate the UPR to ER stress as compared to tunicamycin treated cells. The most straightforward explanation for this observation is that tunicamycin treatment produces many misfolded glycoproteins while VZV infection produces an overabundance of normally folded glycoproteins. The elements of the UPR activated in each situation would likely differ. As compared to the positive control of tunicamycin treated cells, VZV infected cells showed increased transcription of a gene associated with decreased cholesterol synthesis as well as increased transcription of the ER stress sensor and transcription factor, *CREBH*. At the same time, VZV infected cells showed decreased transcription of genes associated with ERAD and apoptosis. We hypothesize that this transcriptional profile is compatible with the infected cell attempting to accommodate the influx of viral glycoproteins by greatly increasing the capacity of the ER. For example, increased transcription of *AMFR* (*gp78*) and *INSIG* is associated with a decrease in cholesterol synthesis via degradation of HMG-CoA reductase, an enzyme necessary for cholesterol synthesis (Jo et al., 2011; Tsai et al., 2012). Decreased cholesterol content would increase the lability of the ER membrane and facilitate expansion of the ER (Mouritsen and Zuckermann, 2004).

We also found that some differences in transcription between tunicamycin treated cells vs. VZV infected cells as measured by the UPR specific PCR array could not be confirmed by qPCR, using individual primers selected by our laboratory. The reason behind these discrepancies is unclear but may center around two possibilities: (i) The choice of primers in the PCR array vs. those used in the qPCR measurements or (ii) the asynchronous nature of VZV infection. In general, there was better agreement between tunicamycin treated values as measured by the UPR-specific PCR

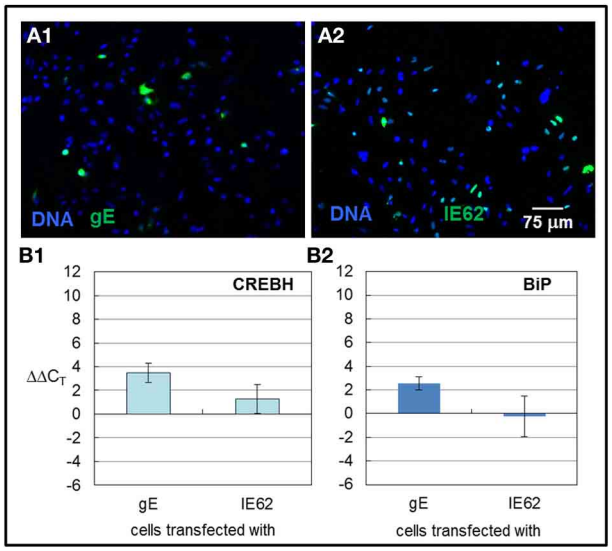

FIGURE 8 | CREBH and BiP transcription were upregulated in cells transfected with VZV gE but not VZV IE62. HeLa cells were grown in six well culture plates with or without glass coverslips and subsequently transfected with plasmids encoding the VZV gE glycoprotein or a non-glycosylated VZV IE62 protein, using either Lipofectamine 2000 or ExtremeGene HP transfection reagents. 6 h after the transfection reagent and plasmid were applied to the cells, the medium was replaced with fresh medium. Some monolayers were processed for microscopy while others were harvested for RNA extraction. The extracted RNA was converted to cDNA and RT-PCR was performed using primers against *CREBH* and *BiP*. **(A1,A2)** Representative images of cells transfected with VZV gE **(A1)** or VZV IE62 **(A2)**. (**B1,B2)** RT-PCR values were normalized to *GAPDH* and then differences to values measured for cells that were only treated with transfection reagent alone were computed.

array and individual qPCR assays. The biggest differences were observed in values measured from VZV infected cells. The latter scenario suggests that asynchronous VZV infection may play a role; for example, the input virus is always extremely low, such that some cells within a monolayer will remain uninfected even at 72 hpi. This same scenario may explain why we have observed similar unexpected differences in experiments to measure protein expression, for example, BiP. Because of increased VZV-induced autophagy, we predicted increased BiP production in infected cells. However, we have observed variable changes in BiP protein expression following VZV infection. Nevertheless, the main conclusions of this report were confirmed by both assays, namely, the significant upregulation of CREBH as well as the more modest upregulation of the cholesterol regulator INSIG.

A key question is whether the infected cell is responding to abundant viral glycoprotein expression through normal mechanisms or alternatively, does the virus encode its own proteins which manipulate the UPR. Recently, Burnett et al found that HSV ICP0 transactivated elements of the UPR and in turn was transactivated by the UPR itself via an ERSE promoter element in the HSV ICP0 gene (Burnett et al., 2012). Similarly, both the human and murine strains of the beta herpesvirus cytomegalovirus (CMV) encode proteins that manipulate the UPR—proteins that the VZV genome does not encode (Isler et al., 2005; Xuan et al., 2009; Qian et al., 2012; Stahl et al., 2013). VZV does encode a homolog of HSV ICP0—ORF61, but its promoter region does not appear to have any of the known UPR promoter elements: ERSE, ERSE-II, and UPRE based on bioinformatics searches (data not shown). It would be interesting to test VZV ORF61 against a luciferase reporter construct containing the BiP promoter element in future experiments.

In further support of our hypothesis that abundant expression of VZV glycoproteins contributes to the activation of the UPR in a specific way that leads to an enlarged ER and increased autophagosome production, we found that transfection of the VZV gE gene led to increased *CREBH* and *BiP* transcription. We observed abundant VZV gE protein in the ER/Golgi after transfection. In contrast, transfection with VZV IE62, a non-glycosylated viral protein, did not lead to increased transcription of either transcript. Obviously, the IE62 protein never enters the ER/Golgi. These results confirm and expand our 2011 report that transfection with VZV glycoprotein genes resulted in increased ER size and increased autophagosome production. The UPR is known to upregulate autophagy (Yorimitsu et al., 2006).

In summary, even though both tunicamycin treatment and VZV infection induced an UPR, the profiles of UPR related genes were different after the two analyses. The UPR in VZV infected cells exhibited greatly increased *CREBH* and cholesterol synthesis regulation transcription and diminished ERAD transcription. The transcription patterns appeared to correlate with increasing ER capacity secondary to increasing viral glycoprotein synthesis in the infected cell. Of importance, the CREBH data were totally unexpected, based on all prior VZV research, and would never have been uncovered in the absence of the UPR array data described in this report.

ACKNOWLEDGMENTS

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ERAD and how viruses exploit it

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Endoplasmic reticulum (ER)-associated degradation (ERAD) is a universally important process among eukaryotic cells. ERAD is necessary to preserve cell integrity since the accumulation of defective proteins results in diseases associated with neurological dysfunction, cancer, and infections. This process involves recognition of misfolded or misassembled proteins that have been translated in association with ER membranes. Recognition of ERAD substrates leads to their extraction through the ER membrane (retrotranslocation or dislocation), ubiquitination, and destruction by cytosolic proteasomes. This review focuses on ERAD and its components as well as how viruses use this process to promote their replication and to avoid the immune response.

Keywords: ERAD, immune response, retrotranslocation, ubiquitination, proteasomal degradation, retrovirus, herpesvirus, polyomavirus

INTRODUCTION

Although endoplasmic reticulum (ER)-associated degradation (ERAD) has been most thoroughly defined in yeast, recent studies in higher organisms have revealed the conservation of this process and its components. Multiple diseases, including Parkinson's, Alzheimer's, cancer, and infectious processes, result from failure of ERAD, confirming its significance for correct cell function. Predictably, viruses have exploited various aspects of this key cellular machinery to further their propagation. Nonetheless, the complexity of ERAD and the number of players involved necessitates a review of its features prior to a description of how viruses have manipulated ERAD to their advantage. In understanding how viruses exploit ERAD, we learn more about the cellular process, but also how we might alter the outcome of viral diseases.

THE ERAD PROCESS

A majority of newly synthesized proteins in mammalian cells are either misfolded or misassembled (Hoseki et al., 2010). Approximately 30% of new proteins are synthesized in association with the ER (Brodsky and Wojcikiewicz, 2009). The ER quality control system both senses and disposes of terminally misfolded proteins by ERAD, a process that is conserved in eukaryotes (Vembar and Brodsky, 2008; Merulla et al., 2013). This process detects misfolded proteins in the ER lumen, and then extracts them through membrane channels in an energy-dependent manner for delivery to cytosolic proteasomes (Olzmann et al., 2013). Protein extraction through ER membrane channels is known as dislocation or retrotranslocation (Hampton and Sommer, 2012). Because protein folding depends on multiple cellular components (Merulla et al., 2013), protein overexpression or the presence of mutant proteins may sequester limiting components, leading to accumulation of misfolded proteins in the ER lumen. A more general failure of the ERAD process may occur if proteins are unable to fold within a reasonable time, resulting in inefficient retrotranslocation and proteasomal degradation. Levels of ERAD-associated factors also may be affected by the intraluminal concentration of misfolded proteins. Inability of the ERAD system to destroy misfolded proteins is associated with more than 60 diseases, including neurological illnesses (Alzheimer's and Parkinson's), cystic fibrosis, infectious diseases, diabetes, and cancer (Guerriero and Brodsky, 2012). Particularly relevant to the subject of this review, viruses can produce large quantities of glycoproteins in a short period of time, which may overwhelm ERAD, leading to the accumulation of misfolded proteins, cell death, and associated pathology (Franz et al., 2014).

Although ERAD is vital to the maintenance of healthy cells, many parts of this process are not well characterized. Multiple aspects of ERAD have been described in yeast (Thibault and Ng, 2012), including the nature of the ER channel and the components needed to identify misfolded proteins during and after translation. Protein translocation across the ER membrane is the prerequisite for ERAD. Translation of many transmembrane proteins involves recognition of a hydrophobic signal peptide (SP) emerging from the ribosome by signal recognition particle (SRP), which is associated with the trimeric Sec61 complex. Many of the SPs are cleaved by signal peptidase, which is associated with the luminal side of the translocon (Auclair et al., 2012). The Sec61 complex provides the aqueous channel for co-translational transfer of proteins across the ER membrane (Loibl et al., 2014).

Recent evidence indicates that translocation across the ER membrane can occur through an SRP-independent process (Denic, 2012; Johnson et al., 2013). Based on recent experiments in yeast, more than 40% of signal-containing proteins fail to use SRP, including tail-anchored (TA) proteins and short secretory proteins (Johnson et al., 2012; Ast et al., 2013). Instead, these proteins are targeted by the GET pathway to the Sec61 translocon that is associated with the Sec 62/63 complex rather than through

docking to the SRP receptor (Rapoport, 2007; Ast et al., 2013). One large class of SRP-independent proteins includes the glycosylphosphatidylinositol (GPI)-anchored proteins, which contain both an N-terminal signal sequence and a C-terminal GPI anchor (Ast et al., 2013). This N-terminal signal is less hydrophobic than typical SRP targets. Furthermore, the Sec61 translocon has been implicated as the channel for retrotranslocation (Kiser et al., 2001), and it has been proposed that protein transfer can be either forward or reverse with respect to the ER lumen (Johnson and Haigh, 2000). Therefore, Sec61 appears to complex with a number of different proteins, leading to a highly flexible and dynamic structure, where association with different proteins/protein complexes leads to transit in or out of the ER (**Figure 1**).

SUBSTRATE RECOGNITION FOR ERAD

Reports in yeast indicate that proteins can be O-mannosylated prior to N-glycosylation (Ecker et al., 2003), and both types of glycosylation are believed to occur co-translationally (Loibl et al., 2014). These glycosylases also have been shown to be associated with the translocon (Chavan and Lennarz, 2006), and experiments indicate competition for different glycosylation sites (Loibl et al., 2014). The protein *O*-mannosyl transferases (PMTs) and the oligosaccharyltransferases (OSTs) are transmembrane proteins, but the latter catalyzes addition of oligosaccharides to nascent polypeptides on asparagine residues (Breitling and Aebi, 2013). The OSTs prefer NxT/S sequences in an unfolded or flexible protein domain, and the unfolded state may be facilitated by the OST complex associated with the translocon (Breitling and Aebi, 2013). Glycosylation near the C-terminal end of the protein is less efficient, perhaps due to competition between OSTs and protein folding (Ben-Dor et al., 2004; Breitling and Aebi, 2013). PMTs also are essential for ERAD in yeast. A Δ*pmt* mutant showed increased degradation of a typical ERAD substrate (Arroyo et al., 2011). Moreover, addition of oligosaccharides can be prevented by nearby cysteines and disulfide bond formation (Allen et al., 1995). Thus, glycosylation is one determinant of the correct folding of a protein in the ER lumen (Breitling and Aebi, 2013; **Figure 1A**).

The oligosaccharides on ER luminal proteins are critical for their correct folding or selection for ERAD. The nascent N-glycosylated protein has a three-branch structure with glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$-asparagine (Aebi et al., 2010; Merulla et al., 2013). Trimming of the first two glucose residues on one branch then allows interactions with two ER-resident chaperone/lectin proteins, calnexin and calreticulin, which may lead to protein folding (Brodsky, 2012). Removal of the third glucose causes release from these lectins and exit from the ER (Smith et al., 2011; Olzmann et al., 2013), but re-addition of this glucose by UDP-glucose:glycoprotein glucosyltransferase allows reassociation (Shenkman et al., 2013). Proteins retry folding until removal of three or four mannose residues triggers ERAD (Lederkremer and Glickman, 2005; Shenkman et al., 2013). Correctly folded proteins leave the ER after one or two mannose residues have been cleaved (Shenkman et al., 2013). Mannose removal is achieved using ER mannosidase I (ERmanI), the ER degradation-enhancing α-mannosidase-like proteins (EDEMs) and/or the Golgi-resident protein Man1C1 (Gonzalez et al., 1999; Hirao et al., 2006; Olivari et al., 2006; Hosokawa et al., 2007). Several lectins, OS-9 and XTP3-B, then interact via their MRH domains with the mannose-trimmed proteins, allowing their association with the retrotranslocon (Bernasconi et al., 2008; Christianson et al., 2008; Hosokawa et al., 2008). OS-9 and XTP3-B also associate with different proteases, LONP2 and carboxypeptidase vitellogenic-like protein (CPVL), respectively, suggesting that some substrates may be partially degraded prior to dislocation (Christianson et al., 2012; Olzmann et al., 2013). Nonetheless, multiple attempts are made to refold proteins before their triage through ERAD. The role of chaperones includes recognition of inappropriate glycosylation as well as refolding efforts, but proteins delivered to the retrotranslocon may require unfolding and partial proteolysis to allow their transit through the narrow membrane channel (Gogala et al., 2014).

Non-glycosylated proteins can be subjected to ERAD, but detection of misfolding of these proteins does not involve calnexin and calreticulin (Brodsky, 2012). Notably, the non-lectin chaperone BiP is involved in ERAD targeting of both types of proteins (Ushioda et al., 2013), yet also serves to prevent leakage of calcium out of the ER lumen (Schäuble et al., 2012). In addition, targeting of unglycosylated proteins to the proteasomes involves EDEM1 (Shenkman et al., 2013), which, like BiP, recognizes misfolded glycoproteins, as well as the transmembrane Herp protein (Usa1p in yeast; Okuda-Shimizu and Hendershot, 2007). Both glycosylated and their non-glycosylated derivatives are recruited to the ER-derived quality control compartment (ERQC) near the nucleus in the presence of a proteasomal inhibitor (Shenkman et al., 2013). Thus, these studies suggest that targeting of misfolded proteins for ERAD is similar for glycoproteins and non-glycosylated proteins (Shenkman et al., 2013).

Interaction of lectin-type and other chaperones with ERAD substrates allows association with members of the protein disulfide isomerase (PDI) family, which generally are characterized by one or more thioredoxin-like motifs (CXXC; Brodsky and Skach, 2011). Interestingly, these proteins can form, break, or rearrange disulfide bonds as well as act as chaperones (Benham, 2012). The yeast PDI family is composed of five members (Pdi1, Mpd1, Mpd2, Eug1, and Eps1), although only Pdi1 is essential (Farquhar et al., 1991). In mammalian cells, PDI is one of the best characterized family members, but there are at least 21 such enzymes (Benham, 2012; Grubb et al., 2012). PDI family proteins are generally confined by a KDEL retention sequence (Benham, 2012) to the ER, which has an oxidizing environment (Costantini et al., 2013). The oxidoreductase ERp57, which is localized near the ER-Golgi intermediate compartment (ERGIC), may provide some protection for proteins that might be routed for ERAD by calnexin (Frenkel et al., 2004). In addition, some PDI members can escape the secretory system and appear at the cell surface (Benham, 2012). For example, a disintegrin and metalloproteinase (ADAM17; also known as tumor necrosis factor alpha-converting enzyme or TACE) has been shown to be regulated by an extracellular activity of PDI (Bass and Edwards, 2010; Willems et al., 2010; Düsterhöft et al., 2013). PDI members also have a role in ERAD, with different requirements for different substrates (Grubb et al., 2012). In hepatic cells, PDI promotes the folding of apolipoprotein B (ApoB)

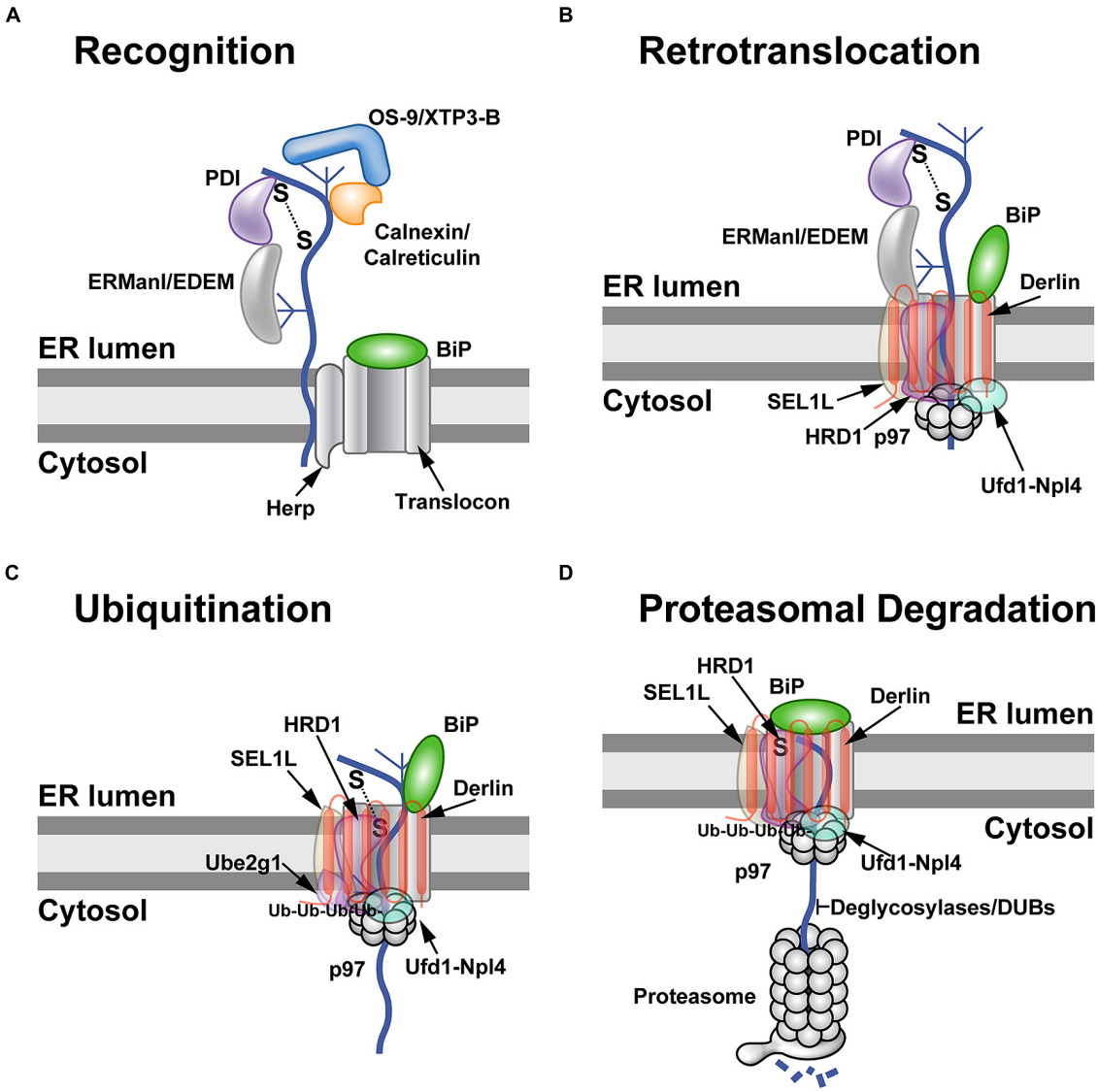

FIGURE 1 | The ERAD process. (A) Substrate recognition. Many nascent polypeptides (curved line) have one or more high-mannose carbohydrates (shown as a branched structure), which must be recognized and processed in a timely manner to allow exit from the ER. Binding of these ER-luminal proteins to substrates is affected by folding to their native conformations. Folding involves formation and breakage of disulfide bonds by members of the PDI family, such as ERp57 and ERp72, and is facilitated by chaperone proteins, such as BiP. Specific carbohydrates are bound by different chaperones/lectins in the ER lumen. These proteins include ERManI, EDEM, OS-9, XTP3-B, calreticulin, and calnexin. Recognition of ERAD substrates probably results in assembly of the retrotranslocon (shown here as Herp and the translocon/BiP complex). Herp is thought to facilitate oligomerization of the Hrd1 E3 ligase. BiP binds to a number of glycosylated and non-glycosylated ERAD substrates and provides a barrier on the ER luminal side of the translocon. **(B)** Retrotranslocation. Recognition of misfolded or misassembled proteins triggers the assembly of the retrotranslocon. Current evidence indicates that multiple types of retrotranslocons are possible (see text). A typical retrotranslocon/dislocon is shown containing Derlin, the E3 ligase Hrd1 and its partner Sel1L, which then recruits the cytosolic ATPase p97. Derlin has 6 transmembrane domains with both the N-terminus and C-terminus in the cytosol. Presumably some or all of the recognition components, such as PDI and ERManI, disengage as the substrate passes through the translocon. All retrotranslocation events appear to involve p97. The retrotranslocon is shown with BiP opening the Sec61 channel for substrate passage into the cytosol. **(C)** Ubiquitination of ERAD substrates. Retrotranslocation exposes ERAD substrates to cytosolic E1 (unknown), E2 (shown here as Ube2g1), and E3 enzymes (e.g., Hrd1). A polyubiquitin chain is produced as the substrate is engaged by the E2 and E3 proteins. Multiple E3s may be responsible for the polyubiquitin chains that then bind to the p97 partner proteins, Ufd1 and Npl4. The substrate is shown moving through the translocon into the center of the p97 hexamer. **(D)** Proteasomal degradation. Once the substrate has been retrotranslocated, the BiP protein seals the luminal side of the translocon. The retrotranslocon may then be disassembled prior to engagement of a new substrate. The retrotranslocated proteins must be modified by removal of carbohydrate and ubiquitin chains for insertion into the narrow channel of the proteasome. It is possible that p97 substitutes for the 19S lid, which provides access to the proteasome channel and the energy for unfolding of substrates. Degraded polypeptides are shown emerging from the 19S lid. This model suggests that there are retrotranslocon-specific proteasomes.

through its chaperone activity, whereas ERp57 or ERp72 expression leads to ERAD (Grubb et al., 2012). Further, various cell types express different PDI proteins, allowing differential regulation of substrates (Benham, 2012; Pescatore et al., 2012) and, presumably, their ERAD targeting.

Protein folding involves both formation of disulfide bonds and cis/trans isomerization of peptide bonds preceding proline residues (Hebert and Molinari, 2007). Certain ERAD substrates appear to be dependent on proline isomerization (Bernasconi et al., 2010b), and such refolding events may be necessary for transit through the retranslocon by elimination of turns in substrate secondary structure (Määttänen et al., 2010). ERAD requirements for peptidyl-prolyl *cis/trans* isomerases (PPIs) depend on whether the substrate is strictly in the ER lumen or is tethered to the ER membrane (Bernasconi et al., 2010b). The PPI protein cyclophilin B was needed for ERAD of a luminal target, but not the same target with a transmembrane domain (Bernasconi et al., 2010b). Requirement for PPIs during ERAD may depend on proline residues in the *cis* configuration (Bernasconi et al., 2010b), potentially by conversion into *trans* peptidyl–prolyl bonds, thus eliminating secondary structures that hinder retrotranslocation (Määttänen et al., 2010).

RETROTRANSLOCATION

Mammalian cells have ERAD factors that are not present in yeast. As observed for other pathways (Tsai and Weissman, 2012), ERAD components identified in yeast have multiple family members in higher eukaryotes; e.g., instead of a single Derlin in yeast (Der1p), mammalian cells have three proteins (Derlin-1, -2, and -3; Oda et al., 2006). Derlins are multiple membrane-spanning domain proteins that have been proposed to be part of the retrotranslocon channel (Ye et al., 2005) and/or regulatory factors for retrotranslocation (Brodsky, 2012; **Figure 1B**). In addition, Derlin-3 has a cell-type specific distribution (Oda et al., 2006), suggesting that recognition of certain substrates may be involved in its function. Derlins are related to rhomboid proteases, such as RHBDL4, which is an ER-resident transmembrane protein that cleaves unstable single-membrane-spanning or polytopic membrane proteins (Fleig et al., 2012). RHBDL4 also is upregulated by ER stress and binds to the cytosolic AAA ATPase p97 (see below; Fleig et al., 2012). In contrast to the rhomboid proteases, the Derlins lack proteolytic activity, suggesting that these proteins bind to ERAD substrates and target them to E3 ligases for ubiquitination and to p97 for membrane extraction (Brodsky, 2012). Cleavage of ERAD substrates by RHBDL4 (Fleig et al., 2012), SP peptidase (SPP; Loureiro et al., 2006), or proteases associated with OS-9 and XTP3-B (Olzmann et al., 2013) may occur prior to retrotranslocation of some substrates (Tsai and Weissman, 2012). On the other hand, it has been proposed that Derlins form a six-transmembrane structure with a gate that allows association and unfolding of substrates or access to other retrotranslocon components, such as p97 (see below; Olzmann et al., 2013). The p97 ATPase (Cdc48 in yeast) is bound to Derlin-1 and Derlin-2 through their SHP domains (Greenblatt et al., 2011).

Suppressor/enhancer of Lin12-like (SEL1L) appears to link luminal factors that recognize misfolding and inappropriate glycosylation, such as OS-9, XTP3-B, EDEMs, ERdj5, and the PDI protein ERp90, to components of the retrotranslocon (Olzmann et al., 2013; Williams et al., 2013). The transmembrane SEL1L protein (Hrd3p in yeast) also participates in regulation of ERAD by sequestering EDEM1 and OS-9 into ER-derived vesicles known as EDEMosomes (Bernasconi et al., 2012a). Inducible knockout of Sel1L in mice leads to death of adult mice from acute pancreatic atrophy (Sun et al., 2014). Sel1L expression is required for stability of the E3 ligase hydroxymethylglutaryl reductase degradation protein 1 (Hrd1), and its loss leads to ER stress and attenuates translation, leading to cell death. Other proteins have been described, such as Erlins 1 and 2 and TMUB1, which may act as adapters between polytopic membrane substrates and E3 ligases (Olzmann et al., 2013).

UBIQUITINATION

The ubiquitin ligases (E3s) have been proposed to be a structural part of the retrotranslocon channel (Brodsky, 2012), but their role is considerably more complex (**Figure 1C**). Several E3 ligases associated with ERAD are multiple membrane-spanning proteins with cytosolic RING domains (Smith et al., 2011; Ruggiano et al., 2014). In yeast, where ERAD has been studied most extensively, a prototypical transmembrane E3, such as Hrd1p (also called SYVN1; Nadav et al., 2003; Kikkert et al., 2004), can promote ERAD of a luminal substrate (ERAD-L). The ERAD process also involves Hrd3p (SEL1L in metazoans) as well as Usa1p and Der1p (Carvalho et al., 2010). Herp may assist with Hrd1 oligomerization (Carvalho et al., 2010), Nevertheless, the other components appear to be dispensable if Hrd1p is overexpressed, consistent with a role for Hrd1p in ERAD substrate transfer across the membrane (Carvalho et al., 2010), although such overexpression may be toxic due to inappropriate protein degradation (Denic et al., 2006). Thus, protein adapters appear to be necessary to achieve substrate specificity (Smith et al., 2011).

Hrd1p-mediated ERAD requires oligomerization and transmembrane domains as well as ubiquitin ligase activity (Carvalho et al., 2010). Overexpression of a dominant-negative RING mutant of the HRD1 ligase prevented ERAD of a non-glycosylated substrate, but a dominant-negative Fbs2 mutant (a component of SCF E3 ligases) did not (Shenkman et al., 2013). Dependence on HRD1 also is affected by tethering of the substrate to the ER membrane. Splice variants of the human beta-site amyloid precursor cleaving enzyme (BACE) with the same deletion mutation in the ectodomain are degraded through HRD1 if they are luminal (ERAD-L_S substrates), but disposal occurs in a HRD1-independent manner if the variant has a transmembrane domain (ERAD-L_M substrates; Bernasconi et al., 2010a). Therefore, HRD1 recognizes substrates for ubiquitination and, perhaps, modifies the translocon in the ER membrane.

Multiple E3 ligases participate in ERAD. These ligases include the transmembrane proteins gp78/AMFR (Fairbank et al., 2009), TRC8 (Stagg et al., 2009), RMA1/RNF5 (El Khouri et al., 2013), MARCH6/TEB4 (Doa10 in yeast; Kreft and Hochstrasser, 2011; Olzmann et al., 2013), and CHIP (Matsumura et al., 2013). An additional 40−50 membrane-spanning E3s may be involved in ERAD (Stagg et al., 2009). Other E3 ligases associated with ERAD

are localized to the cytosol, where they recognize misfolded glycoproteins that already have been retrotranslocated (Yoshida et al., 2005; Shenkman et al., 2013). These ubiquitin ligases are members of the cytosolic SCF (S-phase kinase-associated protein 1 (Skp1)-Cullin 1 (Cul1)-F-box) family, where the F-box components of the SCF complex recognize the N-glycans of the retrotranslocated substrate, e.g., Fbs1 and Fbs2 (Yoshida, 2007). Furthermore, E3s may work together to direct substrates for degradation (Olzmann et al., 2013).

PROTEASOMAL DEGRADATION

The p97 protein (Cdc48 in yeast) is a member of the AAA ATPase family (Erzberger and Berger, 2006) that functions during ERAD in a complex with several cofactors that have a ubiquitin-X (UBX) or UBX-like domain (Schuberth and Buchberger, 2008; **Figure 1**). These cofactors include the heterodimer nuclear protein localization homolog 4 (Npl4)–ubiquitin fusion degradation 1 (Ufd1; Meyer et al., 2012; Wolf and Stolz, 2012), p47, UBXD1, UBXD7, Ufd3/PLAA, VCIP135, and Ataxin-3 (Meyer et al., 2012). The UFD1L and NPL4 proteins are believed to form a heterodimer, where NPL4 is needed to stabilize UFD1L (Nowis et al., 2006). The heterodimer acts as a substrate adapter to the p97 ATPase associated with the retrotranslocon (Bays and Hampton, 2002). UFD1L and NPL4 bind to K48-linked and K63-linked polyubiquitin chains, respectively, which have been added by E3 ligases associated with the retrotranslocon (Ye et al., 2003; Komander et al., 2009).

In yeast, the Cdc48 ATPase binds to the Hrd1 E3 ligase in a RING-dependent manner (Hampton and Sommer, 2012), and the transmembrane Ubx2 (Sel1) protein acts as an adapter using a UBA domain (Neuber et al., 2005; Schuberth and Buchberger, 2005). Several other ubiquitin ligases bind p97 directly or through cofactors (Alexandru et al., 2008). The p97 cofactors act as ubiquitin-binding proteins, although p97 also has ubiquitin-binding activity (Ye et al., 2003; Meyer et al., 2012). The adapter-p97 complexes may recognize different substrates and perform independent functions, such as membrane protein segregation and trafficking, as well as directing substrates to the proteasome (Ritz et al., 2011). Alternatively, other models suggest that Derlins are involved in unfolding of substrates as well as providing contacts with p97 and its associated factors (Greenblatt et al., 2011). The p97 ATPase binds ubiquitin chain editors that can extend shorter chains as well as deubiquitinating enzymes (DUBs; Jentsch and Rumpf, 2007; Sowa et al., 2009). Two ATPase domains (D1 and D2; Meyer et al., 2012) within p97 form two stacked hexameric rings that provide the energy for protein remodeling and substrate extraction from the membrane or through the retrotranslocon (Hampton and Sommer, 2012). Mutations in the D2 domain result in dominant-negative proteins that bind, but fail to release, substrates (Pye et al., 2006). Mutant proteins have been widely used to study p97 function in ERAD and its myriad other activities (Meyer et al., 2012). Cytosolic chaperones, such as Hsp70, also may provide energy for extraction of membrane proteins with misfolded cytoplasmic domains (ERAD-C substrates; Taxis et al., 2003; Hrizo et al., 2007).

Once extraction from the ER membrane has occurred, p97 recruits peptide N-glycanase (PNGase) to cleave N-linked glycans from glycosylated substrates (Hirsch et al., 2003; Li et al., 2006; **Figure 1D**). In addition, p97 binds to a deubiquitinating enzyme YOD1, presumably so that polyubiquitin chains will not interfere with insertion into the proteasome (Ernst et al., 2009). The proteasome is a highly complex structure with a 19S lid that has an ATPase activity very similar to that of p97 (Lipson et al., 2008; Matouschek and Finley, 2012). These enzymes may function synergistically to deliver substrates to the 20S core (Hampton and Sommer, 2012). Alternatively, p97 may deliver certain substrates directly to the proteasome core (Matouschek and Finley, 2012). The proteasome core is composed of 28 subunits arranged into four rings, each composed of seven subunits (Bhattacharyya et al., 2014). Proteolytic activity is sequestered in the center of a narrow chamber formed by the rings and, therefore, only unfolded proteins can enter the chamber (Groll et al., 2000). The 19S lid, p97, or other activators provide docking for substrates and substrate modifying proteins as well as regulated opening of the chamber to allow access of unfolded proteins for degradation in the 26S core (Bhattacharyya et al., 2014).

Many questions remain about ERAD components and how they identify and interact with different substrates. Similar to our analysis of other cellular and molecular biological processes through virology, studies of viruses that use ERAD are likely to prove insightful.

VIRAL MANIPULATION OF THE IMMUNE RESPONSE BY ERAD

The ability of viruses to cause persistent infections is a consequence of downregulation or subversion of the immune response. The herpesviruses are known to cause persistent infections. One well-studied example of herpesvirus manipulation of the immune response is reduced cell expression of major histocompatibility complex class 1 (MHC-I) molecules by the viral proteins US2 and US11 (Wiertz et al., 1996). Both proteins are transmembrane glycoproteins and bind to newly made MHC-I to initiate retrotranslocation. Despite their similar function, US2 and US11 use different pathways for MHC-I degradation (**Figure 2**). US2-mediated degradation of MHC-I is independent of Derlin-1 and involves SPP (Loureiro et al., 2006), which cleaves many SPs following their removal from nascent ER-bound pre-proteins (Voss et al., 2013). Using an siRNA screen, TRC8 was identified as the E3 ligase involved in MHC-I degradation by US2, but knockdown of this transmembrane RING-type E3 had no effect on US11-mediated destruction of MHC-I (Stagg et al., 2009). The US2 cytosolic tail interacts with SPP and the p97 ATPase (Chevalier and Johnson, 2003; Loureiro et al., 2006), whereas TRC8 and US2 bind through their transmembrane domains (Stagg et al., 2009; **Figure 2A**).

Unlike the Derlin-independent mechanism proposed for US2, studies of the US11 protein facilitated identification of Derlin-1 and SEL1L as ERAD components (**Figure 1**; Lilley and Ploegh, 2004; Ye et al., 2004; Mueller et al., 2006). US11 does not require SPP for MHC-I degradation (Loureiro et al., 2006), but appears to interact with the E3 ligase MARCHVII/axotrophin (Flierman et al., 2006). The cytosolic domain of MHC-I is required for US11-mediated ERAD targeting (Story et al., 1999; Barel et al., 2003), and deletion of the C-terminal valine of MHC-I reduced interaction with Derlin-1 (Cho et al., 2013a). The ER luminal domain

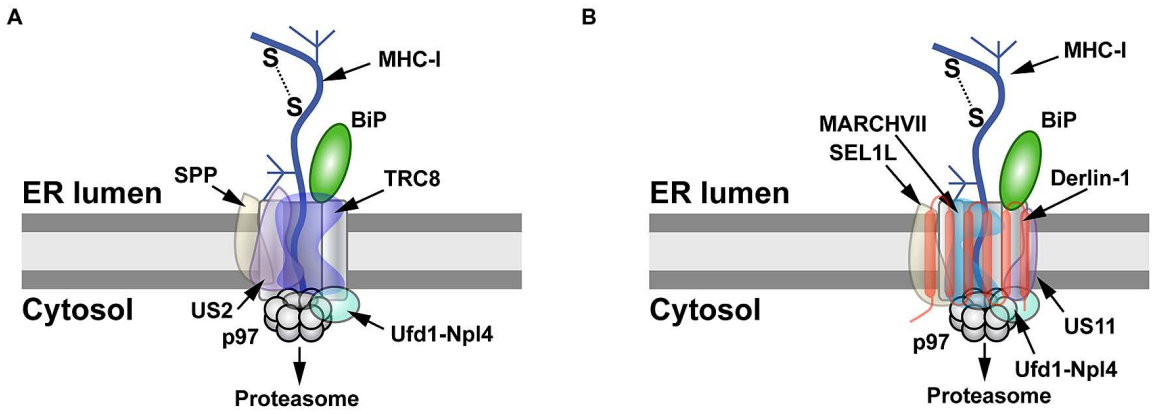

FIGURE 2 | MHC-I degradation by the herpesvirus US2 and US11 proteins. (A) Retrotranslocation of MHC-I by US2. US2 targets MHC-I molecules for retrotranslocation by a process that requires signal peptide peptidase (SPP), the E3 ligase TRC8, and the Ufd1-Npl4-p97 complex. SPP may induce partial degradation of the substrate prior to its proteasomal entry. **(B)** Retrotranslocation of MHC-I by US11. MHC-I is retrotranslocated after US11 recruits SEL1L, Derlin-1, the E3 ligase MARCHVII, and p97. It is not clear whether either degradation of MHC-I by US2 or US11 involves the adapter complex Ufd1-Npl4, which recognizes different types of polyubiquitin chains.

also affects degradation (Barel et al., 2003). In addition, MHC-I substituted with the transmembrane domain of US11 caused interaction with Derlin-1 and proteasomal degradation (Cho et al., 2013b). The p97 ATPase does not appear to interact directly with MHC-I, but requires the interaction of MHC-I cytosolic domain with the C-terminal domain of Derlin-1 (Cho et al., 2013a). Cho et al. speculated that US11 recognizes MHC-I through its cytosolic domain and transfers it to Derlin-1, which then interacts with the p97 ATPase for membrane dislocation (Cho et al., 2013a; **Figure 2B**). Therefore, studies of the herpesvirus US2 and US11 proteins revealed that the same substrate does not always use the same ERAD pathway, and presumably these viral proteins act as adapters that recognize different parts of MHC-I for targeting to the dislocon.

Herpesviruses use another mechanism to decrease levels of MHC-I. The mouse gammaherpesvirus 68 (MHV68) encodes an E3 ligase (mK3) that ubiquitinates newly made MHC-I heavy chains for proteasomal degradation (Boname and Stevenson, 2001). The mK3 ligase also is associated with the transporter-associated with antigen processing (TAP) as well as p97 and Derlin-1 (Wang et al., 2006). Polyubiquitination of MHC-I did not require lysines (Wang et al., 2005), but could occur on serine and threonine residues in the heavy chain C-terminal tail via the recruitment of the Ube2j2 E2 enzyme (see **Figure 1**; Wang et al., 2007, 2009; Herr et al., 2009). These data indicate that multiple ERAD mechanisms can be used by viruses to diminish the adaptive immune response.

Like the herpesviruses, retroviruses also manipulate the immune system through ERAD. Early studies indicated that human immunodeficiency virus type 1 (HIV-1)-infected cells had decreased levels of both CD4 mRNA and protein (Hoxie et al., 1986). CD4 acts as the receptor for binding the viral envelope (Env) protein (McClure et al., 1987). Furthermore, CD4 participates in T-cell activation by binding to both the T-cell receptor and MHC class II molecules on antigen-presenting cells. CD4+ T cells secrete cytokines that control antibody production, phagocytic cell function, and cytotoxic T-cell responses, making them crucial for adaptive immune responses (Tubo and Jenkins, 2014). HIV-1 encodes a number of accessory proteins, including Vpu, which are not required for virus replication in tissue culture, but contribute to viral pathogenesis (Strebel, 2013). Expression of Vpu and CD4 by transient transfection showed dramatic decreases in CD4 levels, and CD4 depletion was dependent on serines 52 and 56 in Vpu (Magadán et al., 2010).

Vpu-induced CD4 degradation has been shown to involve the ERAD system. Knockdown of both β-TrCP1 and β-TrCP2 largely prevented Vpu-mediated CD4 loss (Magadán et al., 2010). β-TrCP1 and β-TrCP2 (also known as FBW1A, FBXW1, FBXW1A, or FWD1) are F-box proteins containing WD40 domains, which are associated with the SCF family of ubiquitin ligases (**Figure 3**). These protein complexes are linked to regulation of multiple pathways involving cell cycle checkpoints, NFκB, and Wnt (Skaar et al., 2013). In addition, knockdown of p97, UFD1L (also called Ufd1) or NPL4 (see **Figure 1C**) blocked depletion of CD4 (Magadán et al., 2010). Mutations that prevented ATP binding or hydrolysis by p97 failed to affect CD4 levels (Magadán et al., 2010). These experiments indicated that Vpu uses ERAD to degrade CD4, but also prevents cell surface expression by retaining CD4 in the ER, probably through transmembrane domain interactions (Magadán et al., 2010). Moreover, Vpu used an atypical E3 ligase to induce ERAD (Margottin et al., 1998), and this process involved $SCF^{\beta-TrCP}$ ubiquitination of the CD4 cytosolic tail on lysine, serine, and threonine residues (Magadán et al., 2010). Thus, Vpu may act as an adapter between CD4, retrotranslocon components, and a cytosolic E3 ligase. CD4 degradation promotes HIV-1 infection by preventing re-infection, facilitating virus release by avoiding Env–CD4 interactions during their trafficking to the cell surface, and minimizing adaptive immune responses (Lanzavecchia et al., 1988; Willey et al., 1992; Argañaraz et al., 2003).

HIV-1 Vpu also targets another cellular protein, tetherin/BST-2, for ERAD (Neil et al., 2008; Mangeat et al., 2009). Tetherin is an unusual type II membrane protein with an N-terminal

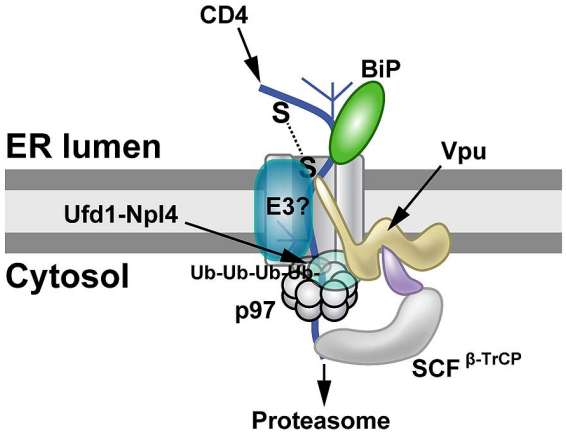

FIGURE 3 | Proteasomal targeting of CD4 by HIV-1 Vpu. The transmembrane protein Vpu recruits the E3 ligase complex $SCF^{\beta-TrCP}$. Knockdown of both β-TrCP1 and β-TrCP1 (shown to be contacting Vpu) can prevent CD4 degradation, suggesting that either F-box protein can provide a functional SCF complex for ubiquitination (Magadán et al., 2010). Another E3 ligase (E3?) also may be involved. The p97 ATPase with the adapters Ufd1 and Npl4 are required for CD4 degradation, but the UFD1L protein recognizes polyubiquitinated CD4. Lysine and serine/threonine residues in the CD4 cytosolic tail are needed for ubiquitination (Magadán et al., 2010).

transmembrane segment and a C-terminal GPI anchor (Kupzig et al., 2003; Sauter, 2014). Moreover, two tetherin monomers are bound together by disulfide bonds (Ishikawa et al., 1995; Kupzig et al., 2003). Using a unique method that only allows biotinylation of retrotranslocated molecules by cytosolic BirA protein, recent experiments indicate that both CD4 and tetherin remain glycosylated and retain disulfide bonds during retrotranslocation (Petris et al., 2014). These data suggest that the typical Sec61 channel used for translocation is insufficiently wide to accommodate retrotranslocation substrates modified with these structures (Petris et al., 2014), but an alternative model involving lipid droplet formation has not been confirmed (Olzmann and Kopito, 2011). Given the large number of proteins that have been implicated, a single mechanism for retrotranslocation is unlikely. Despite common delivery of substrates to the proteasome via the p97 ATPase, each of the previous examples of viral ERAD targeting involves different E3 ligases.

Recent evidence suggests that ERAD can target the retrovirus HIV-1 Env (Zhou et al., 2014), a glycosylated transmembrane protein. Studies of a human CD4+ T-cell line CEM.NKR indicated that HIV-1 replication is restricted in these cells, which also are resistant to natural killer cell-mediated lysis (Howell et al., 1985). Surprisingly, these cells overexpressed a mitochondrial translocator protein, TSPO (Braestrup and Squires, 1977; Papadopoulos et al., 2006), and knockdown or knockout of this protein rescued Env and HIV-1 production (Zhou et al., 2014). Further experiments indicated that drugs inducing ERAD led to recovery of Env levels and viral titers. These results suggested that the ER and mitochondria communicate through juxtaposition of their membranes, so that conditions in the mitochondria influence protein folding and ERAD. In support of this conclusion, gp78 is an ERAD-associated E3 ligase (Fang et al., 2001) localized to mitochondria–ER membrane contacts (Fu et al., 2013). Thus, mitochondria proteins may influence ERAD and modulate HIV-1 Env presentation to the immune system.

Triggering of an innate immune response to viruses is affected by the ERAD process. Some anti-viral signaling is controlled through mitochondria, which also cooperates with the ER for lipid synthesis and calcium-controlled processes at the mitochondrial-associated membrane (MAM; Jacobs et al., 2014). Mitochondrial antiviral signaling protein (MAVS; also called IPS-1, VISA, or CARDIF) binds to different retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) proteins, which sense cytosolic viral RNAs (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The MAVS protein is present in the mitochondrial and peroxisomal membranes, and viral RNA triggers both interferon-dependent or independent responses, respectively, (Jacobs and Coyne, 2013; Jacobs et al., 2014). The levels of MAVS are affected by gp78, an E3 ubiquitin ligase that is localized to the ER-mitochondrial interface (MAM; Jacobs et al., 2014). The gp78 ligase was detected by a high throughput RNAi screen to identify genes that restricted enterovirus replication (Coyne et al., 2011). Downregulation of gp78 was shown to decrease yields of vesicular stomatitis virus (VSV) and to increase type I interferon responses.

Some viruses, such as those inducing hepatitis B (HBV) or C (HCV), use ERAD to reduce the amounts of glycoproteins and particles produced. Interestingly, both viruses partially induce the unfolded protein response (UPR; Li et al., 2007, 2011; Saeed et al., 2011), which then increases the levels of certain ERAD components. HBV, a member of the *Hepadnaviridae*, triggers upregulation of the glycoside hydrolase 47 family enzymes, EDEM 1 and 2. Increased EDEM levels appear to bypass normal ER folding of HBV glycoproteins to result in ERAD (Lazar et al., 2012). HCV, a member of the *Flaviviridae*, induces primarily EDEM1 through the UPR and splicing of X-box binding protein 1. Further experiments suggested that elevated levels of EDEM 1 and 3 increase binding to SEL1L, an adapter to the retrotranslocon (**Figure 1**). Inhibition of EDEM binding to SEL1L interfered with ubiquitination of HCV Env protein, E2 (Saeed et al., 2011). Interestingly, infections by another member of the *Flaviviridae*, Japanese encephalitis virus, did not result in EDEM binding to the Env proteins, indicating that not all viral family members control Env proteins by this mechanism. Overall, manipulation of EDEM levels appears to be a common mechanism to reduce viral glycoprotein levels. Lowered amounts of Env proteins and virus particles then contribute to avoidance of innate and adaptive immunity, leading to chronic infections (Saeed et al., 2011; Lazar et al., 2012).

VIRAL ESCAPE FROM ERAD

A number of pathogens harness the ERAD process to facilitate various replication strategies. The best known examples are the bacterial AB toxins, particularly cholera toxin, which is thought to hijack the ERAD machinery for delivery to the cytosol (Hazes and Read, 1997). Cholera toxin has a catalytic A chain divided into two subunits (CTA1 and CTA2) inside a pore composed of five receptor-binding B subunits (Spangler, 1992). The holotoxin

binds to the ganglioside GM1 on the surface of gut epithelial cells, which then triggers toxin internalization and trafficking through the Golgi to the ER (Fujinaga et al., 2003). The A subunits are bound to the B subunits by disulfide bonds, and the toxin complex interacts with the ER-resident enzyme PDI (**Figure 1**). PDI is a redox-dependent chaperone that unfolds the toxin, which is then released in the oxidized state (Tsai et al., 2001). This unfolding event appears to be required for the ability of CTA1 to retrotranslocate to the cytosol, where it induces the ADP-ribosylation of the Gαs protein and, ultimately, opening of chloride channels leading to massive diarrhea (Muanprasat and Chatsudthipong, 2013).

As noted above, retrotranslocation of ERAD substrates is preceded by a recognition step. The chaperone BiP, which is known to be involved in identification of non-glycosylated ERAD substrates, and an ER-resident ATPase (Torsin A) promote CTA1 retrotranslocation (Tsai et al., 2001; Winkeler et al., 2003; Forster et al., 2006; Moore et al., 2010). Sel1L and ERdj5, a co-chaperone of BiP, also facilitate CTA1 retrotranslocation, where the J domain of ERdj5 is required (Williams et al., 2013). ERdj5 also binds to Sel1L, likely providing interaction with the Hrd1 E3 ligase (see **Figure 1**). Torsin A may provide the link to the membrane-resident Derlin-1 protein (Nery et al., 2011). CTA1 retrotranslocation appears to involve Derlin-1 (Bernardi et al., 2008) and the transmembrane ubiquitin ligases, Hrd1 and gp78 (Bernardi et al., 2010). Thus, multiple low affinity interactions are likely involved in the identification of CTA1 as a substrate and its delivery to the retrotranslocon.

Similar to other retrotranslocated substrates, the cytosolic p97 ATPase participates in CTA1 extraction from the ER membrane (Abujarour et al., 2005; Kothe et al., 2005). Nevertheless, CTA1 subverts the normal ERAD process by avoiding polyubiquitination (Rodighiero et al., 2002). The hypothesis that CTA1 avoids ubiquitination through the absence of lysines targeted for polyubiquitination was not substantiated by mutational analysis (Rodighiero et al., 2002). These results indicate that CTA1 employs many of the typical components used for ERAD targeting, including the E3 ligase, but it is unclear how polyubiquitination and degradation of the substrate are avoided. Therefore, retrotranslocon targeting and substrate extraction from the ER membrane is not necessarily coupled to ubiquitination, although ubiquitination may be required for proteasomal degradation.

Viral pathogens also use ERAD. Mouse mammary tumor virus (MMTV) is a betaretrovirus that subverts the ERAD process to complete its viral replication cycle. All retroviruses synthesize an unspliced viral RNA that requires export from the nucleus to the cytosol for translation or packaging into virus particles (Cullen, 2003). The unspliced RNAs of simple retroviruses have a highly structured *cis*-acting sequence, such as the constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV; Bray et al., 1994). The CTE facilitates RNA export through the typical TAP/NXF1-mediated pathway used by cellular mRNAs (Grüter et al., 1998). In contrast, the complex retroviruses encode an adapter protein, such as the Rev protein of HIV-1 (Hanly et al., 1989), which binds to a structured RNA element near the 3′ end of the genome (Daly et al., 1989; Zapp and Green, 1989). MMTV also produces a Rev-like protein, Rem, for export of unspliced RNA (Mertz et al., 2005), but Rem binding to viral RNA has additional translation-associated functions (Mertz et al., 2009b).

Unlike other complex retroviruses, Rem is made from an internally deleted form of the Env protein, and the export function resides in a long SP of 98 amino acids (Indik et al., 2005; Mertz et al., 2005). Interestingly, Rem is a precursor protein that is directed to the ER membrane for translation, where it appears to be cleaved by signal peptidase into the Rev-like Rem-SP and a C-terminal glycosylated product (Rem-CT) of unknown activity (Byun et al., 2010). Recent evidence indicates that Rem-SP uses retrotranslocation for extraction from the ER membrane, but, like cholera toxin, avoids proteasomal degradation (Byun et al., 2010, 2012).

Dultz et al. (2008) first reported that Rem is directed to the ER membrane for translation and cleavage by signal peptidase. They also suggested that the Rem precursor (the uncleaved protein) could be detected in the nucleus by fluorescence microscopy (Dultz et al., 2008). Byun et al. (2010) showed that mutation of the predicted signal peptidase cleavage site prevented the appearance of Rem-SP as detected by both Western blotting and a highly sensitive reporter assay for Rev-like function (Mertz et al., 2005; Byun et al., 2010). This assay requires binding to a specific RNA element in viral RNA (Müllner et al., 2008; Mertz et al., 2009a). Fluorescence experiments indicated that only the cleaved Rem-SP enters the nucleus, whereas the uncleaved form was highly unstable and localized to the cytosol (Byun et al., 2010). Furthermore, Rem-SP activity was inhibited by expression of a dominant-negative form of the p97 ATPase required for retrotranslocation (Byun et al., 2010). Rem-SP function also was reduced by the expression of a dominant-negative Derlin-1, but not Derlin-2 protein (Byun et al., in preparation). These results strongly suggest that Rem must be cleaved by signal peptidase prior to SP retrotranslocation to the cytosol and import into the nucleus for RNA binding (**Figure 4**).

Experiments indicate that an altered conformation of either the N-terminal Rem-SP in the cytosol or the ER-luminal portion of Rem affect folding and accessibility to signal peptidase, which is associated with translocons (Falk and Gilula, 1998). First, Rem tagging at the C-terminus with green fluorescent protein (Rem-GFP) resulted in a stable protein that was inefficiently cleaved and had little fluorescence (Mertz et al., 2005; Byun et al., 2012). Rem-GFP also had very low functional activity in reporter assays (Mertz et al., 2005). In contrast, Rem tagged at the N-terminus with GFP was cleaved normally, and GFP-Rem-SP localized to the nucleoli, a result typical of other Rev-like proteins (Cullen, 2003; Mertz et al., 2005). Second, deletion mutations of the Rem C-terminus greatly affected stability of the protein (Byun et al., 2012). Removal of the 50 C-terminal amino acids had little effect on the cleavage or stability of the protein, but deletion of 100 or 150 amino acids produced a highly unstable precursor that could be rescued by the proteasomal inhibitor MG-132 (Byun et al., 2012). Reduced cleavage of the precursor also was observed. Surprisingly, further deletion to give only the SP (Rem-SP) again yielded a stable protein (Byun et al., 2012). Third, substitution of the leucine at position 71 in the SP gave a stable precursor protein that was poorly cleaved by signal peptidase (Mertz et al., 2009a; Byun et al., 2010). An independent report indicated that residues

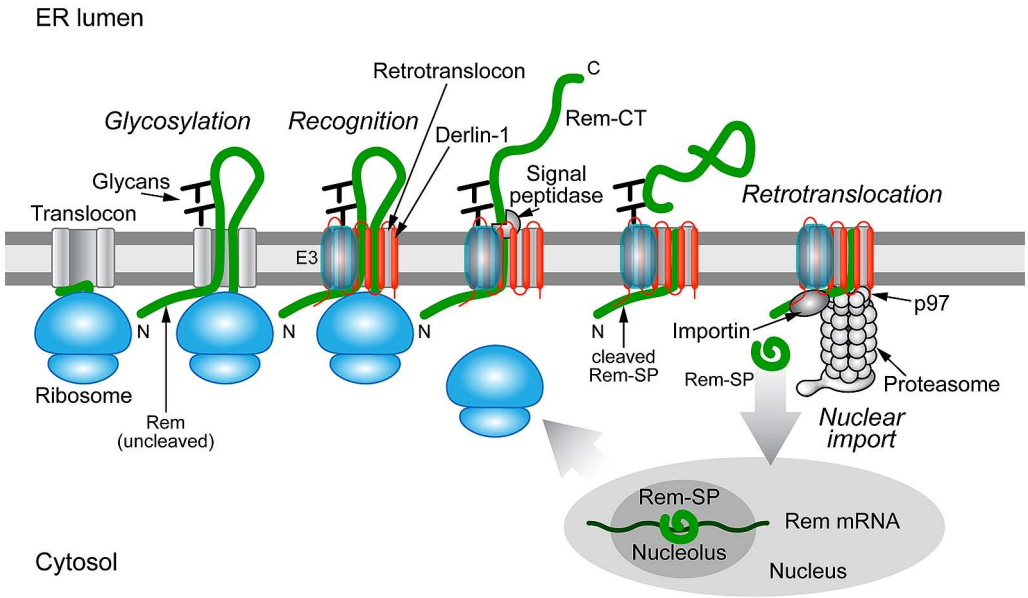

FIGURE 4 | Trafficking of the MMTV Rem protein by subversion of ERAD. Rem is a precursor protein that has an N-terminal signal peptide (Rem-SP) that directs translation to the ER membrane. The Rem-CT enters the ER lumen, where it is modified by N-glycosylation on two different sites. Rem recognition for retrotranslocation is not understood, but appears to involve Derlin-1 and, potentially, an E3 ligase, although ubiquitinated Rem has not been observed. Full-length Rem is cleaved by signal peptidase, and Rem-CT is released into the ER lumen. Similar to other retrotranslocation substrates, Rem-SP is extracted from the ER membrane using the p97 ATPase. Despite its dislocation into the cytosol, Rem-SP escapes the proteasome and translocates into the nucleus for binding of MMTV RNA. This figure is adapted from Byun et al. (2012).

80 through 98 act as the hydrophobic membrane anchor sequence, suggesting that position 71 is localized in the cytosol (Dultz et al., 2008). Recognition of Rem C-terminal sequences in the ER lumen, presumably by their interaction or lack of interaction with specific chaperone proteins, prevent degradation by ERAD.

The ER-luminal chaperone BiP has repeatedly been detected after purification and proteomic analysis of Rem-binding proteins (Gou et al., manuscript in preparation). Our preliminary data indicate that Rem-SP is not ubiquitinated, and it is possible that this feature protects Rem-SP from proteasomal degradation. Since the Rem precursor and C-terminal deletion mutants are subject to ERAD, cleavage and association with specific cellular proteins appear to be critical for avoidance of the degradative process. The idea that viral proteins manipulate E3 enzymes to form alternative complexes (Olzmann et al., 2013) would be consistent with Rem-SP escape from ERAD.

The polyomaviruses have a unique entry method that uses retrotranslocation, while avoiding ERAD. The BK polyomavirus (BKV) first binds to the ganglioside receptors GT1b and GD1b and enters through caveolae (Neu et al., 2009), which are composed of membrane microdomains/lipid rafts that are enriched for sphingolipids and signaling molecules (Head et al., 2014; **Figure 5**). Particle delivery to the cytosol occurs through a pH-dependent step involving endosomal trafficking via microtubules to the ER (Eash and Atwood, 2005; Moriyama and Sorokin, 2008; Jiang et al., 2009). Other members of the Polyomaviridae use caveolae-independent entry for ER delivery (Neu et al., 2009). ER localization of these viruses is necessary to access specific retrotranslocation components.

The VP1 capsid proteins of polyomaviruses form pentamers during assembly that are held together by disulfide bonding (Li et al., 2003). Each pentamer is associated with one molecule of either the minor capsid protein VP2 or VP3 (Barouch and Harrison, 1994), which become accessible to antibodies after exposure to the unique environment of the ER (Norkin et al., 2002). Particle delivery into the ER allows reduction and isomerization of disulfide bonds using ERp29 (mouse polyomavirus; Magnuson et al., 2005) or ERp57 and PDI (SV40; Schelhaas et al., 2007) to allow partial uncoating (Jiang et al., 2009; Tsai and Qian, 2010). The partially uncoated virion then engages the retrotranslocation machinery to allow cytosolic entry similar to cholera toxin (Neu et al., 2009).

Interestingly, different polyomaviruses use distinct Derlin family members for retrotranslocation. SV40 uses Derlin-1 and SEL1L (Schelhaas et al., 2007), whereas mouse polyoma virus uses Derlin-2 (Lilley et al., 2006; **Figure 5**). Additional experiments indicate that exposure of VP2 hydrophobic sequences tethers virus particles to the ER membrane, and that both BiP and BAP31 are needed for dislocation of SV40 to the cytosol (Geiger et al., 2011). BAP31 may serve as a shuttle to the ERQC that has been associated with enriched ERAD components (Kamhi-Nesher et al., 2001; Wakana et al., 2008). Furthermore, use of epoxomicin or eeyarestatin 1, inhibitors of the proteasome or p97 ATPase, respectively, blocked early events of BKV infection (Bennett et al., 2013). Epoxomicin treatment of cells allowed accumulation of BKV in the calnexin-rich, BiP-deficient ERQC (Bennett et al., 2013). These results are consistent with ERAD extraction of polyomaviruses from the ER to the cytosol, although it is has been suggested that

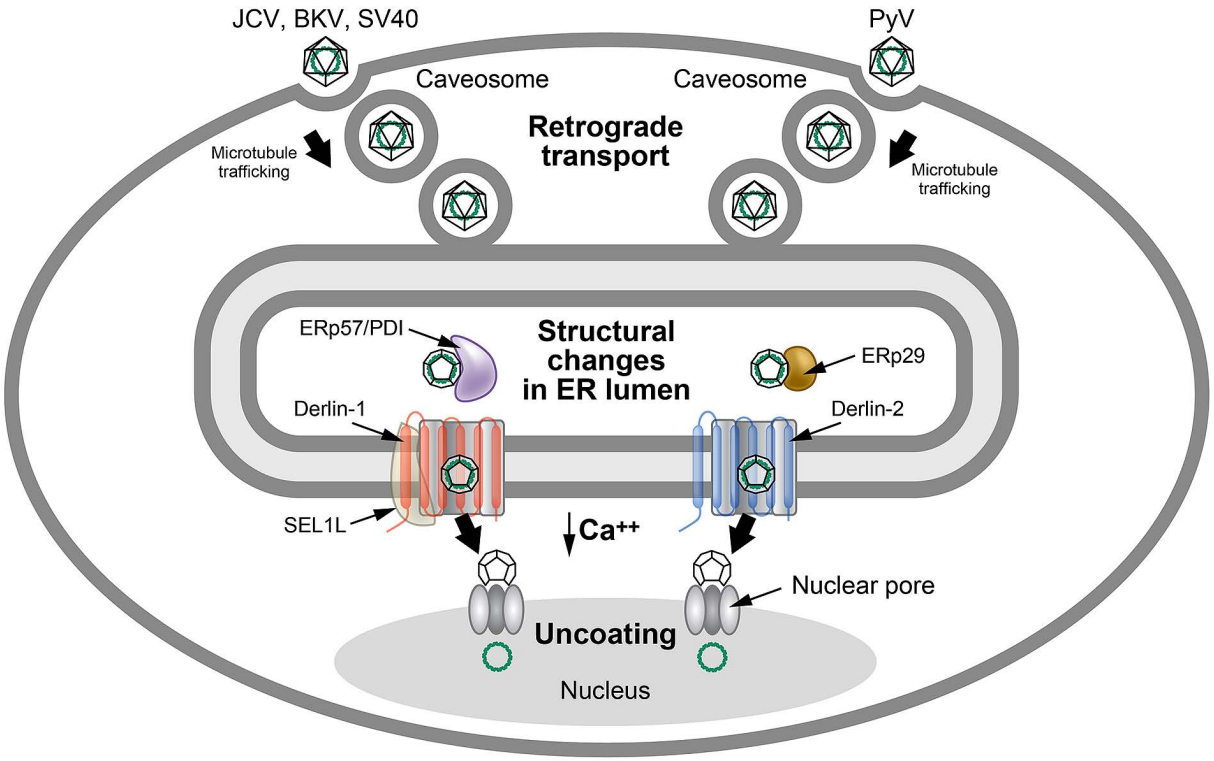

FIGURE 5 | Use of ERAD for polyomavirus uncoating. Many polyomaviruses enter through caveosomes that are enriched for viral entry receptors, triggering particle uptake through endosomes. Using the microtubule network, vesicles traffic the virus to the ER, where the unique environment allows structural changes to the icosahedral capsids. Studies of JCV, BKV, and SV40 indicate that viral particles interact with PDI and ERp57 in the ER lumen to rearrange capsid proteins. In contrast, the related mouse polyomavirus (PyV) uses the PDI family member, ERp29, presumably for a similar function. The altered particles then appear to engage different retrotranslocons (dependent on either Derlin-1 or Derlin-2) to induce retrotranslocation to the cytosol, where the reduced calcium environment produces further capsid rearrangements. These particles then bind to the nuclear pore where uncoating occurs to allow passage of viral DNA into the nucleus. This figure is adapted from Neu et al. (2009).

there are cell-type and virus-specific differences and that direct ER to nuclear transport may occur (Bennett et al., 2013). Low levels of calcium in the cytosol lead to further capsid destabilization and exposure of the nuclear localization signals on capsid proteins. The partially uncoated capsid then transits through the nuclear pores for initiation of viral DNA replication (Neu et al., 2009).

The preceding experiments indicate that ERAD is used by viruses to allow trafficking events that promote replication. MMTV Rem trafficking through the ER allows access to signal peptidase and cleavage of Rem precursor into functional N- and C-terminal proteins. In contrast, the polyomaviruses use ERAD to partially uncoat virions on their path to the nucleus. Importantly, both types of viruses avoid proteasomal degradation during ERAD, although the mechanisms remain unclear.

VIRUSES AND ERAD TUNING

ERAD may be regulated or "tuned" through the rapid turnover of specific components through the proteasomes or autophagosomes/vesicular trafficking to lysosomes (Merulla et al., 2013). Normal secretory vesicles released from the ER are 60–70 nm in diameter and have coatamer proteins, such as COPII, whereas the ER-derived tuning vesicles (EDEMosomes) lack coatamers and are 200–800 nm in diameter (Bernasconi et al., 2012b). Tuning vesicles contain SEL1L, EDEM1, and OS-9, which are transmembrane or luminal proteins involved in ERAD (**Figure 1**; Olzmann et al., 2013). EDEMosomes are believed to reduce ERAD by disposal in acidic organelles (Bernasconi et al., 2012b), favoring the correct folding of polypeptides (Calì et al., 2008). The coronaviruses are known to take advantage of ERAD tuning (Reggiori et al., 2010).

Many plus-stranded RNA-containing viruses manipulate cellular membranes to further RNA replication (Paul and Bartenschlager, 2013). These membrane structures have been divided into invaginated vesicle/spherule type and double-membrane vesicle (DMV) type (two lipid bilayers). Such vesicles allow viruses to concentrate their replication components, to separate distinct viral processes (e.g., translation, transcription, and replication), and to avoid immune detection (Paul and Bartenschlager, 2013). Severe acute respiratory syndrome coronavirus (SARS-CoV) and mouse hepatitis virus (MHV) induce DMVs for targeting their replication and transcription (Reggiori et al., 2010). The DMVs originate from ER membranes and contain the non-structural transmembrane proteins nsp3 and nsp4 and viral double-stranded RNA (Stertz et al., 2007; Reggiori et al., 2010). Nevertheless, DMVs lack markers typical of the ERGIC or the Golgi (Oostra et al., 2007).

Recent experiments indicate that DMVs are coated with microtubule-associated protein light chain 3 [LC3; Atg8 in yeast (Reggiori et al., 2010)], which is a ubiquitin-like modifier (van der Veen and Ploegh, 2012). LC3 can exist in a lipidated form (covalent linkage to phosphatidylethanolamine; also known as LC3-II) or a predominantly cytosolic non-lipidated form (LC3-I). LC3-II is believed to be involved in fusion of autophagosomes to lysosomes (van der Veen and Ploegh, 2012), but coronavirus DMVs display the non-lipidated LC3-I (Reggiori et al., 2010). These ubiquitin-like modifiers recognize specific receptors that target associated vesicles to particular cellular locations (van der Veen and Ploegh, 2012). The coronaviruses appear to be redirecting vesicles destined for autophagosomes to sequestered locations in the cytosol where replication will occur.

The autophagy machinery is not required for coronavirus replication, and no colocalization of viral non-structural proteins was observed with LC3-II-coated autophagosomes (Reggiori et al., 2010). Coronavirus-induced DMVs and EDEMosomes both are coated with the non-lipidated LC3-I protein (Calì et al., 2008; Reggiori et al., 2010), which is not covalently attached to membranes like LC3-II (Kabeya et al., 2000). Induction of autophagy in coronavirus-infected cells with rapamycin decreased the levels of EDEM1 and coronavirus (Reggiori et al., 2010). The virus-containing DMVs had both EDEM1 and OS-9, but not other ERAD-associated chaperones, and virus infection interfered with ERAD tuning by hijacking the EDEMosomes. Nevertheless, LC3-I, but not EDEM1 and OS-9, is necessary for coronavirus infection, and the hijacked EDEMosome cargo is not degraded by proteases in the endosomes/lysosomes (Reggiori et al., 2010). Further, the ERAD transmembrane adapter protein, SEL1L, is needed for DMV formation, capturing the ER-resident EDEM1 and OS-9 proteins (and possibly XTP3-B and EDEM3), while using its proline-rich cytosolic domain to bind to LC3-I. As expected, SEL1L knockdown impairs coronavirus replication (Bernasconi et al., 2012a).

The organizationally similar arterioviruses (classified with coronaviruses, toroviruses, and roniviruses into the order Nidovirales; Gorbalenya et al., 2006) subvert EDEMosome trafficking for their replication, although the size of the vesicles is smaller (Monastyrska et al., 2013). The mechanism for altering EDEM1-containing vesicular trafficking is unclear, but likely involves expression of viral non-structural proteins that span the ER-derived membranes (Monastyrska et al., 2013), perhaps through their interaction with SEL1L. These experiments indicate that viruses hijack EDEMosomes to sequester their double-stranded RNA from cytosolic sensors that will trigger interferon production and innate immunity (Zinzula and Tramontano, 2013). Other components of the ERAD system, particularly chaperone proteins, also participate in the replication and transmission of both plant and mammalian viruses (Verchot, 2014).

CONCLUSION

The ERAD system is a complex and highly regulated process controlling the disposal of misfolded or misassembled proteins that are directed to the ER for translation. Deregulation of this process results in pathogenic conditions, including infectious diseases. Viruses exploit ERAD to decrease overall viral levels and allow establishment of chronic infections by minimizing antigen presentation to the immune system. Trafficking of specific viral proteins or entire virion particles may involve ERAD for refolding or processing in the unique ER environment. Alternatively, viruses can use ERAD-associated components to form isolated lipid vesicles for replication and shelter from immune detection. Virus-mediated subversion of ERAD can lead to degradation of molecules that are involved in innate or adaptive immunity. Continued studies of viruses are certain to provide additional insights into both the ERAD process and the components that regulate it. Further experiments may identify targets for viral therapeutics.

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Can't RIDD off viruses

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The mammalian genome has evolved to encode a battery of mechanisms, to mitigate a progression in the life cycle of an invasive viral pathogen. Although apparently disadvantaged by their dependence on the host biosynthetic processes, an immensely faster rate of evolution provides viruses with an edge in this conflict. In this review, I have discussed the potential anti-virus activity of inositol-requiring enzyme 1 (IRE1), a well characterized effector of the cellular homeostatic response to an overloading of the endoplasmic reticulum (ER) protein-folding capacity. IRE1, an ER-membrane-resident ribonuclease (RNase), upon activation catalyses regulated cleavage of select protein-coding and non-coding host RNAs, using an RNase domain which is homologous to that of the known anti-viral effector RNaseL. The latter operates as part of the Oligoadenylate synthetase OAS/RNaseL system of anti-viral defense mechanism. Protein-coding RNA substrates are differentially treated by the IRE1 RNase to either augment, through cytoplasmic splicing of an intron in the *Xbp1* transcript, or suppress gene expression. This referred suppression of gene expression is mediated through degradative cleavage of a select cohort of cellular RNA transcripts, initiating the regulated IRE1-dependent decay (RIDD) pathway. The review first discusses the anti-viral mechanism of the OAS/RNaseL system and evasion tactics employed by different viruses. This is followed by a review of the RIDD pathway and its potential effect on the stability of viral RNAs. I conclude with a comparison of the enzymatic activity of the two RNases followed by deliberations on the physiological consequences of their activation.

Keywords: unfolded protein response, UPR, RNaseL, OAS, IRE1, Xbp1, RIDD pathway

INTRODUCTION

Establishment of infection by a virus, even in permissive host cells, is beset with a plethora of challenges from innate-antiviral and cell-death pathways. Therefore, the host response to a virus infection might prove to be inhibitory for the viral life cycle in a direct or an indirect manner. The direct mechanism involves expression of multiple anti-viral genes that have evolved to recognize, react, and thereby rid the infected host of the viral nucleic acid (Zhou et al., 1997; Thompson et al., 2011). On the other hand the pathways, e.g., those that culminate in initiating an apoptotic death for the host cell, indirectly serve to limit the spread of virus (Roulston et al., 1999). A major difference between these two mechanisms is that while the former response is transmissible to neighboring uninfected cells through interferon (IFN) signaling, the latter is observed mostly in *cis*. Recent reports, however, have demonstrated transmission of an apoptotic signal between cells that are in contact through gap junctions, although such a signaling from an virus infected host cell to an uninfected one is not known yet (Cusato et al., 2003; Udawatte and Ripps, 2005; Kameritsch et al., 2013). Successful viral pathogens, through a process of active selection, have evolved to replicate and simultaneously evade or block either of these host responses. The viral nucleic acids which could be the genome (positive-sense single-stranded RNA virus) or RNA derived from transcription of the genome [negative-stranded single-sense RNA or double-stranded RNA (dsRNA) or DNA virus], offer critical targets for both detection and eradication. The viral nucleic acid targeting armaments in the host arsenal include those that recognize the associated molecular patterns like toll-like receptors (TLRs), DDX58 (or RIG-1), IFIH1 (or MDA5), IFIT proteins [IFN-stimulated genes (ISG)56 and ISF54], etc. (Aoshi et al., 2011; Bowzard et al., 2011; Jensen and Thomsen, 2012). This is followed by IFN signaling and expression or activation of factors that target the inducer for degradation or modification like OAS/ribonuclease L (RNaseL) system, APOBEC3, MCPIP1, the ZC3HAV1/exosome system and RNAi pathways (Gao et al., 2002; Sheehy et al., 2002; Guo et al., 2007; Daffis et al., 2010; Sidahmed and Wilkie, 2010; Schmidt et al., 2012; Cho et al., 2013a; Lin et al., 2013). In this review we focus on two proteins containing homologous RNase domains, RNaseL with a known direct antiviral function and Inositol-requiring enzyme 1 (IRE1 or ERN1) which has an RNaseL-like RNase domain with a known role in homeostatic response to unfolded proteins in the endoplasmic reticulum (ER) and a potential to function as an antiviral (**Figure 1**; Tirasophon et al., 2000).

DEGRADATION OF VIRAL RNA BY RNaseL AND VIRAL EVASION

In mammalian cells the tell-tale signs of RNA virus infection, like the presence of cytosolic RNA having 5′-ppp or extensive (>30 bp) dsRNA segments are detected by dedicated pathogen associated molecular pattern receptors (PAMPs) or pattern recognition receptors (PRRs) in the host cell, like RIG-1, MDA5, and the IFIT family of proteins (Aoshi et al., 2011; Bowzard et al., 2011; Vabret and Blander, 2013). The transduction of a signal of this recognition results in the expression of IFN genes the products

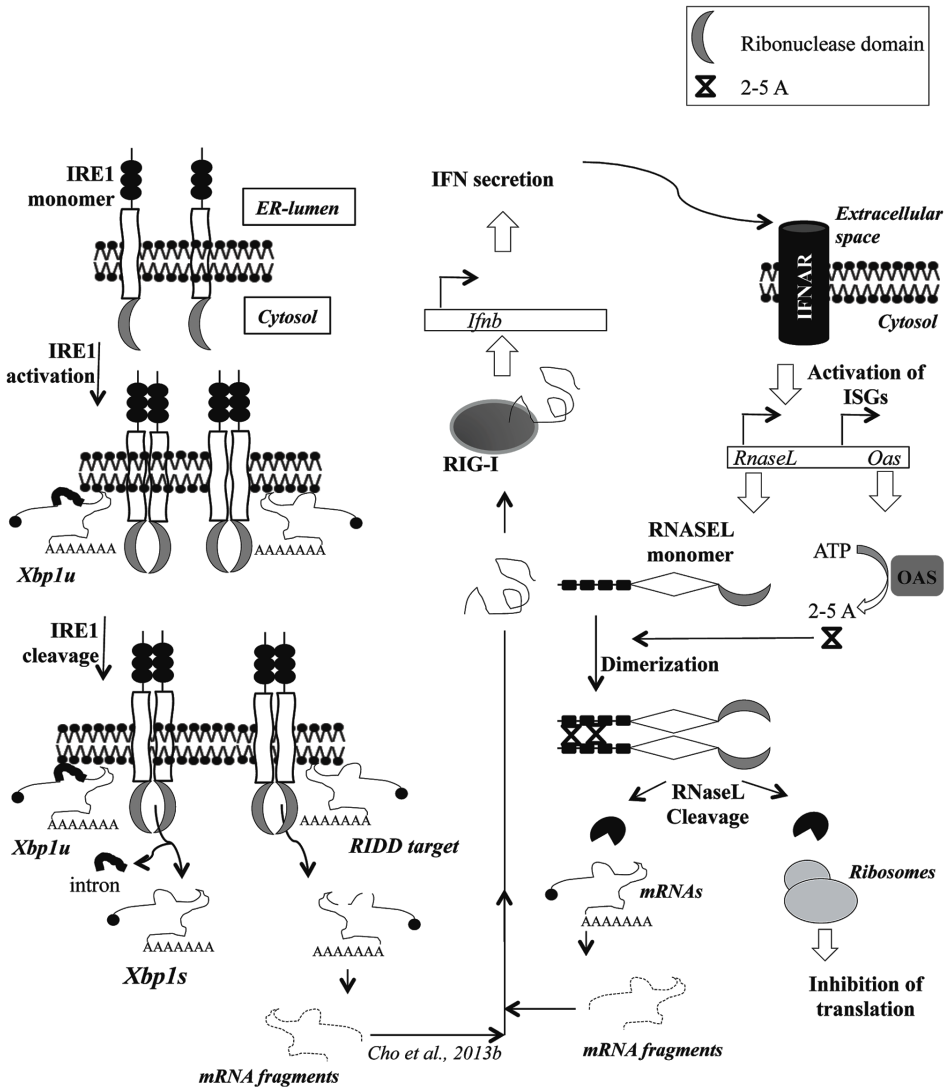

FIGURE 1 | Schematic representation of the ribonuclease activity of IRE1 and RNaseL showing cross-talk between the paths catalysed by the enzymes. The figure shows activation of RNase activity following dimerization triggered by either accumulation of unfolded proteins in the ER-lumen or synthesis of 2–5A by the enzyme OAS, respectively, for IRE1 and RNaseL. The cleavage of *Xbp1u* by IRE1 releases an intron thus generating *Xbp1s*. The IRE1 targets in RIDD pathway or all RNaseL substrates are shown to undergo degradative cleavage. The cleavage products generated through degradation of the respective substrate is shown to potentially interact with RIG-I thereby leading to Interferon secretion and trans-activation of *Oas* genes through Interferon signaling. Abbreviations: RIG-I = retinoic acid inducible gene-I, Ifnb = interferon beta gene loci, IFN = interferons, ISG = interferon-sensitive genes, 2–5A = 2′–5′ oligoadenylates.

of which upon secretion outside the cell bind to cognate receptors, initiating further downstream signaling (**Figure 1**; Randall and Goodbourn, 2008). The genes that are regulated as a result of IFN signaling are termed as IFN-stimulated or IFN-regulated genes (ISGs or IRGs; Sen and Sarkar, 2007; Schoggins and Rice, 2011). Oligoadenylate synthetase or OAS genes are canonical ISGs that convert ATP into 2′–5′ linked oligoadenylates (2–5A) by an unique enzymatic mechanism (**Figure 1**; Hartmann et al., 2003). Further, they are RNA-binding proteins that function like PRRs, in a way that the 2–5A synthesizing activity needs to be induced through an interaction with dsRNA (Minks et al., 1979; Hartmann et al., 2003). In a host cell infected by an RNA virus, such dsRNA is present in the form of replication-intermediates (RI), which are synthesized by the virus-encoded RNA-dependent RNA polymerases (RdRp) and subsequently used by the same enzyme to synthesize more genomic RNA, through asymmetric transcription (Weber et al., 2006). However, the replications complexes (RCs) harboring these RI molecules are found secluded inside host-membrane derived vesicles, at least in positive-strand RNA viruses, a group which contains many human pathogens (Uchil and Satchidanandam, 2003; Denison, 2008). Reports from different groups suggest OAS proteins to be distributed both in the cytoplasm as well as in membrane-associated fractions, perhaps indicating an evolution of the host anti-viral methodologies

towards detection of the membrane-associated viral dsRNAs (Marie et al., 1990; Lin et al., 2009). DNA viruses on the other hand, produce dsRNA by annealing of RNA derived from transcription of both strands in the same viral genomic loci, which are probably detected by the cytoplasmic pool of OAS proteins (Jacobs and Langland, 1996; Weber et al., 2006). Post-activation the OAS enzymes synthesize 2–5A molecules in a non-processive reaction producing oligomers which, although potentially ranging in size from dimeric to multimeric, are functionally active only in a trimeric or tetrameric form (Dong et al., 1994; Sarkar et al., 1999; Silverman, 2007). These small ligands, which bear phosphate groups (1–3) at the 5′ end and hydroxyl groups at the 2′ and 3′ positions, serve as co-factor which can specifically interact with and thereby allosterically activate, existing RNaseL molecules (Knight et al., 1980; Zhou et al., 1997, 2005; Sarkar et al., 1999). As part of a physiological control system these 2–5A oligomers are quite unstable in that they are highly susceptible to degradation by cellular 5′-phosphatases and PDE12 (2′-phosphodiesterase; Silverman et al., 1981; Johnston and Hearl, 1987; Kubota et al., 2004; Schmidt et al., 2012). Viral strategies to evade or overcome this host defense mechanism ranges from preventing IFN signaling which would hinder the induction of OAS expression or thwarting activation of expressed OAS proteins by either shielding the viral dsRNA from interacting with it or modulating the host pathway to synthesize inactive 2–5A derivatives (Cayley et al., 1984; Hersh et al., 1984; Rice et al., 1985; Maitra et al., 1994; Beattie et al., 1995; Rivas et al., 1998; Child et al., 2004; Min and Krug, 2006; Sanchez and Mohr, 2007; Sorgeloos et al., 2013). Shielding of viral RNA from interacting with OAS is possible through enclosure of dsRNA replication intermediates in membrane enclosed compartments as observed in many flaviviruses (Ahlquist, 2006; Miller and Krijnse-Locker, 2008; Miorin et al., 2013).

RNaseL is a 741 amino acid protein containing three predominantly structured region, an N-terminal ankyrin repeat domain (ARD), a middle catalytically inactive pseudo-kinase (PK) and a C-terminal RNase domain (**Figure 2A**; Hassel et al., 1993; Zhou et al., 1993). The activity of the RNase domain is negatively regulated by the ARD, which is relieved upon binding of 2–5A molecules to ankyrin repeats 2 and 4 followed by a conformational alteration (**Figure 1**; Hassel et al., 1993; Tanaka et al., 2004; Nakanishi et al., 2005). In support of this contention, deletion of the ARD has been demonstrated to produce constitutively active RNaseL, although with dramatically lower RNase activity (Dong and Silverman, 1997). However, recent reports suggest that while 2–5A links the ankyrin repeats from adjacent molecules leading to formation of dimer and higher order structures, at sufficiently high *in vitro* concentrations, RNaseL could oligomerize even in the absence of 2–5A (Han et al., 2012). Nonetheless, *in vivo* the RNaseL nuclease activity still seems to be under the sole regulation of 2–5A (Al-Saif and Khabar, 2012). In order to exploit this dependence, multiple viruses like mouse hepatitis virus (MHV) and rotavirus group A (RVA) have evolved to encode phosphodiesterases capable of hydrolysing the 2′–5′ linkages in 2–5A and thereby attenuate the RNaseL cleavage activity (Zhao et al., 2012; Zhang et al., 2013). In addition to 5′-phosphatases and 2′-phosphodiesterases to reduce

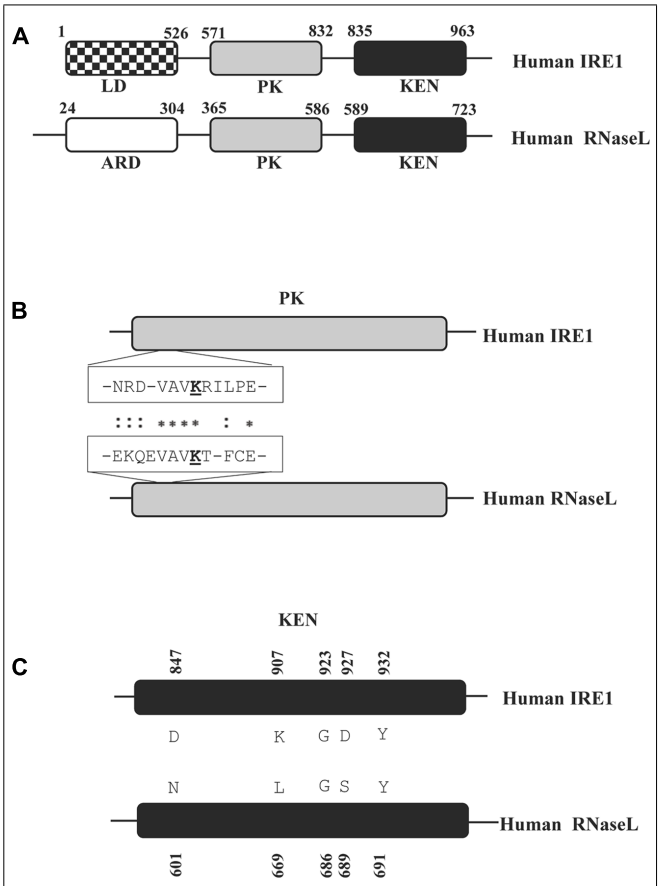

FIGURE 2 | Schematic representation of distinct protein domains in human RNaseL and IRE1. (A) The domains homologous between RNaseL and IRE1 are shaded identically. The domain name abbreviations denote the following: ARD = ankyrin repeat domain; LD = luminal domain; PK = protein kinase domain; KEN = kinase extension nuclease domain. The amino acid positions bordering each domain are numbered. The schematic drawings are not according to scale. **(B)** ClustalW alignment of primary sequence from a segment of the PK domain indicating amino acid residues which are important for interacting with nucleotide cofactors. The conserved lysine residues, critical for this interaction (K599 for IRE1 and K392 in RNaseL) are underlined. **(C)** Alignment of the KEN domains in RNaseL and IRE1. The amino acids highlighted and numbered in IRE1 are critical for the IRE1 RNase activity (Tirasophon et al., 2000).

the endogenous 2–5A levels, mammalian genomes encode post-transcriptional and post-translation inhibitors of RNaseL activity in the form of microRNA-29 and the protein ABCE1 (RNaseL inhibitor or RLI), respectively (Bisbal et al., 1995; Lee et al., 2013). Direct inhibition of RNaseL function is also observed upon infection by Picornaviruses through, either inducing the expression of ABCE1 or exercising a unique inhibitory property of a segment of the viral RNA (Martinand et al., 1998, 1999; Townsend et al., 2008; Sorgeloos et al., 2013).

Once activated by 2–5A, RNaseL can degrade single-stranded RNA irrespective of its origin (virus or host) although there seems to exist a bias towards cleavage of viral RNA (Wreschner et al., 1981a; Silverman et al., 1983; Li et al., 1998). RNA sequences that are predominantly cleaved by RNaseL are U-rich with the cleavage points being typically at the 3′ end of UA or UG or

UU di-nucleotides, leaving a 5′-OH and a 3′-monophosphate in the cleavage product (Floyd-Smith et al., 1981; Wreschner et al., 1981b). A recent report shows a more general consensus of 5′-UNN-3′ with the cleavage point between the second and the third nucleotide (Han et al., 2014). Cellular targets of RNaseL include both ribosomal RNA (rRNA) and mRNAs, the latter predominantly representing genes involved in protein biosynthesis (Wreschner et al., 1981a; Al-Ahmadi et al., 2009; Andersen et al., 2009). Additionally, RNaseL activity can also degrade specific ISG mRNA transcripts and thereby attenuate the effect of IFN signaling (Li et al., 2000). Probably an evolution towards insulating gene expression from RNaseL activity is observed in the coding region of mammalian genes where the UU/UA dinucleotide frequency is rarer (Bisbal et al., 2000; Khabar et al., 2003; Al-Saif and Khabar, 2012). Perhaps not surprisingly, with a much faster rate of evolution, similar observations have been made with respect to evasion of RNaseL mediated degradation by viral RNAs too (Han and Barton, 2002; Washenberger et al., 2007). Moreover, nucleoside modifications in host mRNAs, rarely observed in viral RNAs, have also been shown to confer protection from RNaseL (Anderson et al., 2011). In addition to directly targeting viral RNA, the reduction in functional ribosomes and ribosomal protein mRNA affects viral protein synthesis and replication in an indirect manner. Probably, as a reflection of these effects on cellular RNAs, RNaseL is implicated as one of the factors determining the anti-proliferative effect of IFN activity (Hassel et al., 1993). The anti-viral activity of RNaseL extends beyond direct cleavage of viral RNA, through stimulation of RIG-I by the cleavage product (Malathi et al., 2005, 2007, 2010). A global effect of RNaseL is observed in the form of autophagy induced through c-jun N-terminal kinase (JNK) signaling and apoptosis, probably as a consequence of rRNA cleavage (Li et al., 2004; Chakrabarti et al., 2012; Siddiqui and Malathi, 2012). RNaseL has also been demonstrated to play a role in apoptotic cell death initiated by pharmacological agents extending the physiological role of this pathway beyond the boundary of being only an anti-viral mechanism (Castelli et al., 1997, 1998).

IRE1 AND THE RIDD PATHWAY

The ER serves as a conduit for maturation of cellular proteins which are either secreted or destined to be associated with a membrane for its function. An exclusive microenvironment (high Calcium ion and unique ratio of reduced to oxidized glutathione) along with a battery of ER-lumen resident enzymes (foldases, chaperones, and lectins) catalyse/mediate the necessary folding, disulfide-bond formation, and glycosylation reactions (Schroder and Kaufman, 2005). A perturbation of the folding capacity, due to either physiological disturbances or virus infection, can lead to an accumulation of unfolded proteins in the ER lumen, which signals an unfolded protein response (UPR). UPR encompasses a networked transcriptional and translational gene-expression program, initiated by three ER-membrane resident sensors namely IRE1 or ERN1, PKR-like ER Kinase (PERK or EIF2AK3) and activating transcription factor 6 (ATF6; Hetz, 2012). IRE1 is a type I single-pass trans-membrane protein in which, similar to what is observed with RNaseL, the N-terminal resident in the ER lumen serves as sensor and the cytosolic C-terminal as the effector (**Figure 1**; Chen and Brandizzi, 2013). The IRE1 coding gene is present in genomes ranging from yeast to mammals and in the latter is ubiquitously expressed in all tissues (Tirasophon et al., 1998). Signal transduction by stimulated IRE1 initiates multiple gene regulatory pathways with either pro-survival or pro-apoptotic consequences (Kaufman, 1999). During homeostasis or unstressed conditions the sensor molecules are monomeric, a state maintained co-operatively by the " absence" of unfolded proteins and the "presence" of HSPA5 (GRP78 or Bip, an ER-resident chaperone) molecules bound to a membrane-proximal disordered segment of the protein in the ER-lumen-resident N-terminus (Credle et al., 2005). Accumulated unfolded proteins in the lumen triggers coupling of this domain from adjacent sensor molecules through a combination of (a) titration of the bound HSPA5 chaperone molecules and (b) direct tethering by malfolded protein molecules (Shamu and Walter, 1996; Credle et al., 2005; Aragon et al., 2009; Korennykh et al., 2009). Abutting of the luminal domains juxtapose the cytosolic C-terminal segments, leading to an aggregation of the IRE1 molecules into distinct ER-membrane foci (Kimata et al., 2007; Li et al., 2010). The C-terminal segment has a serine/threonine kinase domain and a RNase domain homologous to that of RNaseL (**Figure 1**; Tirasophon et al., 1998, 2000). A *trans*-autophosphorylation by the kinase domain allosterically activates the RNase domain (Tirasophon et al., 2000; Lee et al., 2008; Korennykh et al., 2009). In fact, exogenous over-expression of IRE1 in mammalian cells lead to activation suggesting that, under homeostatic conditions, the non-juxtaposition of cytosolic domains maintains an inactive IRE1 (Tirasophon et al., 1998). Once activated, IRE1 performs cleavage of a variety of RNA substrates mediated by its RNase domain, in addition to phosphorylating and thereby activating JNK (Cox and Walter, 1996; Urano et al., 2000). Depending on the RNA substrate, the cleavage catalyzed by IRE1 RNase produces differential consequence. Although scission of the *Xbp1* mRNA transcript at two internal positions is followed by splicing of the internal segment through ligation of the terminal cleavage products, that in all other known IRE1 target RNA is followed by degradation (**Figure 1**; Sidrauski and Walter, 1997; Calfon et al., 2002). The latter mode of negative regulation of gene expression is termed as the regulated IRE1-dependent decay (RIDD) pathway (Hollien and Weissman, 2006; Oikawa et al., 2007; Iqbal et al., 2008; Lipson et al., 2008). Gene transcripts regulated by RIDD pathway includes that from IRE1 (i.e., self-transcripts), probably in a negative feedback loop mechanism (Tirasophon et al., 2000). In addition to protein coding RNA, RIDD pathway down-regulates the level of a host of microRNA precursors (pre-miRNAs) and can potentially cleave in the anti-codon loop of $tRNA^{Phe}$ (Korennykh et al., 2011; Upton et al., 2012).

The IRE1 RNase domain cleaves the *Xbp1u* (u for unspliced) mRNA transcript at two precise internal positions within the open reading frame (ORF) generating three segments, the terminal two of which are ligated by a tRNA ligase in yeast and by an unknown ligase in mammalian cells, to produce the *Xbp1s* (s for spliced) mRNA transcript (**Figure 1**; Yoshida et al., 2001). The *Xbp1s* thus generated has a longer ORF, which is created by a frame-shift

in the coding sequence downstream of the splice site (Cox and Walter, 1996; Calfon et al., 2002). A similar dual endonucleolytic cleavage is also observed to initiate the XRN1 and Ski2-3-8 dependent degradation of transcripts in the RIDD degradation pathway (Hollien and Weissman, 2006). The RIDD target transcript genes are predominantly those that encode membrane-associated or secretory proteins and which are not necessary for ER protein-folding reactions (Hollien and Weissman, 2006). The cleavage of *Xbp1* and the RIDD-target transcripts constitute homeostatic or pro-survival response by IRE1 since XBP1S *trans*-activates genes encoding multiple chaperones (to fold unfolded proteins) and the ERAD pathway genes (to degrade terminally misfolded proteins) whereas RIDD reduces flux of polypeptides entering the ER lumen (Lee et al., 2003; Hollien and Weissman, 2006). On the other hand, cleavage of pre-miRNA transcripts which are processed in the cell to generate CASPASE-2 mRNA (*Casp2*) controlling miRNAs, constitutes the pro-apoptotic function of IRE1 (Upton et al., 2012). Another pro-apoptotic signal from IRE1 emanates from signaling through phosphorylation of JNK1 (Urano et al., 2000). Although in the initial phase RIDD activity does not cleave mRNAs encoding essential ER proteins, at later stages of chronic UPR such transcripts are rendered susceptible to degradation promoting apoptosis induction (Han et al., 2009; Bhattacharyya et al., 2014).

Infection of mammalian cells by a multitude of viruses induce an UPR which is sometimes characterized by suppression of signaling by one or more of the three sensor(s; Su et al., 2002; Tardif et al., 2002; He, 2006; Yu et al., 2006, 2013; Medigeshi et al., 2007; Zhang et al., 2010; Merquiol et al., 2011). Among these at least two viruses from diverse families, HCMV (a DNA virus) and hepatitis C virus (a hepacivirus), interfere with IRE1 signaling by different mechanism (Tardif et al., 2004; Stahl et al., 2013). An observed inhibition of any cellular function by a virus infection could suggest a potential anti-virus function for it, which the virus has evolved to evade through blocking some critical step(s). In both the cases mentioned above, stability of the viral proteins seems to be affected by ERAD-mediated degradation, although other potential anti-viral effect of IRE1 activation are not clear yet (Isler et al., 2005; Saeed et al., 2011). Interestingly, host mRNA fragments produced following IRE1 activation during bacterial infection, has been shown to activate RIG-I signaling (**Figure 1**; Cho et al., 2013b). Theoretically, other functions of IRE1 can also have anti-viral effect necessitating its inhibition for uninhibited viral replication. It is, however, still not clear whether IRE1 is able to cleave any viral RNA (or mRNA) in a manner similar to that of other RIDD targets (**Figure 1**). The possibilities of such a direct anti-viral function are encouraged by the fact that all these viruses encode at least one protein which, as part of its maturation process, requires glycosylation and disulfide-bond formation. Such a necessity would entail translation of the mRNA encoding such a protein, which in case of positive-sense single-stranded RNA viruses would mean the genome, in association with the ER-membrane (**Figure 1**; Lerner et al., 2003). Additionally for many RNA viruses, replication complexes are housed in ER-derived vesicular structures (Denison, 2008; den Boon et al., 2010). Considering the proximity of IRE1 and these virus-derived RNAs it is tempting to speculate that probably at some point of time in the viral life cycle one or more virus-associated RNA would be susceptible to cleavage by IRE1. However, studies with at least two viruses have shown that instead of increasing viral titre, inhibiting the RNase activity of activated IRE1 has an opposite effect (Hassan et al., 2012; Bhattacharyya et al., 2014). This implies potential benefits of IRE1 activation through one or more of the following, (a) expression of chaperones or other pro-viral molecules downstream of XBP1S-upregulation or JNK-activation, (b) cleavage of potential anti-viral gene mRNA transcripts by RIDD activity. However, the mode of protection for the viral RNA from RIDD activity is still not clear. It is possible that the viral proteins create a subdomain within the ER membrane, which through some mechanism excludes IRE1 from diffusing near the genomic RNA, thereby protecting the replication complexes (Denison, 2008). It is therefore probably not surprising that single-stranded plus-sense RNA viruses encode a polyprotein, which produces replication complexes in *cis*, promoting formation of such subdomains (Egger et al., 2000). The fact that IRE1 forms bulky oligomers of higher order probably aggravates such an exclusion of the activated sensor molecules from vicinity of the viral replication complexes. The UPR signaling eventually attenuate during chronic ER-stress and since that is what a virus-induced UPR mimics, probably the viral RNA needs protection only during the initial phase of UPR activation (Lin et al., 2007). Since the choice of RIDD target seems to be grossly driven towards mRNAs that encode ER-transitory but are not ER-essential proteins, it is also possible that one or more viral protein have evolved to mimic a host protein the transcript of which is RIDD-resistant (Hollien and Weissman, 2006). Most of the RIDD target mRNA are observed to be ER-membrane associated, the proximity to IRE1 facilitating association and cleavage (**Figure 1**; Hollien and Weissman, 2006). Although ER-association for an mRNA is possible without the mediation of ribosomes, Gaddam and co-workers reported that continued association with polysomes for a membrane-bound mRNA can confer protection from IRE1 cleavage (Cui et al., 2012; Gaddam et al., 2013). This would suggest important implications for the observed refractory nature of Japanese encephalitis virus (JEV) and influenza virus RNA to RIDD cleavage (Hassan et al., 2012; Bhattacharyya et al., 2014). In contrast to Influenza virus, flaviviruses (which include JEV) do not suppress host protein synthesis implying the absence of a global inhibition on translation as would be expected during UPR (Clyde et al., 2006; Edgil et al., 2006). Therefore, a continued translation of viral RNA in spite of UPR activation can in principle confer protection from the pattern of RNA cleavage observed in the RIDD pathway.

COMPARISON OF IRE1 AND RNaseL

IRE1 and RNaseL, in addition to biochemical similarities in protein kinase domain and structural similarities in their RNase domain, share the functional consequences of their activation in initiating cellular apoptosis through JNK signaling (**Table 1** and **Figure 2**; Liu and Lin, 2005; Dhanasekaran and Reddy, 2008). Though initial discoveries were made in the context of homeostatic and anti-viral role for the former and latter, differences between the pathways are narrowed by further advances in research. In the same vein, while inhibition of IRE1 signaling in virus infected cells indicates a potential anti-viral role,

Table 1 | A comparison of the structural and biochemical properties of RNaseL and IRE1, showing similarities and differences.

	Similarities	
	RNaseL	**IRE1**
Inactive state	Monomeric	
Active state	Oligomeric	
Factor driving oligomerization	Catenation of by 2–5A bound to ankyrin repeats of multiple monomers	Titration of HSPA5 bound to luminal domain and catenation of the same from multiple monomers by unfolded proteins
Activation upon exogenous overexpression	Yes (demonstrated *in vitro* for RNaseL)	
Position of ligand–receptor and RNase domain	N- and C-terminal, respectively	
Ribonuclease domain	KEN or kinase-extension homology domain	
Role of PK domain in activating RNase	Nucleotide binding, even in absence of hydrolysis, to conserved residue in protein-kinase like domain is necessary for RNase activity (Tirasophon et al., 1998; Dong and Silverman, 1999; Papa et al., 2003; Lin et al., 2007)	
Nature of RNase substrates	Both 28S rRNA and mRNAs	IRE1β can cleave both 28S rRNA and mRNA while IRE1α substrates include only mRNAs (Iwawaki et al., 2001)
Dissimilarities		
Autophosphorylation	No	Yes
Cleavage substrates	Beside 28S rRNA, predominantly cleaves mRNAs encoding ribosomal proteins (Andersen et al., 2009)	*Xbp1u* and other mRNAs in addition to microRNA precursors which are targeted as part of the RIDD pathway
Selection of cleavage site	Cleaved between 2nd and 3rd nucleotide positions of UN/N sites (Han et al., 2014)	RNA sequence with the consensus of 5′-CUGCAG-3′ in association with a stem-loop (SL) structure essential for recognition of *Xbp1u* and other mRNAs (Oikawa et al., 2010)

association of RNaseL mutations with generation of prostate cancer extends the ambit of influence of this anti-viral effector to more non-infectious physiological disorders (Silverman, 2003). Biochemically, the similarity in their RNase domains does not extend to the choice of either substrates or cleavage point, which are downstream of UU or UA in RNaseL and downstream of G (predominantly) for IRE1 (**Figure 2C**; Yoshida et al., 2001; Hollien and Weissman, 2006; Upton et al., 2012). Further, while RNaseL cleaves pre-dominantly in single-stranded region, IRE1 seems to cleave equally well in single- and double-stranded region (Upton et al., 2012). However, a recent report suggested a consensus cleavage site with the sequence UN/N, in RNaseL targets and in those mRNAs that are cleaved by IRE1 as part of the RIDD pathway (Han et al., 2014). Access to potential cleavage substrate for RNaseL is conjectured to be facilitated through its association with polyribosomes, while no such association is known for IRE1 (Salehzada et al., 1991). Possibilities exist that IRE1 would have preferential distribution in the rough ER which, upon activation, would give it ready access to mRNAs for initiating the RIDD pathway.

In the context of a virus infection, the pathway leading from both these proteins have the potential to lead to cell death. Notwithstanding the fact that this might be an efficient way of virus clearance, it also portends pathological outcomes for the infected organism. Future research would probably lead to design of drugs targeting these proteins based on the structural homology of their effector domains, regulating the pathological denouement of their activation without compromising their anti-viral or potential anti-viral functions.

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A new paradigm: innate immune sensing of viruses via the unfolded protein response

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The immune system depends upon combinations of signals to mount appropriate responses: pathogen specific signals in the context of co-stimulatory "danger" signals drive immune strength and accuracy. Viral infections trigger anti-viral type I interferon (IFN) responses by stimulating endosomal and cytosolic pattern recognition receptors (PRRs). However, viruses have also evolved many strategies to counteract IFN responses. Are there intracellular danger signals that enhance immune responses to viruses? During infection, viruses place a heavy demand on the protein folding machinery of the host endoplasmic reticulum (ER). To survive ER stress, host cells mount an unfolded protein response (UPR) to decrease ER protein load and enhance protein-folding capacity. Viruses also directly elicit the UPR to enhance their replication. Increasing evidence supports an intersection between the host UPR and inflammation, in particular the production of pro-inflammatory cytokines and type I IFN. The UPR directly activates pro-inflammatory cytokine transcription factors and dramatically enhances cytokine production in response to viral PRR engagement. Additionally, viral PRR engagement may stimulate specific pathways within the UPR to enhance cytokine production. Through these mechanisms, viral detection via the UPR and inflammatory cytokine production are intertwined. Consequently, the UPR response is perfectly poised to act as an infection-triggered "danger" signal. The UPR may serve as an internal "co-stimulatory" signal that (1) provides specificity and (2) critically augments responses to overcome viral subterfuge. Further work is needed to test this hypothesis during viral infections.

Keywords: unfolded protein response, viruses, type I IFN, innate immunity, XBP1, ER stress, pattern recognition receptors

INTRODUCTION: TUNING AN APPROPRIATE IMMUNE RESPONSE

Inappropriate activation of the immune system, as evident by toxic shock and autoimmune diseases, reveals an incredibly potent force that can wreak havoc on the human body. Thus multiple safeguards are in place to ensure self-tolerance, including activation induced cell death, anergy, ignorance, regulatory cytokine networks, and T-regulatory cells (Walker and Abbas, 2002; Bluestone and Bour-Jordan, 2012). However, in the face of a foreign invader, the immune system must respond quickly and dynamically. Much investigative emphasis has been placed on combinations of signals that ramp up the adaptive immune response to infectious challenges. Conserved structural components of the pathogens provide essential immune stimulatory signals. These pathogen-associated molecular patterns (PAMPs; e.g., lipopolysaccharide (LPS), peptidoglycan, flagellin, zymosan) are recognized by cell surface pattern recognition receptors (PRRs) on immune cells. One class of PRRs, the Toll-like receptor (TLR) family, responds to a broad spectrum of pathogens. Endogenous products produced during concomitant tissue destruction during infection, so called "danger associated molecular patterns" (DAMPs) also stimulate PRRs (Matzinger, 1994; Bianchi, 2007; Tang et al., 2012). Engagement of PRRs on macrophages and dendritic cells enhances antigen presentation, expression of T cell co-stimulatory molecules, and provides an inflammatory cytokine milieu. Through these combinations of stimuli, cells are poised to respond appropriately to external threats.

However, not all immune stimuli remain extracellular. Also, infected cells must cope until an effective adaptive immune response can be mobilized. Intracellular pathogens such as viruses excite immune responses by triggering endosomal and cytosolic PRRs. Host cells detect viral dsRNA via endosomally localized TLR3, cytosolic RNA-helicases such as retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation associated 5 (MDA-5), and interferon induced sensors such as protein kinase R (PKR). Additionally, endosomal TLR7/8 responds to ssRNA, TLR9 senses CpG oligodinucleotides, and a variety of cytosolic PRRs (e.g., DAI, AIM2 etc.) recognize DNA (Thompson et al., 2011; Goubau et al., 2013; Szabo and Rajnavolgyi, 2013). Early during viral infection, engagement of PRRs leads to the transcription of type I IFN genes that are regulated by the transcription factor interferon regulatory factor 3 (IRF3), including IFN-β and limited species of IFN-α (Hiscott, 2007). This initial wave of IFN serves as an "alarm signal": binding of early IFN to the type I IFN receptor (IFNAR) triggers Janus kinase 1/tyrosine kinase 2 – signal transducers and activators of transcription 1/2 (JAK1/Tyk2–STAT1/2) signaling, and thus an anti-viral transcriptional program (Levy et al., 2003).

IFNAR-regulated genes include IRF7, which induces transcription of multiple IFN-α genes, the dsRNA sensor PKR, and other interferon-stimulated genes (ISGs) that enhance viral recognition and interfere with multiple steps of viral replication (Sato et al., 2000). This PRR-elicited anti-viral transcriptional program plays a critical role in controlling infection.

There are several challenges to the generation of an effective anti-viral program following PRR engagement, including specificity, strength of signal, and viral sabotage. It is not completely clear how the host differentiates between ssRNA, dsRNA, and dsDNA of host and pathogen origin. MDA5 can distinguish a ribose 2′ *O*-methylation found on host mRNA (Zust et al., 2011). However, NS5 of flaviviruses such as Dengue virus (DENV) cap viral RNA with 2′ *O*-methylation to evade detection (Dong et al., 2012). Another potential mechanism to resolve host and pathogen resides in the compartmentalization of host nucleic acids and corresponding PRRs. Stimulation of PRRs with purified agonists alone, such as LPS or the synthetic dsRNA polyI:C, leads to weak, barely detectable amounts of IFN in macrophages (Smith et al., 2008). Engagement of multiple types of PRRs by different motifs on a complex pathogen may be required to synergize (Nasirudeen et al., 2011; Szabo and Rajnavolgyi, 2013). Finally, viruses have evolved numerous strategies to combat IFN signaling at multiple levels, from production of early IFN to IFNAR signaling. For instance, Coronavirus antagonizes a molecule in the DNA-sensing pathway, Stimulator of Interferon Gene (STING/MITA) by disrupting its association with the IRF3-activating kinase tank binding kinase 1 (TBK1)/IKKε (Ishikawa et al., 2009; Sun et al., 2012). Respiratory syncytial virus (RSV) disrupts association between IRF3 and the transcriptional co-activator CREB binding protein (CBP)/p300 (Ren et al., 2011). Vesicular stomatitis virus (VSV) and Hepatitis C virus (HCV) targets the IFNAR receptor for degradation (Liu et al., 2009). DENV cleaves STING, blocks Tyk2 phosphorylation, impairs STAT1 phosphorylation, and targets STAT2 for proteosomal degradation (Green et al., 2014). Paramyxovirus induces degradation of STAT1 and STAT2 (Horvath, 2004). In the face of all these challenges to the PRR-induced anti-viral program, might there also be *intracellular* co-stimulatory or "danger" signals that provide context and critically augment the immune response to ensure success?

VIRUSES AND ER STRESS

Production of high numbers of new virions within a host cell places inordinate stress on the protein folding machinery of the host endoplasmic reticulum (ER). To survive ER stress, the host cell mounts a response known as the "Unfolded Protein Response" or UPR (Schroder and Kaufman, 2005). In the co-evolutionary dance between host and invader, viruses have manipulated this host stress response to enhance viral reproduction. However, in the past decade it has become apparent that the UPR, or specific pathways within the UPR, can promote inflammatory cytokine production. Thus, the UPR may be poised to serve as an internal "danger" signal, complementing PRRs in alerting a cell to invasion and boosting subsequent immune responses (Dalod and Pierre, 2011). The case for UPR as viral-triggered immune stress signal will be reviewed below.

UPR PATHWAYS

The ER controls vital cell functions including protein folding, post-translational modifications, calcium storage, and lipid membrane biosynthesis. Physiologic stresses (increased protein secretion, misfolding proteins) and environmental perturbations (e.g., nutrient starvation, calcium dysregulation, hypoxia etc.) may derail ER function. The UPR is an evolutionarily conserved stress response that maintains ER homeostasis (Hetz et al., 2011; Walter and Ron, 2011). In the unstressed state, UPR initiation molecules residing in the ER membrane are held in check through association with the folding chaperone BiP/GRP78. During ER stress, BiP is released from three primary stress-transducers, activating transcription factor (ATF6), inositol requiring kinase 1 (IRE1), and PKR-like endoplasmic reticulum kinase (PERK), thus activating downstream signaling pathways (**Figure 1**). This activation step may involve multiple potential mechanisms, including competitive sequestration of BiP by misfolded proteins (PERK and IRE1), direct sensing of misfolded proteins by the IRE1 (and by analogy PERK) luminal domains, as well as active dissociation of BiP from ATF6 through an undefined mechanism (Ron and Walter, 2007; Shen et al., 2005).

(1) Dissociation of BiP from ATF6 uncovers a Golgi localization signal, enabling egress from the ER. Upon transit to the Golgi, site-specific proteases (S1P and S2P) cleave ATF6 to release the active transcription factor, which then induces UPR target genes (Adachi et al., 2008). (2) IRE1 has dual functions as both kinase and endonuclease (Hetz et al., 2011). The only known specific mRNA target for the endonuclease function is the transcription factor X-box binding protein 1 (XBP1). IRE1 cleaves 26bp from the XBP1 mRNA, thus removing a premature stop codon. The unconventionally spliced XBP1 mRNA encodes the full length XBP1 containing a transcriptional transactivation domain. Coordinately and independently ATF6 and XBP1 regulate chaperones and other proteins involved in folding and ER-associated protein degradation (ERAD; Lee et al., 2003; Adachi et al., 2008). XBP1 also critically regulates lipid synthesis, promoting expansion of the ER (Sriburi et al., 2004). In addition to XBP1 splicing, IRE1 endonuclease activity also regulates multiple microRNAs, including miR-17, thus relieving translational repression of molecules involved in apoptosis such as Caspase-2 (Upton et al., 2012). Finally, IRE1 has a non-specific nuclease activity that degrades ER membrane associated mRNAs encoding mostly secretory proteins in a process known as regulated IRE1 dependent decay (RIDD; Hollien and Weissman, 2006; Hollien et al., 2009). Related to its kinase activity, IRE1 forms a multi-molecular complex ("UPRosome") with TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) that triggers multiple signaling pathways and cellular processes, including jun N-terminal kinase (JNK) signaling, autophagy, and the regulation of apoptosis vs. survival (Woehlbier and Hetz, 2011). (3) Upon release of BiP, PERK dimerizes, and auto-transphosphorylates to activate its kinase activity. PERK in turn phosphorylates eIF2α, resulting in global translational attenuation apart from select open reading frames. One of the primary targets for this selective translation is the transcription factor ATF4, which regulates amino acid transport, protection against oxidative stress, and

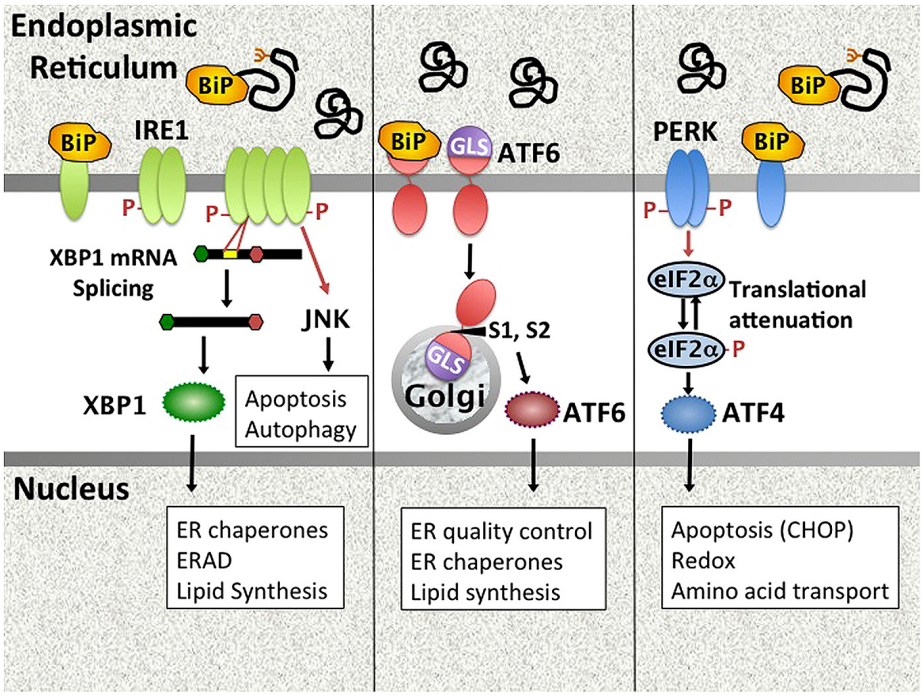

FIGURE 1 | Mammalian UPR pathways. The UPR encompasses signaling pathways triggered by the activation of ER stress transducers IRE1, ATF6, and PERK. In unstressed cells, these molecules associate with the folding chaperone BiP. Upon accumulation of unfolded proteins in the ER, PERK, and IRE1 release BiP and oligomerize. IRE1 is both a kinase that phosphorylates targets such as JNK, and an endonuclease that splices 26bp from the XBP1 mRNA, removing a premature stop codon. Dissociation of ATF6 from BiP uncovers a Golgi localization signal. ATF6 traffics to the Golgi, where site-specific proteases (S1, S2) cleave it to an active transcription factor. PERK phosphorylates eIF2α, resulting in global translational attenuation apart from select open reading frames (e.g., ATF4). UPR gene targets (e.g., CHOP) and UPR regulated cellular processes are in boxes. ERAD = ER associated degradation. GLS = Golgi localization signal.

apoptosis via CHOP (Walter and Ron, 2011). ATF4 induced growth arrest and DNA damage inducible 34 (GADD34) associates with protein phosphatase 1 to mediate dephosphorylation of eIF2α, thus turning off the PERK pathway in a negative feedback loop. As another example of cross talk between pathways, XBP1-induced p58^{ipk} binds PERK and inhibits its kinase activity (Lee et al., 2003; van Huizen et al., 2003). Translational attenuation decreases ER client load, but the transitory duration ensures cell survival.

Together, these three primary effector-dependent biochemical pathways induce a gene transcriptional program that enables cells to cope with stress by enhancing protein folding and decreasing protein load in the ER. In addition to regulating protein synthesis, the UPR exerts a profound effect on multiple cellular processes including autophagy, apoptosis, ER and Golgi biogenesis, Redox status, and lipid synthesis. If ER stress remains unresolved despite these adaptive measures, the UPR initiates apoptosis. Related to its role in supporting protein production, the UPR is physiologically active in highly secretory cells such as pancreatic acinar cells, hepatocytes, and Paneth cells (Lee et al., 2005; Kaser et al., 2010). However, the UPR also apparently plays a critical role in immune cell homeostasis, being required for plasma cell development from B-lymphocytes and the development and survival of both myeloid and plasmacytoid dendritic cells (Iwakoshi et al., 2003, 2007).

VIRUSES AND THE UPR

In order to replicate, viruses must utilize host ER to produce greatly increased quantities of viral protein, inducing ER stress. Although the increased folding capacity of the UPR should benefit viruses, translational attenuation, ERAD, and host apoptosis could all potentially limit viral replication. Thus perhaps it is not surprising that many viruses have evolved strategies to manipulate different aspects of the host UPR (He, 2006). Viruses induce the UPR in various ways, including greatly increasing protein synthesis, elaboration of misfolded proteins (e.g., hemagglutinin) and direct interaction with BiP, as seen with the US11 protein of human cytomegalovirus (HCMV; Hurtley et al., 1989; He, 2006; Hegde et al., 2006). The extent of UPR induction varies between viruses and reports describing individual viruses have also varied over the years, complicating interpretation of the literature. For instance reports investigating HCV have commented on isolated ATF6 cleavage, ATF6, and XBP1 splicing (but inhibition of downstream XBP1 target induction), or induction of all three major arms of the UPR (Tardif et al., 2002, 2004; Ke and Chen, 2011; Merquiol et al., 2011). Some of these discrepancies may arise from investigations of individual viral protein vs. whole cell infections, as well as choice of host cell. Some viruses selectively induce parts of the UPR. For instance, HCMV US11 induces XBP1 splicing (without downstream EDEM induction) but does not lead to ATF6 cleavage (Isler et al., 2005). West Nile virus activates XBP1 and

ATF6 but inhibits PERK activity (Ambrose and Mackenzie, 2011). Lymphocytic choriomeningitis virus selectively activates ATF6, but not PERK or IRE1 (Pasqual et al., 2011). Epstein Barr virus (EBV) appears to induce all three axes, with a feed forward loop of EBV LMP protein activating PERK and the PERK-dependent ATF4 inducing viral LMP (Lee and Sugden, 2008). Viruses may also activate different arms of the UPR at different times following infection. For instance, one report on DENV describes early PERK activation followed by inhibition, XBP1 induction mid-infection and ATF6 activation late in infection (Pena and Harris, 2011). In this case, CHOP induction did not lead to activation of caspases and apoptosis. PERK inhibition appears to be a common thread between different viruses. One of the most notable examples is the Herpes simplex virus (HSV) protein $\gamma_1$34.5/ICP34.5 that acts analogously to the GADD34 target to relieve translational inhibition (He et al., 1997; Cheng et al., 2005). Induction of the UPR, or parts of the UPR, appears to be essential for promoting viral lifestyle. Consequently, blockade or knockdown of the UPR pathways adversely impact viral replication and increase cytopathic effects (Yu et al., 2006; Ke and Chen, 2011; Ambrose and Mackenzie, 2013).

The direct induction of the UPR by viral proteins, as well as the host response to increased protein load in the ER both position the UPR well to serve as an intracellular "danger signal" alerting the cell to infection. Interestingly, multiple UPR pathways appear to share evolutionary history with dedicated anti-viral pathways. PERK is evolutionarily related to the interferon induced PKR (as PERK's name implies). PKR responds directly to dsRNA by phosphorylating eIF2α (analogously to PERK) in an effort to halt viral protein synthesis (He, 2006). GCN2, a third eIF2α kinase family member responsive to amino acid starvation is induced by Sindbis virus and inhibits replication (Berlanga et al., 2006). IRE1 is related to the anti-viral molecule RNAse-L both in structure and function (>40% similarity; Chakrabarti et al., 2011; Martinon and Glimcher, 2011). Like RNAse-L, the non-specific endonuclease activity of IRE1 generates small RNA species with 5′OH and cyclic 2′3′ phosphodiester 3′ ends that can be recognized by RIG-I (Cho et al., 2013). Thus perhaps it is not merely coincidence that the UPR should be engaged during viral infection. How then does this stress response interact with host immune, and more specifically anti-viral responses?

THE INTERSECTION OF ER STRESS AND INFLAMMATION

Beyond its role in supporting immune cell development, the UPR has become increasingly implicated in various inflammatory conditions ranging from obesity and atherosclerosis to diabetes, neurodegenerative diseases, arthritis, and inflammatory bowel disease (Zhang and Kaufman, 2008; Wang and Kaufman, 2012; Claudio et al., 2013). Is the UPR an inflammatory instigator or byproduct of the inflammatory state (or both)?

DIRECT INFLAMMATORY SIGNALING BY THE UPR

Over the past decade, it has become apparent that the UPR directly triggers inflammatory signal transduction pathways, including mitogen activated protein (MAP) kinase (ERK1/2, p38, and JNK) signaling, and activates key inflammatory transcription factors such as nuclear factor kappa-light chain enhancer of activated B cells (NF-κB; Zhang and Kaufman, 2008; Hotamisligil, 2010; Hasnain et al., 2012). In unstimulated cells, NF-κB family members (p50, p52, p62, RelB, and c-Rel) are sequestered in the cytoplasm by association with an inhibitory molecule inhibitor of κB (e.g., IκBα). Upon stimulation (e.g., PRR engagement), IκB kinase (IKK) phosphorylates IκBα, targeting it for ubiquitination and proteosomal degradation. Dissociation from IκBα allows NF-κB to transit to the nucleus where it can induce cytokines such as TNF-a and IL-6 (Hayden and Ghosh, 2008). In Li et al. (2005), reported that free cholesterol-induced MAP kinase signaling and NF-κB activation in macrophages required transit of the cholesterol to the ER and induction of ER stress. Other examples of non-infectious UPR-related inflammation have since been described: the oxidized phospholipid-stimulated UPR regulates cytokine production by human endothelial cells (Gargalovic et al., 2006). Pharmacologic agents that induce the UPR such as tunicamycin (N-linked glycosylation inhibitor) or thapsigargin (SERCA pump inhibitor) also stimulate low-level inflammatory cytokine production (e.g., IL-6; Martinon et al., 2010; Peters and Raghavan, 2011).

Multiple UPR pathways participate in NF-κB activation. In the free cholesterol-loaded macrophages, CHOP was apparently necessary for full induction of ERK1/2 phosphorylation and IL-6 production (Li et al., 2005). The mechanism remains unclear but may involve CHOP mediated antagonism of a negative regulator of NF-κB, peroxisome proliferator activator gamma (PPARγ; Park et al., 2010). PERK has also been proposed to activate NF-κB via translational attenuation, related to the relatively short half-life of IκBα compared to NF-κB (Deng et al., 2004). A second major arm of the UPR, stemming from IRE1 activation, also activates NF-κB. The IRE1–TRAF2 complex recruits IKK, potentially supporting basal activation of IKK, and thus contributing to NF-κB activation (Tam et al., 2012). IRE1–TRAF2 also stimulates JNK signaling via ASK1, leading to the activation of other cytokine-regulatory transcription factors belonging to the activator protein-1 (AP1) family (Urano et al., 2000; Nishitoh et al., 2002). Subtilase toxin induced activation of ATF6 also results in NF-κB activation, although the mechanism is not clear (Yamazaki et al., 2009). In addition to the three canonical UPR signaling pathways, ER stress (or ER "overload") activates NF-κB through the generation of reactive oxygen species (ROS) and ER calcium release (Pahl and Baeuerle, 1997; Zhang and Kaufman, 2008). Mitochondria participate in this process, enhancing ROS production and ER calcium leak. In a positive feedback loop, the resulting inflammatory cytokines can trigger further ER stress through induction of more ROS (oxidative stress) and increasing release of calcium from the ER, interfering with chaperone function (Zhang and Kaufman, 2008).

Another potential feed-forward loop has been described in the liver. During ER stress, other molecules besides ATF6 undergo site directed proteolysis, including SREBP, CREBH, CREB4, Luman, and OASIS, possibly in a cell-specific, or context-specific manner (Bailey and O'Hare, 2007). In liver cells, the UPR leads to proteolytic activation of CREBH, which then induces key proteins in the acute phase response, serum amyloid protein and C-reactive protein (Zhang et al., 2006). Interestingly, TLR4 stimulation and inflammatory cytokines such as IL-6 can in turn induce the UPR in liver cells (Zhang et al., 2006). Hepatocytes are not

unique in cytokine-triggered UPR activation: oligodendrocytes also exhibit modest BiP and CHOP upregulation upon stimulation with IFN-γ, consistent with an integrated stress response (Lin et al., 2005). Further, PERK activation may protect mature oligodendrocytes during demyelinating diseases (Lin et al., 2007).

UPR–PRR SYNERGY AND IFN PRODUCTION

As this work on "sterile" inflammation occurred, other lines of investigation suggested a strong partnership between the UPR and infectious signals. In the field of rheumatology, it was noted that the molecule most strongly linked to spondyloarthritis, the MHC allele HLA-B27, misfolded, bound BiP excessively, and induced a UPR (Dangoria et al., 2002; Turner et al., 2005). Further, macrophages from diseased HLA-B27 transgenic rats showed transcriptomic evidence of both UPR (increased CHOP, BiP, Erp70, etc.) and IFN gene signature (Best5, MX1, Oas1, STAT2, Gbp2, IRF7, CXCL10, etc.; Turner et al., 2005). The association between IFN signature and UPR has been observed in other rheumatologic diseases, including systemic sclerosis and possibly specific types of myositis (Nagaraju et al., 2005; Gherardi, 2011; Lenna et al., 2013).

At first the link between UPR and type I IFN was not clear, as treatment of cells with UPR inducing pharmacologic agents such as tunicamycin and thapsigargin triggered virtually undetectable type I IFN (Smith et al., 2008). However, if cells undergoing an acute UPR were then treated with LPS (TLR4 agonist), poly I:C (TLR3) or transfected with poly I:C (MDA-5), the amount of IFN-β was augmented log-fold or more over the PRR agonist alone (Smith et al., 2008; Hu et al., 2011). In addition to IFN-β, the UPR augmented the specific production of other pro-inflammatory cytokines including IL-6, TNF-α, and IL-23, a cytokine implicated in the generation of pathogenic Th17 responses (Smith et al., 2008; DeLay et al., 2009; Martinon et al., 2010). It is not clear what portion of synergistic IFN-α or CXCL10 production reflected IFNAR signaling by primarily increased IFN-β(Smith et al., 2008; Hu et al., 2011). This phenomenon of synergy was not only observed upon pre-treatment with pharmacologic agents: macrophages from HLA-B27 transgenic rats also responded to TLR agonists such as LPS with greatly augmented IFN-β production (Smith et al., 2008). As another example, cells expressing the misfolding α-1 antitrypsin respond to LPS with greater cytokine production (Carroll et al., 2010). Further, relieving ER stress with agents such as chemical chaperones (e.g., 4-phenylbutyric acid, tauroursodeoxycholic acid), which aid in protein folding, can ameliorate LPS induced inflammation (Kim et al., 2013). Synergistic cytokine production has been observed in multiple culture cell types, as well as human macrophages, mouse macrophages, and dendritic cells (Smith et al., 2008; Hu et al., 2011). The synergism is inflammatory-mediator specific, in that it does not extend to all cytokines and chemokines. For instance, IL-1β and RANTES are not synergistically induced by TLR ligation and concomitant UPR (Smith et al., 2008; Martinon et al., 2010). PRR specificity may depend upon cell type: in macrophages, synergism occurs with stimulation of TLR2, TLR3, TLR4, and MDA-5 but not TLR7 and TLR9 (Smith et al., 2008; Martinon et al., 2010). However, in cells where these TLR7 and TLR9 are more prominently engaged, such as plasmacytoid dendritic cells, synergy is readily detected (Hu et al., 2011).

Synergism between environmental stimuli and ER stress made teleological sense for spondyloarthritis for several reasons: in the HLA-B27 rat model, disease does not develop in germ free animals, but reconstitution with limited colonic flora was sufficient, suggesting the need for an infectious trigger (Taurog et al., 1994). A specific type of spondyloarthritis, reactive arthritis, is classically initiated by Gram-negative infections of the gastrointestinal and genitourinary tract. Finally, spondyloarthritis patients often develop overt or subclinical inflammatory bowel disease, another manifestation linking UPR, microbial triggers, and inflammation (Mielants et al., 1988).

MECHANISMS UNDERLYING UPR-PRR SYNERGY

IFN and inflammatory cytokine production is largely regulated by the nuclear availability and activation status of critical transcription factors. As described above, the ability of PERK-eIF2α and IRE1-kinase pathways to enhance the activation of NF-κB and AP1 should potentiate cytokine production by PRR agonists. However, it was not clear why the UPR–PRR interaction was synergistic rather than just additive. A requirement for cooperative transcription factor binding provides one possible explanation (Panne et al., 2007). Further investigation into the mechanisms underlying synergy revealed the involvement of other UPR pathways as well as more direct interaction between UPR-specific transcription factors and cytokine/IFN gene regulatory elements. Studies employing XBP1 gene knockdown, XBP1 deficient MEFs, and macrophages from conditional XBP1 knockout mice, together confirmed a critical role for the IRE1-dependent XBP1 transcription factor in synergistic cytokine production. XBP1 was essential for augmented IFN-β, ISG15, IL-6, TNF-α, and IL8 in response to combined ER stress and PRR signaling (Mielants et al., 1988; Smith et al., 2008; Martinon et al., 2010; Zeng et al., 2010). Indeed, XBP1 apparently plays a role in basal TLR-dependent cytokine production, even in the absence of UPR induction (discussed below). Chromatin immunoprecipitation (ChIP) studies revealed binding of XBP1 to IL-6 and TNF-α promoters as well as a TNF-α enhancer element (Martinon et al., 2010). A similar experimental approach revealed binding of another UPR-regulated transcription factor, CHOP to the IL-23 p19 promoter in dendritic cells (Goodall et al., 2010). The mechanism underlying synergistic IFN-β production, however, proved more elusive.

Regulation of the IFN-β encoding *ifnb1* gene has been intensively investigated and elegantly elucidated (Agalioti et al., 2000). The core *ifnb1* enhancer at −102 to −51 contains a series of tightly packed binding sites for members of the NF-κB family, AP1, IRF3, and IRF7 transcription factors (Panne et al., 2007). Following PRR stimulation, these transcription factors bind cooperatively to the site, forming an "enhanceosome" (Merika and Thanos, 2001). IRF3 associates with a histone acetyltransferase, CREB binding protein (CBP)/p300, thus bringing this transcriptional co-activator to the enhancer. Assembly of the enhanceosome results in sequential recruitment of chromatin modifying factors and basal transcription machinery. As a result of this process, an inhibitory nucleosome slides downstream, away from the TATA box, thus enabling transcription of IFN-β (Agalioti et al., 2000). Binding of IFN-β to the IFNAR receptor then results in new transcription of IRF7, which strengthens IFN-β transcription and leads

to the production of multiple IFN-α genes and other ISGs (Sato et al., 2000).

IRF3 is absolutely required for initial LPS-induced IFN-β expression and early viral-induced IFN (Sato et al., 2000; Sakaguchi et al., 2003). Besides IFN-β and IFN-α4 (IFN-α1 in human), IRF3 regulates a subset of other ISGs, including ISG54, ISG56, and RANTES independently of IFNAR signaling (Grandvaux et al., 2002). IRF3 can also induce apoptosis through association with pro-apoptotic Bax (Chattopadhyay et al., 2010). In unstimulated cells, IRF3 resides in the nucleus. Upon stimulation, TBK1/IKKε family kinases phosphorylate IRF3 at multiple serines and threonines, enabling IRF3 dimerization, nuclear translocation, association with the CBP/p300 co-activator and DNA-binding activity (Hiscott, 2007). During viral infection, phosphorylation at IRF3 S385/S386 plays an important role in regulating phosphorylation in the 396–405 Ser/Thr cluster and strengthens the association with CBP (Chen et al., 2008). Partial phosphorylation will result in some of the activation steps leading from cytosol to nucleus, but will not permit full IRF3 transcriptional activity (Lin et al., 1999).

There are no XBP1 binding consensus sequences in the well-characterized *ifnb1* promoter/enhancer and direct binding of XBP1 to promoter was not detected by ChIP. However, a search of the neighboring chromosomal DNA for XBP1 consensus sites revealed a sequence ∼6 kb downstream of *ifnb1* that does bind XBP1, IRF3, and CBP during concomitant ER stress and LPS signaling and appears to be an ER stress-responsive enhancer element (Zeng et al., 2010). Interestingly, LPS stimulation of macrophages undergoing a UPR resulted in increased recruitment of IRF3 and CBP to the canonical *ifnb1* enhancer/promoter. XBP1 belongs to the CREB family of transcription factors and thus may directly interact with CBP/p300 as suggested by overexpression studies with tagged constructs. Interactions between XBP1 and CBP might strengthen factor recruitment to the *ifnb1* regulatory elements (Zeng et al., 2010). However, the precise relationship between XBP1 and increased IRF3 remained unclear.

Further investigation revealed that ER stress alone was sufficient to induce nuclear localization of IRF3, in an XBP1 independent manner ER stress resulted in phosphorylation of IRF3 at S386, but LPS was required for S396 phosphorylation (and thus presumed oligomerization, CBP-association, DNA-binding, and transactivation; Chen et al., 2008; Liu et al., 2012). How ER stress leads to IRF3 initial phosphorylation and nuclear translocation appears to depend upon the type of ER stress. ER stress that involves calcium dysregulation (thapsigargin treatment, calcium ionophore A23187, oxygen–glucose deprivation) appears to depend upon STING and TBK1. Through unclear mechanisms, induction of ER stress mobilizes the ER-resident STING, inducing its co-localization with TBK1 (Liu et al., 2012). Another group working in an alcohol steatosis model found that alcohol induced both XBP1 splicing and IRF3 phosphorylation in a STING-dependent manner, though the relationship between ER stress and STING activation was not directly assessed (Petrasek et al., 2013). Other forms of UPR induction (e.g., tunicamycin treatment) activate IRF3 in a STING-independent, but S1/S2 protease inhibitor sensitive process (Liu et al., 2012). This work emphasizes that not all types of UPR induction triggers the same pathways.

These results raise some intriguing questions. If the UPR activates NF-κB, AP1, and nuclear translocation of IRF3, why then is it such a poor inducer of IFN-β? The answer may lie in the particular requirements for full IRF3 activity. Given the enabling role for phosphorylation at S386, ER stress may synergize with PRR activation of IRF3 by increasing S396 phosphorylation, but the PRR signal remains indispensible. If the UPR and PRR agonists cooperate in IRF3 activation, why are certain IRF3-regulated genes not synergistically induced (e.g., RANTES)? UPR transcription factor binding sites have been found in gene regulator elements for IL-6, TNF-α, IFN-β (XBP1 binding), and IL-23 (CHOP binding; Goodall et al., 2010; Martinon et al., 2010; Zeng et al., 2010). The restriction in IRF3-regulated genes may relate to lack of binding sites for UPR-transcription factors; however this hypothesis would need to be confirmed experimentally. A requirement for both PRR stimulus and UPR-factor binding site might preserve specificity for situations involving both infection and stress, and also underlie the observed synergistic (rather than additive) degree of cytokine enhancement.

SELECTIVE UPR PATHWAY ACTIVATION IN INNATE IMMUNE SENSING

The UPR stimulates cytokine production directly and dramatically synergizes with PRR signaling to augment IFN and other inflammatory mediators. It has become apparent that pathogen triggered PRRs may also engage UPR molecules or parts of UPR pathways to induce cytokine production, independently of a global UPR. Indeed multiple examples have been described where PRR engagement actually suppresses canonical UPR activity. For instance, LPS suppresses ATF6 and PERK pathway signaling, as evident by decreased ATF6 cleavage, BiP, ATF4, and CHOP induction (Woo et al., 2009). Yet engagement of TLR2 and TLR4 (but not TLR3, 7, or 9) in macrophages stimulates IRE1-dependent XBP1 splicing (Martinon et al., 2010). It was not clear whether the TLR specificity reflected endosomal vs. surface locations, cell type, or specific signaling pathways. Traditional XBP1 targets such as ERdj4 were not induced by TLR engagement, yet the spliced XBP1 was essential for optimal TLR stimulation of multiple cytokines and inflammatory mediators, including IL-6, ISG15, TNF-α, IFN-β, and COX2. TLR mediated IRE1 activation and XBP1 splicing appears to proceed through the NADPH oxidase NOX2 pathway (Martinon et al., 2010).

As another example of selective pathway engagement, cytosolic stimulation of PKR by dsRNA results in eIF2α phosphorylation, selective ATF4 translation, and GADD34 induction. However, in comparison to the effect of GADD34 during the UPR, polyI:C-stimulated global translational inhibition was not relieved upon the dephosphorylation of eIF2α. However, certain transcripts, including those for IL-6, IFN-β, and PKR itself continue to be translated in a GADD34 dependent manner (Clavarino et al., 2012b). Although this PKR pathway induces CHOP at the transcriptional level, CHOP translation is inhibited. As an example of how this pathway affects viral responses, Chikungunya virus-induced IFN-β was severely compromised in the absence of GADD34 (Clavarino et al., 2012a). Interestingly, engagement of this pathway by both cytosolic polyI:C and soluble polyI:C (signaling through TLR3) largely depended upon the adaptor signaling molecule TRIF. The authors propose that the

TRIF–PKR–GADD34 pathway might work in parallel with the MDA-5 pathway for dsRNA sensing.

Cholera toxin sensing also coopts another specific pathway within the UPR. The cholera toxin A (CTA) protein transits into the ER and activates the RNase portion of IRE-1 to initiate RIDD. However, CTA does not activate the ATF6 or PERK pathways. RIG-I senses the small RNA fragments generated by RIDD leading to activation of NF-κB and inflammatory cytokine production. This signaling pathway is both PERK and XBP1 independent. IRE1 endonuclease activity was also required for full induction of IL-6 and IL-8 by Shiga toxin and SV40 virus, which both transit to the ER (Cho et al., 2013).

These examples of selective engagement of XBP1 splicing, GADD34 induction and IRE1 RIDD activity by immune sensors of microbial infection reveal that UPR molecules may be coopted without engagement of the full UPR. Thus the infected cell may utilize stress-signaling pathways without engaging unwanted consequences of the UPR such as apoptosis. This activation of UPR-related molecules and limited UPR pathways by PRR engagement has led to the proposal of a distinct "Microbial Stress Response" (Claudio et al., 2013). However such a response would not necessarily be exclusive of a role for the UPR in initiating or supporting inflammation.

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The multiple pathways by which the UPR supports inflammation, and more specifically IFN production, would render it a potentially hazardous response for a virus to induce, even in support of its own replication. However, as an evolutionary counter, viruses have also coopted the UPR to suppress the antiviral program. For instance, activation of the PERK pathway by VSV and HCV results in phosphorylation and consequent ubiquitination of the IFNAR1 chain, decreasing IFN responsiveness. PERK$^{-/-}$ cells were actually more resistant to VSV infection (Liu et al., 2009). Coronavirus 3a protein sabotages IFNAR signaling in a similar fashion (Minakshi et al., 2009). HCV antagonizes IFN-β production via CHOP and subsequent autophagy activation (Ke and Chen, 2011). Continued study of UPR–pathogen–cytokine relationships is likely to reveal further layers of complexity.

CONCLUSION AND FUTURE PERSPECTIVES

Even as viruses utilize the host UPR to enhance virus production and host cell survival, the invoked UPR in turn has the potential to augment anti-viral responses. Multiple mechanisms intertwining the UPR and inflammatory/IFN responses have been described, from direct activation of cytokine transcription factors to UPR–PRR synergy and selective UPR pathway induction in a "microbial stress response" (Claudio et al., 2013). These pathways are not necessarily exclusive, but may cooperate to ultimately boost the immune response beyond the threshold needed to counteract viral subterfuge (**Figure 2**).

Several reports suggest this proposed danger signal is not just limited to the infected cell, but may be transmitted to neighboring cells. ER stress can lead to the surface expression of calreticulin, thus enhancing inflammatory cytokine production and phagocytosis of the stressed (infected) cell (Peters and Raghavan, 2011). ER stressed tumor cells can "transmit" stress to macrophages by some undefined soluble factor, resulting in macrophage UPR and cytokine production (Mahadevan et al., 2011). Might this also be true for ER stressed infected cells? The effect of infection-triggered

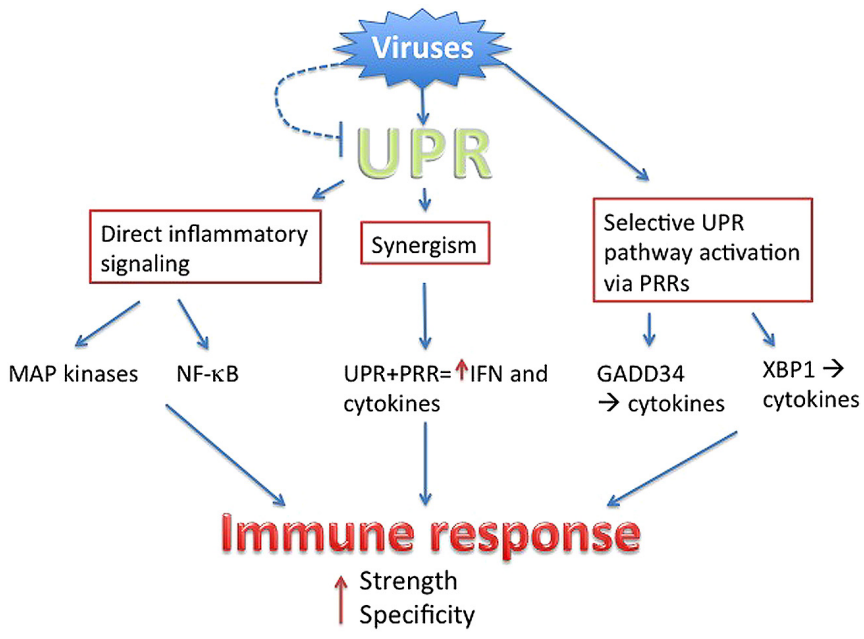

FIGURE 2 | Summary. Viruses directly or indirectly trigger the UPR, but have evolved to antagonize parts of the UPR (dashed line). Through PRR signaling, viruses also mobilize specific parts of UPR pathways. The UPR intersects with inflammatory activation through multiple mechanisms, ultimately boosting the strength of anti-viral IFN and cytokine production. Sensing of the virus via UPR and UPR-related pathways provides context, ensuring specificity.

UPR on non-infected cells adds another interesting dimension for future potential investigations.

While the model for UPR as virus sensing "danger" signal is attractive, current evidence for relevance during viral infection is limited. XBP1 has been reported to be critical for control of VSV by dendritic cells, related to elaboration of type I IFN (Hu et al., 2011). Neonatal GADD34$^{-/-}$ mice demonstrated greater susceptibility to infection with Chikungunya virus (Clavarino et al., 2012a). There is more experimental support for the interaction of pathogens, UPR, and cytokine production from the bacterial literature. XBP1 is critical for control of *Francisella* infection in mice (Martinon et al., 2010). The UPR also apparently plays a role in macrophage immune sensing of intracellular *Brucella* infection: specifically the IRE1 pathway promotes IL-6 production (de Jong et al., 2013). More work is clearly needed to elucidate the role of the UPR in viral sensing and cytokine production in defined *in vitro* and *in vivo* models.

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Cellular unfolded protein response against viruses used in gene therapy

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Viruses are excellent vehicles for gene therapy due to their natural ability to infect and deliver the cargo to specific tissues with high efficiency. Although such vectors are usually "gutted" and are replication defective, they are subjected to clearance by the host cells by immune recognition and destruction. Unfolded protein response (UPR) is a naturally evolved cyto-protective signaling pathway which is triggered due to endoplasmic reticulum (ER) stress caused by accumulation of unfolded/misfolded proteins in its lumen. The UPR signaling consists of three signaling pathways, namely PKR-like ER kinase, activating transcription factor 6, and inositol-requiring protein-1. Once activated, UPR triggers the production of ER molecular chaperones and stress response proteins to help reduce the protein load within the ER. This occurs by degradation of the misfolded proteins and ensues in the arrest of protein translation machinery. If the burden of protein load in ER is beyond its processing capacity, UPR can activate pro-apoptotic pathways or autophagy leading to cell death. Viruses are naturally evolved in hijacking the host cellular translation machinery to generate a large amount of proteins. This phenomenon disrupts ER homeostasis and leads to ER stress. Alternatively, in the case of gutted vectors used in gene therapy, the excess load of recombinant vectors administered and encountered by the cell can trigger UPR. Thus, in the context of gene therapy, UPR becomes a major roadblock that can potentially trigger inflammatory responses against the vectors and reduce the efficiency of gene transfer.

Keywords: gene therapy, UPR, ER-stress, ER-homeostasis, viral vectors, chaperones

INTRODUCTION

One of the important functions of cellular metabolism is protein folding. Endoplasmic reticulum (ER) is the site where all the proteins (secreted, membrane bound, and organelle targeted proteins) are typically processed and folded in eukaryotes (Kaufman et al., 2002; Naidoo, 2009). This accumulates a very high concentration of proteins in the ER which can lead to co-aggregation between proteins and/or polypeptides (Stevens and Argon, 1999). Therefore, the lumen of the ER needs a unique environment that promotes processing of proteins but prevents their aggregation (Anelli and Sitia, 2008; Kim et al., 2008; Hetz et al., 2011; Hetz, 2012). Sometimes, due to a high demand in protein synthesis due to various physiological reasons, the processing capacity of the ER can be challenged (Zhang and Kaufman, 2006; Marcinak and Ron, 2010; Hetz et al., 2011). This results in an imbalance in the ER environment, which is referred to as ER stress (Liu and Howell, 2010; Marcinak and Ron, 2010; Hetz et al., 2011; Iwata and Koizumi, 2012). Altered protein folding leading to ER stress can be induced by various factors such as glucose deprivation, aberrant calcium regulation, viral infection and hypoxia. Normally, cells ensure that proteins are correctly folded using a combination of molecular chaperones namely, the foldases and lectins (Naidoo, 2009). If unfolded or misfolded proteins continue to accumulate, eukaryotes induce the UPR. The basic goal of UPR is to recover the (lost) homeostasis (adaptation), reduce stress within the ER compartment and prevent any cytotoxic effect that might be caused by misfolded proteins *via* adaptive mechanisms as well as by blocking mRNA translation (Xu et al., 2005; Kim et al., 2008; Ye et al., 2011). During adaptation, the UPR tries to correct folding homeostasis *via* induction of chaperones that promote protein folding (Meusser et al., 2005; Kim et al., 2008). However, when proper folding cannot be restored, incorrectly folded proteins are targeted to ER Associated Degradation (ERAD) pathways for processing (Kaufman et al., 2002). UPR is also known to trigger several molecules of the innate immunity pathway, most notably mitogen- activated protein kinases, p38 and nuclear factor-κB (NF-κB) which collectively trigger the UPR induced alarm signal (Ron and Walter, 2007; Kim et al., 2008; Tabas and Ron, 2011) to remove translational block and down-regulate the expression and activity of pro-survival factors such as the B-cell lymphoma 2 (Bcl2) protein. However, if the function of the ER cannot be re-established, UPR eliminates the damaged cells by apoptosis or autophagy (Bernales et al., 2006; Kamimoto et al., 2006; Yorimitsu et al., 2006; Hoyer-Hansen et al., 2007; Kouroku et al., 2007). Apart from such a response against *de novo* synthesized proteins in a cell, the massive accumulation of exogenous proteins intra-cellularly as in the case of viral infection is also known to contribute to ER stress responsive pathways (Zhang and Wang, 2012).

For a virus to successfully infect mammalian cells, it has to undergo several aspects in its life-cycle-their attachment to cell surface receptors, endocytosis, intracellular trafficking, polypeptide synthesis and genome replication (Balakrishnan and Jayandharan, 2014). Viruses are naturally evolved to utilize host cell machinery to successfully complete their life cycle and during this process they produce several viral proteins within host cells. As a natural response to these foreign proteins, the cell in turn can activate the UPR and interferon response. Thus, a potential mechanism that can limit viral replication is the UPR. It is not surprising that viruses have also evolved mechanisms to manipulate UPR pathways to facilitate their infection (Zhang and Wang, 2012). This generally involves regulation of stress response proteins and several molecular chaperones to modulate UPR and increase ER folding capacity or by induction of translational attenuation to repress the UPR pathways (Zhang and Wang, 2012). Several viruses like adenovirus (Ad), adeno-associated virus (AAV), dengue virus, cytomegalovirus, respiratory syncytial virus, simian virus-5, Tula virus, rota virus African swine fever virus, herpes simplex virus type 1 (HSV-1), hepatitis C virus, corona virus, influenza virus amongst others have been shown to regulate host cell UPR machinery to promote their infection and persistence in the host (Bitko and Barik, 2001; Netherton et al., 2004; Isler et al., 2005; Paradkar et al., 2011; Pena and Harris, 2011; Zhang and Wang, 2012). For example, rotavirus interrupts the inositol requiring protein-1 (IRE1) and activating transcription factor 6 (ATF6) UPR pathways by translational inhibition through its non-structural protein NSP3 (Trujillo-Alonso et al., 2011). Hepatitis C virus (HCV) has been shown to suppress the IRE1-XBP1 pathway to promote its expression and persistence in the liver (Tardif et al., 2004). Likewise cytomegalovirus uses the viral protein M50 to downregulate IRE1 leading to suppression of UPR (Stahl et al., 2013). This article reviews the tug of war that is initiated by the cell through its UPR signaling against viruses used in gene therapy and dissects how this information can be helpful to improve gene delivery strategies.

UPR PATHWAYS

Three branches of the UPR have been characterized, which are mediated by ER-located transmembrane proteins: IRE1, protein kinase RNA-like ER kinase (PERK) and ATF6. The binding immunoglobulin protein (BiP) is the master regulator of the UPR. All the three arms of UPR are held in an inactive state by the binding of the BiP to their N-terminal region of IRE1, PERK and ATF6 proteins. When the cell encounters stress, BiP is released due to competitive binding of the misfolded proteins and thus leading to activation of UPR signaling (**Figure 1**) (Xu et al., 2005).

IRE1 PATHWAY

IRE1, the most evolutionarily conserved branch of UPR (Cox et al., 1993) initiates both the pro-survival and pro-apoptotic components in the presence of misfolded proteins. In mammals two isoforms of IRE1 have been identified, IRE1α and IRE1β; IRE1α is expressed in a variety of tissues (Tirasophon et al., 1998), whereas IRE1β is primarily found in the intestine and lung (Bertolotti et al., 2001; Martino et al., 2013). Mechanistically, when there is an increase in unfolded or misfolded protein load, the BiP molecule interacts with the N-terminus of IRE1, located in the ER lumen. This sensing leads to dimerization of IRE-1 and activates two distinct signaling arms of the IRE-1 pathway. The early signaling occurs through the cleavage of a 26-nucleotide intron from the XBP1-mRNA (Shen et al., 2001; Yoshida et al., 2001; Lee et al., 2002; Malhotra and Kaufman, 2007) generating a 41 kDa frameshift variant (sXBP1). sXBP1 acts as a potent transcription factor that regulates the expression of several protein degradation related genes (Rao and Bredesen, 2004; Malhotra and Kaufman, 2007). The late signaling sensor of IRE1 is initiated when the cytosolic IRE1α dimers interact with molecules like the tumor necrosis factor receptor-associated factor 2 (TRAF2) which activates the signal-regulating kinase (ASK1) and further activation of cJUN NH2-terminal kinase (JNK) and p38MAPK (Urano et al., 2000). These proteins in turn trigger a proapoptotic signal through pro-apoptotic molecules such as Bim and caspase-3 leading to cell death.

PERK PATHWAY

PERK is an ER-localized type I transmembrane protein containing a catalytic kinase domain homologous to other kinases of the eukaryotic translation initiation factor 2 (eIF2) such as general control non-depressible-2 (GCN2), heme-regulated inhibitor (HRI) and protein kinase R (PKR) (Harding et al., 1999). The luminal stress sensor domain of PERK is structurally and functionally homologous with the luminal domain of IRE1α, implicating very similar stress-sensing mechanisms between PERK and IRE1α (Bertolotti et al., 2000). The PERK branch of UPR transduces both the pro-survival as well as pro-apoptotic signals following the accumulation of unfolded or misfolded proteins in the ER. However, its main function is to modulate translation. During initial stages of ER stress, PERK oligomerizes in the ER membrane and induces autophosphorylation (He, 2006). Activated PERK phosphorylates eIF2α at S51 (Harding et al., 1999; Raven et al., 2008) leading to global attenuation of translational machinery, thus reducing the trafficking of newly synthesized proteins into the already stressed ER compartment. The accumulated protein load is then cleared off from the ER by ERAD pathway with simultaneous expression of pro-survival genes like activating transcription factor 4 (ATF4) (Harding et al., 2003). ATF4 is not affected by the global eIF2α translational block because of the presence of internal ribosome entry site (IRES) sequences in the 5′ untranslated regions (Schroder and Kaufman, 2005). However ATF4 can drive the cell toward apoptosis by inducing expression of factors like C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible protein 34 (GADD34) (Zinszner et al., 1998; Novoa et al., 2003).

ATF6 PATHWAY

ATF6 is a type II ER transmembrane protein belonging to the bZIP family of transcription factors. The ER luminal domain acts as the sensor for ER stress due to the protein overload while the cytoplasmic domain acts as a transcription factor (49). ATF6 has two homologs- ATF6α (Hai et al., 1989; Haze et al., 1999) and ATF6β (Min et al., 1995; Khanna and Campbell, 1996; Haze et al., 2001) with redundant roles in UPR. Upon dissociation of BiP from the N-terminus of ATF6 following ER stress, it translocates

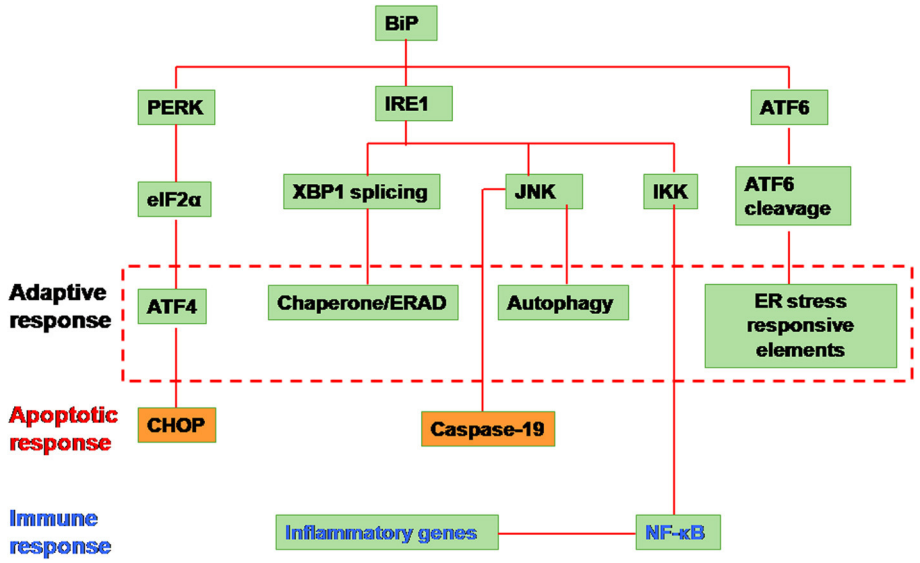


FIGURE 1 | Unfolded protein response signaling. The signaling is initiated by the activation of the proximal sensors of the unfolded protein response (UPR) namely, (1) protein kinase R (PKR)-like ER kinase (PERK), (2) activating transcription factor (ATF) 6 and (3) inositol-requiring enzyme 1 (IRE1). A protein called immunoglobulin heavy chain binding protein (BiP) functions as the master regulator. BiP under normal conditions remains attached to all the three sensors in the luminal domain of the endoplasmic reticulum (ER). Upon encountering any stress like accumulation of misfolded/unfolded proteins or a massive inflow of any exogenous proteins into the ER, the stress sensors, PERK, IRE1, and ATF6, are activated by the release of BiP from the sensors leading to any of the three distinct pathways. (1) When PERK is activated, it dimerises and autophosphorylates leading to phosphorylation of the eukaryotic translation initiation factor (eIF) 2α. Activated eIF2α represses global protein translation of the cell. However the downstream protein called ATF 4 can escape translational repression since it has upstream open reading frames leading to its activation. The activated ATF4 translocates into the nucleus activating a set of target genes to restore cellular homeostasis (adaptive response). However in situations when the cellular homeostasis cannot be restored, C/EBP homologous protein (CHOP) is activated leading to apoptosis. (2) When IRE1 is activated, it dimerizes and autophosphorylates leading to the activation of its endoribonuclease activity. This leads to an unusual splicing of XBP1 (X-box binding protein 1) cleaving 26 nucleotide intron within. The Spliced XBP1 (sXBP1) protein translocates to nucleus transcribing chaperones and unfolded protein response elements (UPREs) to restore cellular homeostasis. In some cases, the IRE1 activates the cellular JNK through phosphorylation. This activated JNK either leads to apoptosis by activaton of caspase 19 or leads to autophagy. Alternatively, IRE1 activates IKK by interacting with tumor necrosis factor receptor-associated factor 2 (TRAF2) which phosphorylates IκB. This releases nuclear factor (NF)-κB. The activated NF-κB translocates into the nucleus and transcribes inflammatory genes. (3) Activation of the third sensor of UPR, ATF6 leads to its translocation into the Golgi complex. In the golgi complex, ATF6 will be cleaved by proteases such as site-1 protease (S1P) and S2P. This cleaved ATF6 fragment further transcribes chaperones and UPRE to cope with the cellular stress and restore homeostasis (Yoshida et al., 2001; Lee et al., 2002; Harding et al., 2003; Novoa et al., 2003; Wu et al., 2007; Yamamoto et al., 2007; Raven et al., 2008).

from the ER to the golgi where it is cleaved by resident proteases like site 1 protease (S1P) and site protease (S2P) (Hetz et al., 2011) to release its cytoplasmic DNA binding fragment called ATF6f. ATF6f increases degradation of unfolded proteins as well as induces the activity of several ER chaperone proteins like BiP, protein disulfide isomerase (PDI) and ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) (Wu et al., 2007; Yamamoto et al., 2007).

GENE THERAPY

In the last two decades, gene therapy has been immensely popular to treat various inherited as well as acquired disorders (Kay, 2011; Misra, 2013). Gene therapy involves either replacing a mutated gene with a healthy copy or introducing a new gene into the cells to help protect against the disease. Despite significant success seen in the treatment of diseases such as lipoprotein lipase deficiency (Gaudet et al., 2012), haemophilia B (Manno et al., 2006; Nathwani et al., 2011), Leber's Congenital Amaurosis (Simonelli et al., 2010) or severe combined immunodeficiency (SCID) (Cavazzana-Calvo et al., 2000), the safety and efficacy of this novel modality of treatment recognizably needs to be improved. For a clinically relevant gene therapy protocol, the efficient delivery and optimal expression of the gene of interest are very important. Since viruses are naturally evolved to efficiently infect and transfer DNA into the host, engineered (gutted) viruses are the most desirable as gene delivery vehicles. Viral vectors currently available for gene therapy can roughly be categorized into integrating and non-integrating vectors. Vectors based on retroviruses (including lentivirus and foamy virus) have the ability to integrate their viral genome into the chromosomal DNA of the host cell, which can theoretically achieve life-long gene expression. Vectors based on Ad, AAV and HSV-1 represent the non-integrating vectors (**Table 1**). These vectors deliver their genomes into the nucleus of the target cell, where they continue to remain episomal. Viral vectors derived from retroviruses, Ad, AAV and HSV have been employed in the majority of gene therapy clinical trials (**Table 2**). Recognizing the activation and basis of cellular events like UPR in response to a virus

Table 1 | Characteristics of the common viruses used in gene therapy.

Viral Vector	Description	Associated disease	Maximum transgene capacity	Host genome integration	Transduction of cells	Advantages	Disadvantages
Adenovirus	36 kb dsDNA, non-enveloped, icosahedric, 70–90 nm in diameter	Yes	∼30 kb	No	Both dividing and non-dividing	Easy production of high titres, ability to infect a wide range of cell types, capacity to carry large transgene	Adverse host humoral and cellular immune response, transient gene expression
Retroviruses (retrovirus and lentivirus)	7–10 kb ssRNA, enveloped, ∼100 nm diameter	Yes	∼8 kb	Yes	Both dividing and non-dividing	High infection efficiency, stable and permanent gene transfer	Insertional mutagenesis causing cancer, high immunogenicity
Adeno associated virus (AAV)	4.7 kb ssDNA, Icosahedric, non-enveloped, ∼22 nm diameter	No	∼4.7 kb	No	Both dividing and non-dividing	Low immunogenicity, non-infectious	Limited transgene carrying capacity, not suitable to target rapidly dividing cells
Herpesvirus-HSV-1	∼152 kb dsDNA, icosahedric enveloped, ∼125 nm diameter	Yes	∼150 kb	No	Only dividing cells	Large transgene carrying capacity, production of high titres	Host immune response, short term gene expression

used in gene therapy is important to further optimize gene delivery.

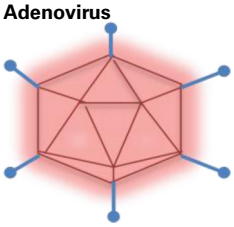
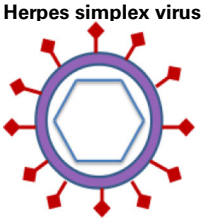
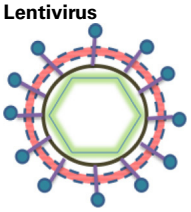
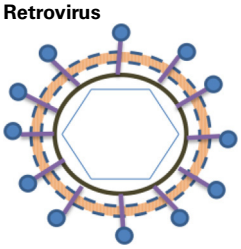
HSV AND UPR

HSV-1 is a large (∼152 kb) fast replicating, enveloped, double stranded (ds) DNA virus. The mature viral particle consists of 3 components- an external envelope made of about 13 glycoproteins which helps the virus to bind and enter the host cell; a second layer called tegument which contains 20 different structural and regulatory proteins and finally an icosohedral capsid containing the genetic material. HSV is an attractive choice as a gene therapy vector for various reasons, including its broad tropism, host range and its cellular receptors (Arii et al., 2009; Fan et al., 2009; Wang et al., 2009b), their ability to infect non-dividing cells with high efficiency, high production titers for recombinant particles and a stable/long-term expression of therapeutic genes especially in neurons (Norgren and Lehman, 1998). Three types of HSV-1 vectors are currently in use in gene therapy- replication-defective, replication-competent vectors and amplicons. Deleting one or more genes involved in the lytic cycle creates a replication-defective vector. Replication-competent viruses are attenuated for genes that are not essential for replication *in vitro* (Hu and Coffin, 2003; Post et al., 2004). The amplicons are derived from engineered plasmids, which contain both the HSV packaging recognition sequence (pac) and the origin of replication (ori). These amplicons can be efficiently packaged in mammalian cells as concatamers with the help of HSV helper elements. Also, amplicons are non-toxic and can carry very large DNA fragments of upto 152 kb (Epstein, 2009). Both replication defective and replication competent HSV vectors have been used in gene therapy of several neurological disorders (**Table 1**). Replication defective HSV vectors have been shown to efficiently transduce both dividing and non-dividing cells including tumors. Taking advantage of this property, HSV vector have been engineered to deliver anticancer transgenes into tumour cells such as melanoma (Krisky et al., 1998; Niranjan et al., 2003), gliosarcoma (Moriuchi et al., 2002; Niranjan et al., 2003) or glioblastoma (Niranjan et al., 2000).

One of the major factor that negatively affects HSV mediated gene delivery is the host immune response directed against it, including the innate and adaptive responses (Ryan and Federoff, 2009). As a first line of defense, innate immunity is a major rate-limiting factor in HSV transduction. One of the principal effector underlying anti-HSV innate defense, is the process of autophagy that is initiated through the cellular UPR pathway (Lee et al., 2009).

During replication of HSV, there is a rapid generation of large amount of viral proteins that may induce UPR and consequently necessitate modulation of the cellular stress response (**Figure 2A**). Indeed, a number of HSV-1 proteins have been shown to block phosphorylation of eIF2α, an important stress response mechanism of the cell, which leads to the attenuation of protein synthesis (He et al., 1996; Cassady et al., 1998; Mulvey et al., 2003, 2006, 2007). Cassady et al. (1998) and Mulvey et al. (2003) showed that a HSV viral protein, US11 can repress two kinases (eIF2α, PKR) and PERK upon HSV infection (**Figure 2A**) (Cassady et al., 1998; Mulvey et al., 2003). He et al., demonstrated that a late protein $\gamma_1$34.5 can dephosphorylate eIF2α with the help of the cellular phosphatase PP1α (He et al., 1996). This inhibition resulted in a 1000-fold increase in the replication efficiency of HSV1 (Talloczy et al., 2006). It has been shown that HSV-1 infection does not activate PERK as well as IRE and was also highly resistant to acute ER stress (Mulvey et al., 2007).

Table 2 | Viral vectors used in clinical trials (Last date of access—24th March, 2014).

Viral vectors	Disease target	Clinicaltrials.gov identifier	Last update
Adenovirus 	Cystic fibrosis	NCT00004779	June 23, 2005
	Ovarian cancer	NCT00964756;	February 11, 2013;
		NCT00562003	January 25, 2011
	Metastatic breast cancer	NCT00307229;	May 31, 2012;
		NCT00197522	October 31, 2012
	Lung cancer	NCT00776295	January 16, 2013
	Brain tumor	NCT00004080	February 6, 2009
	melanoma	NCT01397708	March 11, 2014
	Bladder cancer	NCT00003167	January 22, 2013
Adeno-associated virus (AAV) 	Retinal disease	NCT01482195	November 29, 2011
	Pompe disease	NCT00976352	December 13, 2013
	Late infantile neuronal ceroid lipofuscinosis	NCT01161576	November 5, 2013
	Leber congenital amaurosis	NCT00749957;	March 6, 2013;
		NCT00643747;	December 13, 2013;
		NCT00999609;	January 13, 2014;
		NCT00516477	January 13, 2014
	Alpha-1 antitrypsin deficiency	NCT01054339;	March 6, 2013;
		NCT00377416;	December 20, 2013;
		NCT00430768	December 20, 2013
	Cystic fibrosis	NCT00004533	June 23, 2005
	Idiopathic Parkinson's disease	NCT00985517	December 10, 2012
	Hemophilia B	NCT01687608;	September 19, 2013;
		NCT01620801;	December 20, 2013;
		NCT00076557;	April 2, 2007;
		NCT00515710;	December 20, 2013;
		NCT00979238	December 20, 2013
	Duchenne muscular dystrophy	NCT00428935	February 4, 2013
	Lipoprotein lipase deficiency	NCT01109498;	September 29, 2011;
		NCT00891306	September 28, 2011
Herpes simplex virus vectors 	Melanoma, liver cancer, pancreatic cancer, lung cancer	NCT01935453	August 30, 2013
	Refractory non-central nervous system (non-CNS) solid tumors	NCT00931931	November 5, 2013
	Head and neck cancer or solid tumors	NCT01017185	February 18, 2013
Lentivirus 	Lymphoma	NCT00569985	January 6, 2014
	Acute myeloid leukaemia	NCT00718250	July 16, 2008
	ADA-deficient severe combined immunodeficiency (ADA-SCID)	NCT01852071	January 14, 2014
	X-linked severe combined immunodeficiency (X-SCID)	NCT01306019	March 14, 2014
	Fanconi anaemia	NCT01331018	March 6, 2014
	Wiskott—aldrich syndrome	NCT01515462	January 18, 2012
	AIDS-related non-hodgkin lymphoma	NCT01961063	October 30, 2013
Retrovirus 	Chronic granulomatous disease	NCT00778882	January 15, 2014
	CNS tumors	NCT00005796	October 22, 2009
	X-Linked severe combined immunodeficiency (X-SCID)	NCT00028236	July 26, 2011
	ADA-deficient severe combined immunodeficiency (ADA-SCID)	NCT00598481;	December 12, 2013;
		NCT00599781;	January 23, 2008;
		NCT00794508	February 7, 2013
	Leukocyte adherence deficiency	NCT00023010	December 14, 2010
	Chronic granulomatous disease (CGD)	NCT00564759;	November 27, 2007;
		NCT00001476	December 14, 2010
	Gaucher's disease	NCT00001234	March 3, 2008
	Sickle cell anaemia and β-thalassemia	NCT00669305	December 20, 2013
	Mild hunter synrome	NCT00004454	June 23, 2005
	HIV infection	NCT00001535	February 7, 2008
	Gyrate atrophy	NCT00001735	March 3, 2008

This resistance of PERK toward activation by ER stress in HSV-1 infected cells is attributed to the glycoprotein B (gB) associated with the luminal region of PERK (**Figure 2A**). This study also showed a genetic association between PERK and gB which could regulate the viral protein load in infected cells (Mulvey et al., 2007). To further understand how HSV1 modulates cellular UPR, Burnett et al., reported that HSV-1 can deactivate UPR in the early stages of infection (Burnett et al., 2012). The study observed early

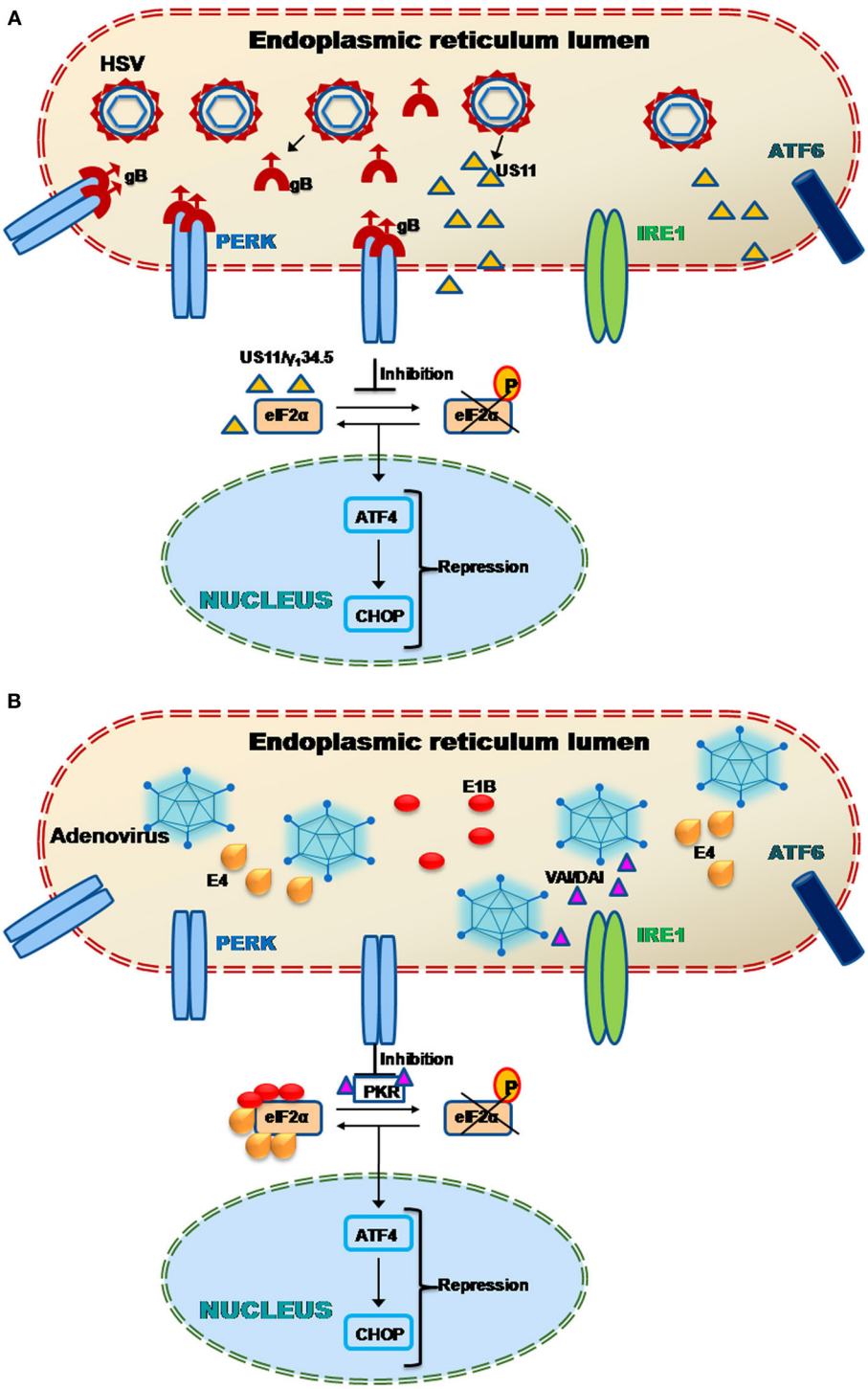


FIGURE 2 | Continued

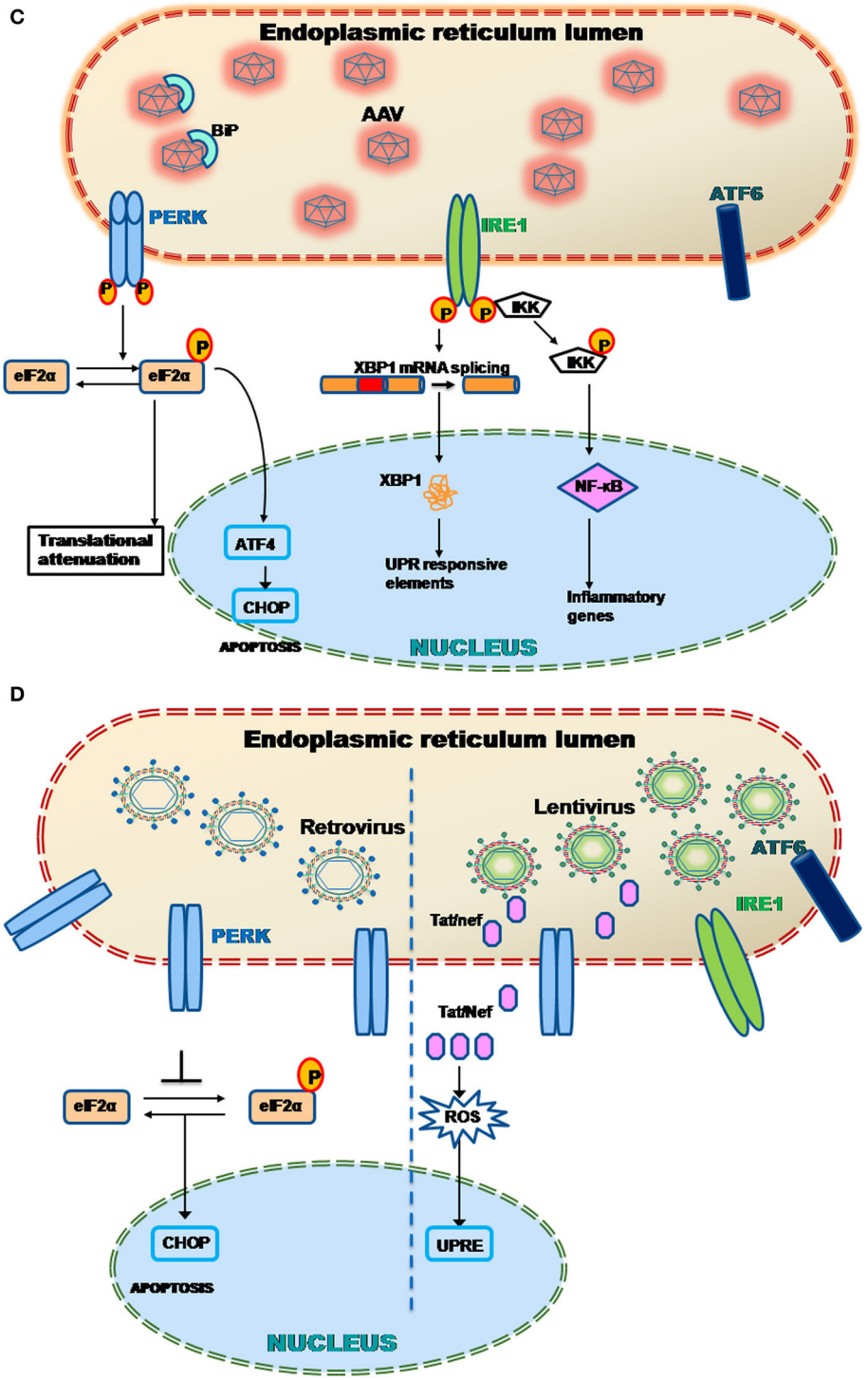


FIGURE 2 | (A) Herpes simplex virus (HSV-1) and UPR. HSV-1 produces proteins such as glycoprotein B (gB) and US11 that have been shown to evade the host UPR mechanism (Mulvey et al., 2003, 2007). In particular, the protein gB specifically binds to the PERK proteins preventing their phosphorylation. This leads to PERK inactivation and hence the downstream effector protein eIF2α could not get activated leading to ATF4 repression. Alternatively another viral protein called US11 represses the eIF2α phosphorylation by directly binding to it. The late HSV viral protein $\gamma_1$34.5 also induces dephosphorylation of eIF2α with the help of the cellular phosphatase PP1α (He et al., 1996). This leads to early repression of ATF4 and CHOP genes downstream. Thus the host UPR response is attenuated and leads to successful viral transduction. **(B)** Adenovirus (Ad) and UPR. Adenoviruses during their late phase of their infection, try to overcome the cellular stress response by preventing the shutdown of protein translation through PKR mediated inhibition of eIF2α phosphorylation *via* viral associated

(Continued)

FIGURE 2 | Continued
RNA molecule I (VAI RNA) as well as double-stranded RNA-activated inhibitor (DAI) (Huang and Schneider, 1990; Mathews and Shenk, 1991; McKenna et al., 2006). Other Ad proteins such as E1B and E4 has also been found to directly bind to the eIF2α, thus preventing its phosphorylation and activation of downstream UPR related genes like ATF4 and CHOP (Spurgeon and Ornelles, 2009). **(C)** Adeno associated virus (AAV) and UPR. When the cellular ER encounters AAV particles, specific stress sensors, PERK and IRE1 gets activated (Balakrishnan et al., 2013). PERK phosphorylation leads to the activation of the eIF2α through phosphorylation. The phosphorylated eIF2α further activates the activating transcription factor 4 (ATF4) the protein of which translocates into the nucleus transcribing UPR responsive genes necessary to cope up with the cellular stress. The phosphorylated eIF2α also arrests the translation of cellular proteins to maintain homeostasis. It has been noted that the AAV particles also activates IRE1 which induces the unusual splicing of X-box binding protein 1 (XBP1) mRNA downstream. The XBP1 protein translocates into the nucleus activating a set of UPR responsive elements. The IRE1 also activates the IKK leading to NF-κB upregulation. The activated NF-κB further activates the inflammatory genes thus inducing an inflammatory response (Jayandharan et al., 2011; Balakrishnan et al., 2013). **(D)** Murine leukemia virus (MLV) and UPR. MLV based γ-retroviral vectors, which are the most common used in gene therapy, has been showed to induce neuropathogenecity in astrocytes (Dimcheff et al., 2003). Later in NIH3T3 cells it was shown that the murine retroviruses induce the ER stress related genes such as CHOP/GADD153 which leads to apoptosis (Dimcheff et al., 2003). On the other hand, the lentiviral proteins such as Tat and Nef have been shown to activate unfolded protein response elements (UPRE) by increasing ROS (Tiede et al., 2011; Abbas et al., 2012).

repression (less that 24 h post infection) of ATF4 and CHOP due to inhibition of phosphorylation of eIF2α. ICP0, an immediate-early Ad gene product known to have transcription factor capabilities (Yao and Schaffer, 1994), was found to be the primary factor triggering activation of the UPR enhancers during HSV-1 replication, thus helping the virus to sense it at an early stage. Consistent to a previous finding (Mulvey et al., 2003), XBP1(for the IRE1 signaling pathway) remained inactive in this study as well.

Ad AND UPR

Ads are non-enveloped DNA viruses whose genome is comprised of a linear 36 kb double-stranded DNA. The recombinant Ad vectors were first used as a gene transfer agent in 1985 (Ballay et al., 1985; Yamada et al., 1985) and since then have been used as a vehicle for various monogenic diseases (Porteus et al., 2006). For example, Ad vectors expressing cystic fibrosis transmembrane conductance regulator CFTR have been used in phase I clinical studies to treat cystic fibrosis (Zuckerman et al., 1999). Muscular dystrophy is another disease where attenuated Ad vectors have been used to deliver dystrophin cDNA into muscle tissue (Clemens et al., 1996; Haecker et al., 1996; Floyd et al., 1998). Improved Ad vectors have also been used to deliver human coagulation factors VIII and IX for phenotypic correction of hemophilia in preclinical animal models (Zhang, 1999). These vectors have been used in the treatment of several other metabolic and genetic diseases like lysosomal storage disease, phenylketonuria and glycogen storage disease (Amalfitano et al., 1999; Nagasaki et al., 1999; Ziegler et al., 1999; Eto and Ohashi, 2000; Stein et al., 2000; Zingone et al., 2000), neurological disorders like Parkinson/Alzheimer's as well as cardiovascular diseases like atherosclerosis, cerebral ischemia and in cancer therapy (Donahue et al., 2000; Papadopoulos et al., 2000; Choi and Yun, 2013).

As is the case of any foreign protein, Ad vectors are also subject to severe inflammatory response, which lead to their clearance and achieves only transient gene expression in the target tissue. One of the major transcription factor that serves as the mediator of inflammatory response is NF-κB, which can be activated by various pathological stimuli like bacterial/viral infection and the inflammatory cytokines. It has been shown previously that accumulation of protein load in the ER can activate NF-κB *via* the PERK and IRE-1 pathway (Tam et al., 2012). It has been demonstrated that the Ad E3/19 K protein can activate NF-κB mediated by Ca^{2+} release from the ER following a protein overload, *in vitro*. As a result, NF-κB activates inflammatory cytokines and interferons that constitute the initial anti-viral response of the cells (Pahl and Baeuerle, 1995). As with most viruses, in the late phase of productive infection, viral protein synthesis is promoted in Ad-infected cells while simultaneously inhibiting the cellular protein synthesis. One of the ways Ad does this, is by inhibition of PKR-mediated eIF2α phosphorylation along with the activation of a protein kinase called double-stranded RNA-activated inhibitor (DAI) (Huang and Schneider, 1990). It has also been shown that the non-coding Ad associated RNA molecule I (VAI RNA) can suppress PKR activation by directly binding to it (Mathews and Shenk, 1991; McKenna et al., 2006). VAI RNA is highly expressed during the late phase of Ad infection when it is transcribed by a RNA polymerase III (Soderlund et al., 1976; Thimmappaya et al., 1982; Svensson and Akusjarvi, 1984). It plays a crucial role in preventing shutdown of cellular translational apparatus by inhibiting eIF2α phosphorylation and the activation of PKR, although the precise mechanism remains unclear (Huang and Schneider, 1990). Spergeon et al., have also shown the role of Ad E1B 55-Kd and E4 Open Reading Frame 6 Proteins in promoting its infection in the late phase. These Ad proteins were shown to limit phosphorylation of eIF2α phosphorylation and PKR activation (**Figure 2B**). This process requires the functioning of the Cul5-mediated E3 ubiquitin-protein ligase of the E1B-55K/E4orf6 complex which is independent of the cytoplasmic levels of VAI RNA (Spurgeon and Ornelles, 2009).

AAV AND UPR

Naturally occurring AAV is small (∼22 nm), non-enveloped and contains single-stranded DNA (∼4.7 kb). It belongs to the family *Parvoviridae*, and the genus *Dependovirus* (Balakrishnan and Jayandharan, 2014). The genome contains two open reading frames encoding the genes, *rep* (responsible for replication) and *cap* (encodes capsid specific proteins) flanked by a 145 base pair long inverted terminal repeat (ITR) sequence. AAV enters the infective cycle only in presence of other helper virus such as Ad or HSV (Daya and Berns, 2008). Recombinant AAV is produced by stuffing the transgene of interest between the flanking ITRs while the *rep* and *cap* genes are supplied *in trans* along with helper function genes (Wright, 2009). Currently, AAV is the choice vector for many inherited and non-inherited diseases

because of its non-pathogenic nature. Another major advantage of using AAV as a gene therapy vector is its low immune profile when compared to Ad vectors (Asokan et al., 2012). So far 12 AAV serotypes (AAV1-12) has been used as gene therapy vectors although several other serovars are known to exist. AAV is naturally hepatotrophic that makes them an attractive choice for liver targeted gene therapy for the treatment of diseases such as hemophilia and alpha1 antitrypsin deficiency (Flotte et al., 2011; Nathwani et al., 2011). However several other alternate serotypes like AAV1, AAV5, AAV9, and rh10 have shown significant promise in targeting tissues like the muscle and the central nervous system (Zincarelli et al., 2008; Tang et al., 2010; Rafi et al., 2012; Gray et al., 2013). Unfortunately, the vector dose-dependent immune response and the presence of pre-existing neutralizing antibody against AAV capsids can limit persistent gene expression in humans (Manno et al., 2006; Boutin et al., 2010). It is known that AAV, after receptor mediated endcocytosis, undergoes trafficking through the endocytic compartments followed by retrograde transport to the Golgi or the ER (Ding et al., 2005). It has been shown earlier that intracellular trafficking of AAV is negatively regulated by components of the ER stress response (Duan et al., 1999; Douar et al., 2001; Ding et al., 2003). For example AAV mediated human factor (F).VIII gene expression improved by ∼300–600% upon inhibiting the proteasomal machinery by using pharmacological agents like bortezomib in preclinical animal models of haemophilia (Monahan et al., 2010). Thus, it is quite logical to note that during intracellular trafficking, ER stress could play an inhibitory role in AAV life cycle. Indeed, we have recently shown the role of UPR in AAV infection (Balakrishnan et al., 2013). In this study self-complementary (sc) AAV2 was shown to activate the PERK and IRE-1 pathway in HeLa cells with peak activation 12 h post-infection. ATF6 however was not induced by scAAV2. Interestingly, single-stranded (ss) AAV2 did not induce UPR effectors as dominantly as scAAV2 although it modestly activated PERK and IRE-1. The activation of PERK and IRE-1 was further confirmed by an increased expression of downstream signaling molecules like CHOP and spliced XBP-1, respectively. Inhibiting PERK and or IRE-1 expression in *in vitro* (using shRNA against PERK/IRE-1) and *in vivo* (metformin, i.p) led to a modest increase in gene expression from scAAV2 vectors (**Figure 2C**). Interestingly, this study also found that alternate AAV serotype vectors like AAV1 and AAV6 can activate distinct arms of UPR. For example, scAAV6 had a comparable effect on PERK activation but not on IRE-1 as scAAV2 vectors. Another observation was the ablation of innate immune response markers following UPR inhibition *in vivo*. This clearly points to the link between UPR activation and clearing of the vectors through innate immune response. It has been shown previously that AAV can activate the classical NF-κB pathway during the acute phase of infection and trigger downstream inflammatory markers like TNF-α, IL1a, IL6, and leading to the activation of the adaptive immune response (Jayandharan et al., 2011). It is also known that UPR caused by protein overload can activate cellular NF-κB in the early phase while it is inhibitory in the late phase (Kitamura, 2011) (**Figure 2C**). Thus, the UPR pathway becomes an important target to reduce inflammatory response in the early stages of AAV infection and to further enhance the persistence and gene expression from AAV vectors. Interestingly, the efficiency of AAV transduction is also known to improve under general cellular stress as shown earlier in cellular models of cystic fibrosis (Johnson et al., 2011).

RETRO-/LENTI-VIRUS AND UPR

Historically, vectors based on retrovirus which were the first viral vector system described in the early 1980s (Douar et al., 2001) have been the most preferred in clinical gene therapy due to their properties of efficient host DNA integration and persistent gene expression. However, in the clinical trial involving infants with X-SCID, 4 out of 9 patients developed leukemia due to random retroviral integration, this remains a major concern with retrovirus based gene therapy (Cavazzana-Calvo et al., 2000; Kohn et al., 2003). Lentivirus, that belongs to the retroviridae family is also known to facilitate stable integration of the viral genome into the host chromosome. Over the past decade, more than 30 patients with different immunodeficiency disorders have been treated successfully using murine leukemia virus (MLV)-based γ-retroviral vectors to transfer therapeutic genes to autologous hematopoietic cells (Aiuti et al., 2002; Aiuti and Roncarolo, 2009). However, random integration of the lentiviral vectors is also known (Wang et al., 2009a). Although not many studies have been conducted to understand if and how retro- or lenti-viruses combat UPR, there is some evidence that retroviruses can induce ER stress. In Shikova et al. (1993) first showed in cultured astrocytes that neuropathogenicity of MLV viruses may be related to protein misfolding in the ER (Shikova et al., 1993). In Dimcheff et al. (2003) demonstrated that a mouse retrovirus $FrCas^E$ is able to induce ER stress related genes like CHOP/GADD153 and Bip *in vitro* in NIH3T3 cells as well as *in vivo* which correlated with the induction of spongiform neurodegeneration (**Figure 2D**) (Dimcheff et al., 2003). Similarly, mink cell focus-forming murine leukemia virus (MCF13 MLV) has been shown to trigger UPR in mink cells following large accumulation of the viral protein MLV $gPr80^{env}$ *via* upregulation of CHOP proteins (Nanua and Yoshimura, 2004). A Lentivirus-HIV-1 protein called the trans-activator of transcription (Tat) has been reported to induce UPR by increasing reactive oxygen species (ROS) in primary rat striatal neurons indicating that ER stress response could be a critical parameter to control during HIV infection (**Figure 2D**) (Tiede et al., 2011). Another HIV viral protein called Nef, known to increase infectivity and replication in lymphocytes and macrophages has been shown to directly interact with the eukaryotic elongation factor (eEF)-1α resulting in its cytoplasmic relocalization and the inhibition of stress-induced apoptosis. Conversely, the nuclear re-localization of the Nef/eEF1α complex can decrease mitochondrial cytochrome c release, thereby inhibiting the caspase activation. This mechanism demonstrates how the lentivirus (HIV) can prevent cell death under conditions of stress condition yet is able to create an environment favoring optimal viral replication (Abbas et al., 2012) (**Figure 2D**). Another unique retrovirus called Foamy viruses (FVs) have also been extensively studied as a gene therapy vector due to their lack of pathogenicity, broad tissue tropism and the ability to carry large (minimum ∼9.2 kb) transgenes (Heneine et al., 2003; Trobridge, 2009). Hematopoietic stem cell (HSC) gene therapy is one area

where FVs have been extensively evaluated with considerable success (Josephson et al., 2004; Bauer et al., 2008). However there are no published evidence which have studied the interaction between FVs and cellular UPR. However it is possible that like other retro-/lenti- viruses, FVs would have developed mechanism to either counteract or utilize the UPR machinery to enhance its own replication in the infected cells.

OTHER VIRAL VECTORS AND UPR

In addition to the commonly used viral vectors described above, attempts have been made to utilize other viruses as vectors for certain disease conditions. For example, *Vaccinia viral vectors* have been in use as a potential therapeutic for cancer gene therapy (Yu et al., 2009; Seubert et al., 2011) mainly because of its efficient infection and gene expression in a wide range of difficult to transduce tumors (Yu et al., 2004) as well as their inability to integrate into the chromosome (Shen and Nemunaitis, 2005). Also, the safety profile of Vaccinia virus as a therapeutic agent is well understood due to its long and widespread use as a vaccine for small pox in humans. Like most viruses, vaccinia virus also regulates the cellular UPR machinery to facilitate its infection. For example, a vaccinia viral protein K3L which has ∼28% sequence identity with eIF-2a is thought to function as a pseudo substrate for its kinase, thus blocking the PKR activity and leading to the inhibition of PERK and eIF2α molecules (Sood et al., 2000). Following ER stress response, another Vaccinia protein called F1L can indirectly inhibit the activation of the apoptotic protein *Baxby* by interacting with the proapoptotic BH3-only proteins through Bak and Bax (Taylor et al., 2006).

Varicella zoster virus (VZV), the causative agent of varicella (chickenpox) and zoster (shingles) and a member of the *Herpesviridae* family has also been tested as a cancer gene therapy vector (Degreve et al., 1997). VZV has been shown to induce cellular UPR through ER stress *in vitro.* It has been shown to activate both the IRE-1 and the CHOP pathway and ultimately leads to autophagy (Carpenter et al., 2011). This study also confirmed that the VZV structural glycoproteins—gE (ORF68), gI (ORF67), gH (ORF37), and gL (ORF60) were enough to induce UPR during an active viral infection.

Epstein–Barr virus (EBV) is a member of the *Herpesviridae* family that has a natural tropism for B cells. This property of the virus has been utilized to deliver GM-CSF to human B cells from B-cell chronic lymphocytic leukemia (B-CLL) patients as a potential immune therapy (Hellebrand et al., 2006). However, since EBV is associated with a number of human malignancies, rigorous vector modification and validation is called for prior to its application as a gene delivery vehicle in humans. The latent membrane protein 1 (LMP1) oncogene of EBV is shown to induce the phosphorylation of eIF2α by activating all the three arms of UPR, the PERK, IRE-1, and ATF6 pathways. This activation in turn up-regulates LMP1, which leads to induction and maintenance of the proliferating B lymphocytes (Lee and Sugden, 2008). Thus it seems that the UPR pathway is required for EBV to enter into its lytic stage toward maintaining its proliferative and infectious life-cycle (Taylor et al., 2011).

Sendai virus (SeV) is a negative strand RNA virus which utilizes sialic acid residue or a sialoglycoprotein as their receptor for cell entry (Markwell et al., 1981). The major advantages of using recombinant SeV as a gene therapy vector is its non-pathogenicity and the ability to be generated in high titre during packaging process. Preclinical studies have shown that SeV can transduce different cell types like vascular tissue (Masaki et al., 2001), skeletal muscle (Shiotani et al., 2001), airway epithelial cells (Yonemitsu et al., 2000) and synovial cells (Yamashita et al., 2002), quite efficiently. Further, it has been shown that SeV vector can efficiently transfer its cargo to $CD34^+$ cell and $CD34^+$ cell subpopulations derived from human cord blood (Jin et al., 2003). In addition, SeV was also able to get stable gene expression in myeloid, erythroid or mixed progenitor cells (Jin et al., 2003). However, since SeV induces cytopathic effects in infected cells, toxicity concerns remain. SeV has been shown to upregulate CXCL2 protein following ER stress, which can lead to cell death via activation of caspase-8 and caspase-3 mediated apoptosis (Bitzer et al., 1999; Versteeg et al., 2007). It is also thought that this virus can activate eIF2α kinases like PERK and PKR to induce IFN regulatory factor (IRF) 7, a major player in host antiviral innate response. ATF4, another key regulator of cellular response to viral infection can be upregulated *via* phosphorylation of eIF2α and the activation of IRF7 ultimately helping in cellular recovery (Liang et al., 2011).

Alphavirus vectors based on Sindbisvirus (SINV) and Semliki Forest virus (SFV) is another group of virus that has been evaluated as a gene therapy vector because of advantages like broad host range, efficient replication in the cytoplasm and the capacity to produce high levels of recombinant proteins. Several preclinical studies have been conducted so far to evaluate the efficiency of alphaviruses as a gene transfer vehicle. For example, SFV was shown to transduce cardiovascular cells as well as human tumor cells to deliver IL-12 (Roks et al., 1997; Zhang et al., 1997). Alphaviral vectors have also been looked upon as a potential delivery vehicle for DNA-based vaccines (Berglund et al., 1997, 1998). More recently, SFV was used for gene transfer into the central nervous system but was also toxic (Graham et al., 2006). In mammalian cells, SFV envelope glycoproteins activate the UPR response through induction of CHOP proteins and its consequent upregulation of caspase-3, caspase-8, and caspase-9 apoptotic enzymes (Barry et al., 2010). SFV has also been shown to have delayed RNA synthesis in the presence of Brefeldin A, a potent UPR inducer (Molina et al., 2007). Another report suggests that PERK can suppress SFV viral replication at an early stage by eliciting strong interferon response in the mouse brain (Barry et al., 2009). One study revealed that the alpha virus SINV could activate PERK and IRE-1 but not the ATF6 within 48 h of infection *in vitro*. In this study, SINV uncontrollably activated the UPR by phosphorylation of eIF2α and leading to apoptosis (Rathore et al., 2013). Moreover SINV has been shown to promote autophagy in neuronal cells both in *in vitro* and *in vivo* probably via activation of the UPR pathways, thus limiting the spread of viral infection (Orvedahl et al., 2010; Shi and Luo, 2012).

STRATEGIES TO INHIBIT UPR AGAINST VIRUSES USED IN GENE TRANSFER

One of the ways to inhibit UPR is through small molecule inhibitors, which can repress the cellular proteasomal machinery.

For example, the use of pharmacological agents that inhibit proteasomes like metformin, MG-132, and ricin has been previously shown to reduce cellular UPR (Lee et al., 2003; Parikh et al., 2008; Amanso et al., 2011; Theriault et al., 2011). We have previously shown that scAAV2 upregulates PERK and IRE-1α genes in murine liver, ∼24 h post vector administration (Balakrishnan et al., 2013). This effect was reversed when animals were pretreated with metformin (250 mg/kg). More importantly, it was also found that attenuation of the UPR response against AAV also inhibited the expression of various inflammatory cytokines and chemokines like Chemokine (C-C motif) ligand 12 (Ccl12), Chemokine (C-C motif) ligand 11 (Ccl11), Chemokine (C-C motif) ligand 22 (Ccl22), Chemokine C-X-C motif ligand 13 (CXCL13), Chemokine (C-C motif) ligand 24 (Ccl24), Chemokine C-C motif receptor 2 (Ccr2) and chemokine C-X-C motif ligand 15 (CXCL15) (Balakrishnan et al., 2013). Thus, inhibiting the UPR by proteasomal repressors could potentially reduce innate immune response against AAV leading to higher and probably persistent gene expression. However, it is a known fact that systemic administration of proteasomal inhibitors can have adverse effects (Rajkumar et al., 2005). It is however conceivable that transient inhibition of UPR pathways prior to gene transfer and during the initial period of viral infection might lead to improved gene transfer efficiency. Another way to repress UPR could be by the use of silencing (si) RNAs against specific components of the UPR pathway. Our study had previously shown a modest increase in transgene expression from AAV vectors when the PERK and IRE-1α pathways were inhibited by specific siRNA *in vitro* (Balakrishnan et al., 2013). shRNAs against UPR components can also be potentially tested *in vivo*. Such shRNAs can be delivered under inducible promoters to avoid adverse effects caused by long term suppression of UPR machinery.

To avoid immune mediated clearance of viral vectors during gene therapy, ideally the vector dose should be kept to the minimum. This would allow the vectors to not only to escape the host immune surveillance before entering the target cells, but may also reduce cellular stress. To this end, vector bioengineering becomes a very important tool by which novel, optimized vectors can be created as described earlier with AAV (Markusic et al., 2010; Qiao et al., 2010; Gabriel et al., 2013; Sen et al., 2013a,b) to achieve efficient gene transfer at lower vector doses.

CONCLUSIONS

In higher eukaryotes, UPR is a beneficial process that protects the cell from undue stress. Cellular UPR strives to reduce the burden on the ER by enhancing its capacity with the help of several stress response chaperones. If this process becomes futile, UPR can induce apoptosis of the host cell. Most viruses reprogram the cellular translational machinery to facilitate the generation of their proteins, but this process can also trigger the UPR pathways, which consequently may lead to cell death. For successful gene therapy, the survival of the transduced cells is very important to achieve sustained gene expression. In this scenario, transient inhibition of UPR prior to gene transfer, by strategies discussed above, provides an attractive alternative to improving the safety and efficiency of viral gene therapy. However, further detailed understanding of the sub-cellular processes that activate UPR against such viral vectors is also necessary to tailor specific strategies and to shift the balance in favor of virus persistence in the host without compromising either of their survival.

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