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RECEPTOR-INDEPENDENT/ -ASSOCIATED VIRAL TROPISM

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Akio Adachi, Masako Nomaguchi, Mikako Fujita and Yasuyuki Miyazaki

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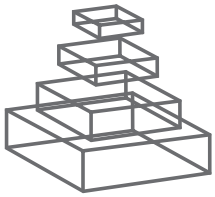

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RECEPTOR-INDEPENDENT/ -ASSOCIATED VIRAL TROPISM

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One of the most important and outstanding characteristics of viruses is their cellular and host tropism. As parasitic entities, viruses have to compromise with numbers of positive and negative factors present in target cells for their survival. In the absence of an appropriate interaction with cells, they do not replicate at all. Viral tropism can be therefore determined at each replication step, from the entry to progeny production in target cells. There are two major types of viral tropism, that is, the receptor-dependent and -independent tropisms. Restriction of viral replication occurs on the cell surface (receptor-dependent viral entry step) and/or intracellularly (receptor-independent post-entry replication steps). Viruses have acquired some mechanisms through adaptive mutations and/or recombinations to counteract a wide variety of cellular restriction factors, or to correctly interact with numerous cellular factors necessary for replication. They thereby can replicate, spread and survive in certain cell lineages, tissues, organs and finally in host individuals. This evolutional process/pressure would have generated profound effects on the biological properties of viruses. Recently, many cellular anti-viral factors with unique action mechanisms in addition to co-viral factors have been discovered by extensive studies on molecular genetics of viruses. Researches of these factors would lead to the effective clinical applications, as well as the increase of basic biological knowledge.

In this Research Topic, we focus on the receptor-independent and uniquely associated viral tropism other than the strictly receptor-dependent or -mediated one. By presenting a series of centered articles, we describe here the unique properties of various virus species. Any types of the tier 1 article would be accepted and included in this Topic.

Table of Contents

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Viral tropism

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One of the most important and outstanding characteristics of viruses is their cellular and host tropism (Levine and Enquist, 2007). As parasitic entities, viruses have to compromise with numbers of positive and negative factors present in target cells for their survival. In the absence of an appropriate interaction with cells, they do not replicate at all. Viral tropism therefore can be determined at each replication step, beginning with the entry into cells and ending with the progeny production from cells. There are two major types of viral tropism, that is, the receptor-dependent and -independent tropisms. Restriction of viral replication occurs on the cell surface (receptor-dependent viral entry step) and/or intracellularly (receptor-independent post-entry replication steps).

In this Research Topic, a number of basic studies on both types of animal virus tropism have been published as either reviews, mini-reviews, or an original research article. Kajitani et al. (2012) have efficiently summarized a unique and complex lifecycle of human papilloma viruses (double-stranded DNA virus). Ohka et al. (2012) have concisely described the receptor-dependent and -independent tropism of poliovirus (positive sense single-stranded RNA virus). Ramadhany et al. (2012) have presented an original study on the mutations in hemagglutinin gene of influenza virus (negative sense single-stranded and segmented RNA virus) and have discussed their effects on the viral tropism. Multiple cellular receptors for measles virus (negative sense single-stranded and segmented RNA virus) have been clearly described by Sato et al. (2012). Takada (2012) has proposed models of filovirus (ebola and Marburg viruses; negative sense single-stranded RNA virus) entry into cells. Yasuda (2012) has discussed the interaction of *ebolavirus* and anti-viral cellular factor tetherin/BST-2. Species tropism of human and simian immunodeficiency viruses (HIV and SIVs; positive sense single-stranded RNA viruses containing reverse transcriptase), a representative of the receptor-independent tropism, has been systematically summarized by Nakayama and Shioda (2012). Sakuma and Takeuchi (2012) has provided insight into the mechanism for species-specific SIV replication. Nomaguchi et al. (2012) have focused on the function of viral accessory proteins, and discussed the bases for HIV-1 species tropism. Tani et al. (2011) have described the use of vesicular stomatitis virus (negative sense single-stranded RNA virus) as tools in various research and medical fields. Finally, Uchiyama (2012) has reviewed the rickettsia tropism as an example of non-viral microbes (obligate intracellular parasitic bacterium).

The authors in this Research Topic have highlighted the importance of viral tropism by presenting biological phenomena and their underpinning host cellular and molecular bases. The biologically unique phenomenon of viral tropism is not only of interest to virologists to bring progress to their basic science but also highly relevant to clinical studies with the goal to design new anti-viral strategies. Accordingly, "Viral Tropism" must be considered a sibling to another Research Topic in Frontiers in Virology entitled "Receptor usage and pathogenesis in acute and chronic viral infection."

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Productive lifecycle of human papillomaviruses that depends upon squamous epithelial differentiation

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Human papillomaviruses (HPVs) target the stratified epidermis, and can causes diseases ranging from benign condylomas to malignant tumors. Infections of HPVs in the genital tract are among the most common sexually transmitted diseases, and a major risk factor for cervical cancer. The virus targets epithelial cells in the basal layer of the epithelium, while progeny virions egress from terminally differentiated cells in the cornified layer, the surface layer of the epithelium. In infected basal cells, the virus maintains its genomic DNA at low-copy numbers, at which the viral productive lifecycle cannot proceed. Progression of the productive lifecycle requires differentiation of the host cell, indicating that there is tight crosstalk between viral replication and host differentiation programs. In this review, we discuss the regulation of the HPV lifecycle controlled by the differentiation program of the host cells.

Keywords: HPV, differentiation, epithelial cell, keratinocyte

INTRODUCTION

Human papillomavirus (HPV) infections of the anogenital organs are a very common "sexually transmitted disease (STD)." Although the incidence of cancer progression is low, a HPV infection is frequently detected in anogenital cancers. As for cervical cancer, HPV DNA is detected in more than 90% of cases. Approx. 5.5 million new cases of HPV infection are reported and there are c.a. 450,000 diagnoses of cervical cancer per year worldwide, leading to approximately 200,000 deaths each year, which ranks second among cancers in women (Parkin and Bray, 2006). HPV infections have also been associated with the head and neck squamous cell carcinomas (HNSCCs).

Human papillomavirus is categorized as a small virus containing DNA. More than 120 types of HPV have been identified and one-third of them target mucosal membranes, the remainder target the cutaneous membranes. Mucosa-tropic HPVs can be classified into two types based on their association with malignant carcinomas: a high-risk type (such as HPV type 16, 18, 31) and a low-risk type (such as HPV6 and 11; Howley, 1996). Prophylactic vaccines for HPV16 and18, Cervarix (GlaxoSmithKline), and for HPV6, 11, 16, and 18, Gardasil (Merck & Co.), have been developed recently and effectively prevent primary infections. They, however, cannot be used as therapeutic vaccines, indicating the importance of a Pap smear and the development of effective treatment strategies (Carter et al., 2011). In order to inhibit HPV-induced cancer, an understanding of the molecular basis of the infection and the characteristics of the infected lesions is important.

GENOME ORGANIZATION OF HPV AND FUNCTIONS OF VIRAL PROTEINS

Human papillomaviruses have a common gene organization (**Figure 1**): an early region encoding non-structural genes, the late region for structural genes, and a regulatory region (long control region: LCR).

The functions of each viral protein are summarized in **Table 1**. E1 and E2 are cooperatively involved in the initiation of viral DNA replication. E2 also functions as a transcriptional transactivator. E6 and E7 modulate the cell cycle control and contribute to viral genome maintenance (Frattini et al., 1996; Stubenrauch et al., 1998; Thomas et al., 1999). They also contribute to cancer development (Münger et al., 2004). Though E4 and E5 are speculated to modulate the productive phase of the HPV lifecycle, their biological roles remain unclear (Fehrmann et al., 2003; Genther et al., 2003; Nakahara et al., 2005; Wilson et al., 2005, 2007; Fang et al., 2006). Both L1 and L2 are capsid proteins.

HPV LIFECYCLE

The target of a HPV infection is the stratified epithelium. In the normal stratified epithelium, the cell attached to the basal membrane (basal cell) is the only cell that has the potential to proliferate. The basal cell divides into a new basal cell and a daughter cell that is detached from the basal membrane, and the daughter cell launches its differentiation process. The daughter cells exit from the cell cycle and change their gene expression pattern, proceeding to terminal differentiation, then peel off from the epithelium (Jones et al., 2007). The lifecycle of HPV is tightly regulated by the differentiation program of the host cells (**Figure 2**). In this section, the differentiation-dependent lifecycle of HPV is briefly summarized.

ENTRY OF HPV INTO THE BASAL CELLS OF STRATIFIED EPIDERMIS

Human papillomavirus virions invade through damaged areas of the epithelium and infect the basal cells. Although the receptor for the HPV infection has not been fully characterized, the following

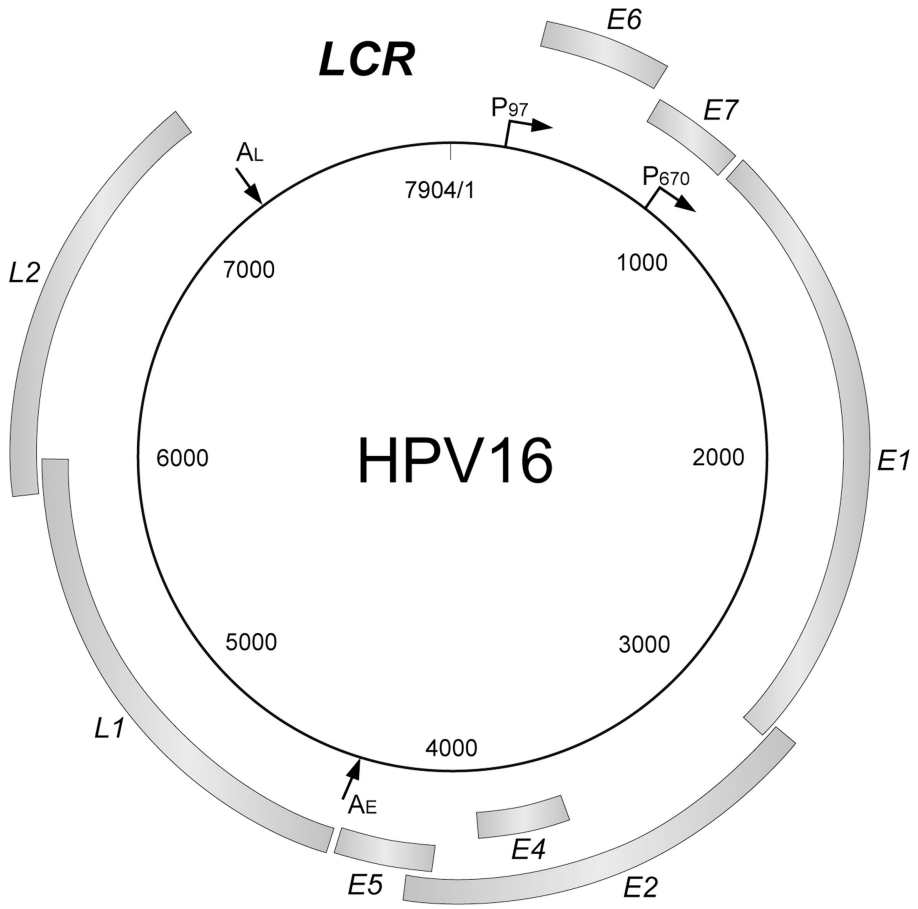

FIGURE 1 | The genome organization of HPV16. The HPV genome has a circular double-stranded DNA structure. The viral genes are transcribed in a single direction (clockwise). There are genes coding for non-structural proteins (E1, E2, E4, E5, E6, and E7) and structural proteins (L1, L2), and a transcriptional control region (long control region; LCR). LCR contains a DNA replication origin and functions as the regulator for the DNA replication. The major promoters and polyadenylation signals are indicated (P97, P670, AE, AL).

model has been postulated; virions initially attach to the heparan sulfate proteoglycan (HSPG) on the basal membrane, and transfer to the receptor expressed on the keratinocytes moving on the basal membrane in the wound-healing process, then enter the cells (Kines et al., 2009).

LOW-LEVEL EXPRESSION OF VIRAL GENES AND GENOME MAINTENANCE IN THE BASAL LAYER

Following viral entry and uncoating, HPV genomic DNA is transported into the nucleus and maintained at a low-copy number in the basal cells (50 ∼ 100 copies per cell; in the basal layer, **Figure 2**; Moody and Laimins, 2010). Genome maintenance as episomal status is essential for the establishment of the early phase of the viral lifecycle (McBride et al., 2006).

PRODUCTIVE REPLICATION OF HPV IN THE DIFFERENTIATED CELLS

After leaving the basal membrane, the infected cells initiate the differentiation program. Because HPV does not encode DNA polymerase activity for viral genome replication, the host DNA replication machinery is required. However, the DNA replication activity is suppressed in the differentiated cells that exit from the cell division cycle. To ensure that the viral genome is replicated, HPV needs to reactivate cell division among the differentiation-initiated cells. E6 and E7 inactivate p53 and retinoblastoma protein (pRb), respectively, which enables the cells to maintain their DNA replication potential (Münger et al., 2004).

In the upper layers of the stratified epithelium (in the spinous layer, **Figure 2**), the expression of viral genes that are required for viral genome replication is markedly accelerated (Hummel et al., 1992; Ozbun and Meyers, 1997), inducing viral genome amplification to thousands of copies per cell (Bedell et al., 1991). Following the genome amplification, in the terminally differentiated cells, the synthesis of capsid proteins is triggered. The capsid proteins assemble into virions that encapsidate viral genomic DNA. The progenitor virions are released externally with peeled keratinocytes.

DIFFERENTIATION-DEPENDENT CONTROL OF HPV LIFECYCLE

The differentiation-dependent lifecycle of HPV is controlled of multiple levels, such as transcription, post-transcriptional processing, translation, and DNA replication. In the following sections, each regulatory mechanism is summarized.

Table 1 | Summary of HPV gene functions.

Function in viral lifecycle	Activities	Target factor
E1		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
E2		
Transcription of viral genes Replication of viral genome Maintenance of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChIR1
E6		
Reactivation of cellular replication mechanisms Proliferation, immortalization, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	p53, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
E7		
Reactivation of cellular replication mechanisms Proliferation, genomic instability, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	RB, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
E4		
Unknown	Destruction of keratin network, induction of G_2M arrest of cell cycle	Cytokeratin 8/18
E5		
Possibly involved in proliferation and/or inhibition of apoptosis	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
L1		
Major capsid protein		
L2		
Minor capsid protein		

TRANSCRIPTIONAL REGULATION OF VIRAL GENES

Human papillomavirus has two major promoters, the early promoter and the late promoter. In HPV16, P97, and P670 have been identified as the early and late promoters, respectively (**Figure 1**). Transcriptional activity is mainly controlled by the LCR. A transcriptional enhancer is located within the LCR, with which various cellular transcription factors can associate (**Figure 3**).

The binding sites for the viral transcriptional regulator, E2, are found in HPV16 LCR. Viral gene expression is regulated by the occupancy status of the E2-binding sites (E2BSs; **Figure 3**), which is partly defined by the E2 expression level controlled by cellular differentiation status (Steger and Corbach, 1997; Hadaschik et al., 2003).

E2 functions in viral genome segregation by tethering the viral DNA to the mitotic chromatin, in which a cellular protein, bromodomain-containing protein 4 (Brd4), has been reported to be involved (McPhillips et al., 2006). Interaction between E2 and Brd4 is also required for the E2-mediated transcriptional activation and repression (McPhillips et al., 2006; Wu et al., 2012).

A ubiquitous transcription factor, Sp1 is a well-known regulator for HPV gene expression. The Sp1-binding site partially overlaps with one of the E2BSs (E2BS#2), and a TATA box element is located close to the promoter–proximal E2BS (E2BS#1; **Figure 3**). The binding of E2 to those E2BSs, therefore, interferes with the assembly of the transcriptional initiation complex, resulting in a suppression of E6/E7 expression that is governed by the early promoter activity (Tan et al., 1992). It was also reported that Sp1 altered the chromatin structure of HPV16 LCR, offering the accessibility of transcription factors (Stünkel and Bernard, 1999).

TRANSCRIPTIONAL CONTROL IN THE UNDIFFERENTIATED CELLS

Transcripts of viral early genes are expressed in the infected basal cells, which is essential for the viral DNA replication (Dürst et al., 1992). It was reported that a unique promoter, P14, was utilized for E1 expression and the E2BSs were considered as necessary for the P14 activity (Lace et al., 2008). The transcript initiated from P14 is a poly-cistronic mRNA containing E6, E7, and E1, in which the shunting in ribosomal scanning process enables the translation of E1 (Remm et al., 1999). The regulatory mechanism for E2 expression has not been clarified. The early promoter is used for E6 and E7 expression, in which several transcription factors, including AP-1, glucocorticoid receptor, NF1, Oct-1, Sp1, YY-1, and CDP, are involved (**Figure 3**; Desaintes and Demeret, 1996).

TRANSCRIPTION IN THE DIFFERENTIATED CELLS

The early promoter is activated in association with the differentiation process, increasing the E1/E2 expression (Hummel et al., 1992; Ozbun and Meyers, 1997). Although levels of E6 and E7 also

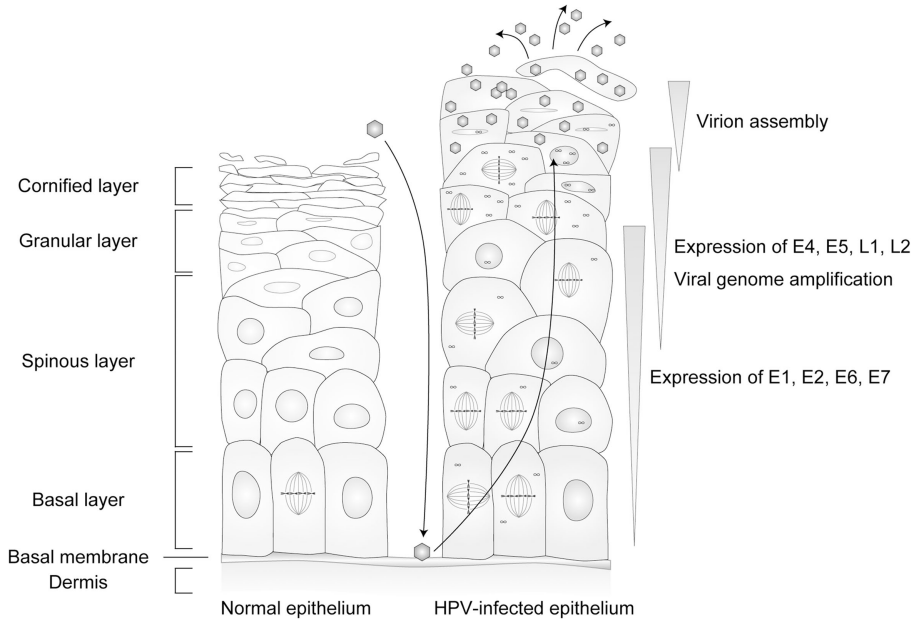

FIGURE 2 | The lifecycle of human papillomaviruses (HPVs). HPVs infect specifically the cells in the basal layer of the stratified epithelium through lesions. Viral genomes are maintained as episomal DNA in the nuclei of infected cells. The viral lifecycle is strictly controlled by host cell differentiation, and the late lifecycle (productive lifecycle) occurs in upper layers of the epithelia that are terminally differentiated, and the progenitor virions are released from the cornified keratinocytes.

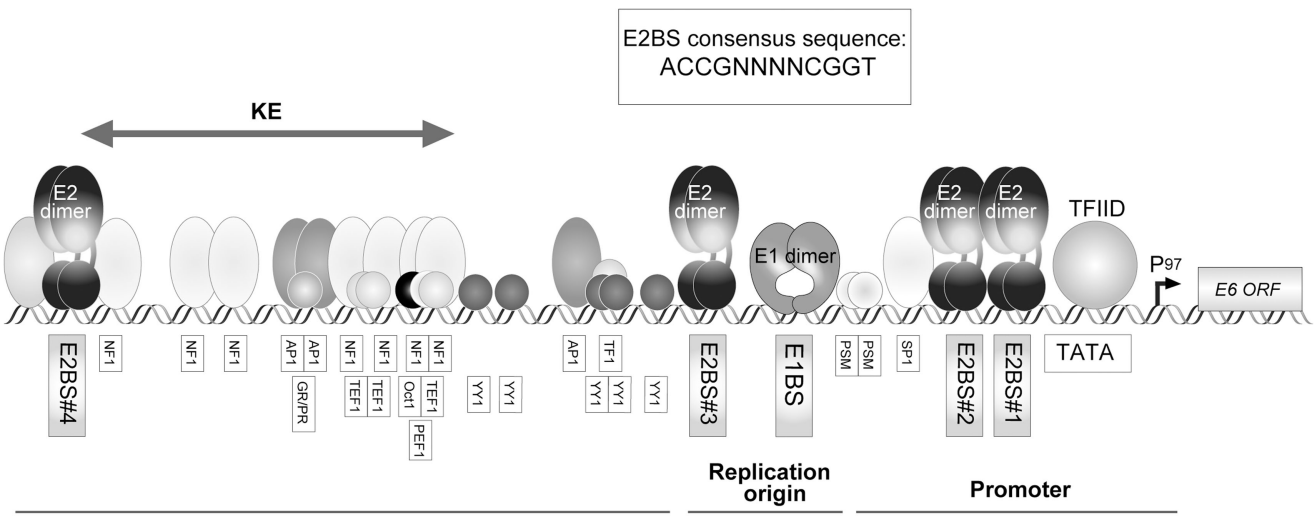

FIGURE 3 | The structure of HPV16 LCR (region of the control of early promoter P97). The early promoter P97 and replication origin are located in LCR, which are regulated by various cellular factors. Activity of P97 is regulated by AP-1, NF1, SP1, TFIID, TF1, Oct-1, PSM, and the viral transcription factor E2. Four E2-binding sites (E2BS) have been identified in HPV16 LCR and the consensus sequence for E2BS is shown in an inset. A glucocorticoid receptor and progesterone receptor (GR/PR) recognition element was also identified in the LCR. The existence of a keratinocyte-specific enhancer (KE) has been proposed (Desaintes and Demeret, 1996).

increase with the early promoter's activation, the E2 overexpressed in the upper layer is thought to suppress their transcription via the mechanism mentioned above. E6 and E7 are important in maintaining infected cells in an undifferentiated state, but terminal differentiation is required for the productive replication of HPV. The inhibition of E6/E7 expression by E2 might promote cellular differentiation, and the cells undergo terminal differentiation, which is suitable for the viral productive lifecycle.

AP-1, a heterodimer composed of Fos and Jun, is considered to be involved in the differentiation-dependent transcriptional

control in keratinocytes; there are reports that the expression profiles of Fos and Jun family members were modified, and that the interaction between AP-1 and KRF-1, a keratinocyte-specific transcription factor (Mack and Laimins, 1991), was strengthened in the differentiation process (Desaintes and Demeret, 1996; Thierry, 2009). Several transcriptional factors were reported to be involved in the differentiation-dependent control of LCR function; EPOC-1/Skn-1a, C/EBP-α, -β, c-Myb, NF1, NFATx, Pax5, and WT1 (Desaintes and Demeret, 1996; Thierry, 2009).

The late promoter is specifically activated in the differentiated layers of epithelium. The late promoter activity is suppressed by CDP (CCAAT displacement protein) and YY-1, whose binding potential was reported to be decreased in differentiated keratinocytes (Ai et al., 1999, 2000). There was also a report that the expression ratio of a transcription factor, Sp1 and its antagonist, Sp3, was altered through the differentiation, which activated the late promoter activity (Apt et al., 1996). The binding of hSkn-1a and C/EBPα to the proximal region of the late promoter contributes to the control of the late promoter activity (Kukimoto and Kanda, 2001; Wooldridge and Laimins, 2008). The involvement of E7 in the regulation of the late promoter activity was also described (Bodily and Laimins, 2011; Bodily et al., 2011). It still remains necessary to clarify the regulatory mechanism for the late promoter in the differentiation of epithelial cells.

METHYLATION OF THE HPV GENOME DURING THE CELL DIFFERENTIATION PROCESS

HPV gene expression is controlled by the methylation of HPV genomic DNA. As E2BSs contain CpG dinucleotides (see inset in **Figure 3**), they can be modified by DNA methylation in the host cell. E2BSs are reported to be highly methylated in undifferentiated cells, inhibiting E2-binding, and demethylation at the E2BSs occurs in association with the cell differentiation (Kim et al., 2003; Vinokurova and von Knebel Doeberitz, 2011).

RNA PROCESSING

For conversion of the gene expression profile from the early to late phase of viral replication, RNA processing is considered critical. The primary transcript of HPV encodes multiple viral genes, and precise RNA processing is essential to produce the mRNA for each viral gene at an appropriate stage of cell differentiation (Schwartz, 2008).

In the early phase of the viral lifecycle, the primary transcription initiated by the early promoter is terminated at the early poly(A) signal, AE (**Figure 1**), and the transcript is processed by using the early splicing signals, which produces the mRNAs encoding the viral early genes. In the differentiated cells, the transcripts for the late genes are expressed from the late promoter and utilize a late poly(A) signal, AL (**Figure 1**), and late splicing signals. The early and late splicing signals compete for the splicing factors, so their usages are generally exclusive.

Multiple splicing signals are found in the HPV genome, which are utilized for the expressions of various viral genes (**Figure 4**). These splicing signals can be categorized into three groups; early phase-specific signals (DS226, SA409, SA526, SA742 in HPV16), late phase-specific signals (SD 3632 and SA5639), and non-specific signals (SD880, SA2709, SA3358; Schwartz, 2008).

Early splicing events have three major roles; regulation of the expression ratio of early genes, production of splicing variants of viral genes, and suppression of late gene expressions. The early

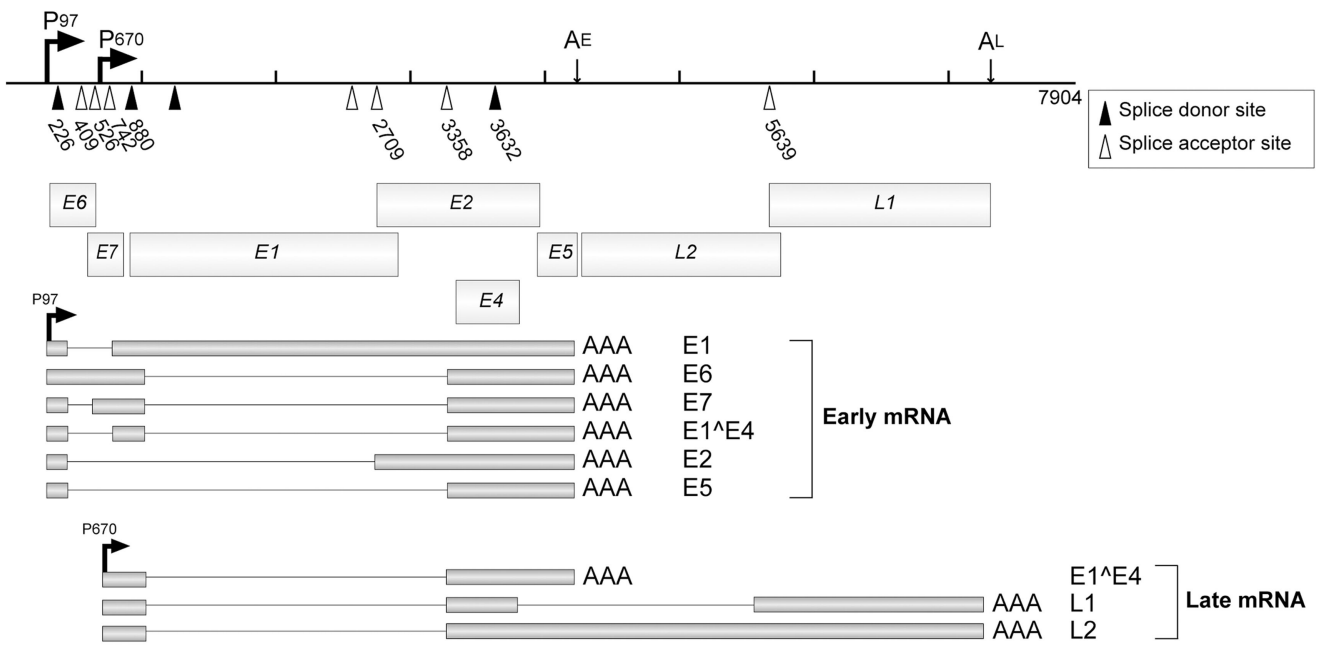

FIGURE 4 | The splicing signals and the transcripts of HPV16. P97 and P670 are the early and late promoters, respectively, for HPV16. AE and AL indicate the early and late polyadenylation signals, respectively. Open triangles indicate splicing acceptors and filled triangles indicate splicing donors. Major mRNAs and their products are indicated. This figure is cited from a review (Schwartz, 2008).

splicing sites of HPV16, SD226, SA409, and SA526, are located in the E6 ORF, which enhances the translation efficiency of E7. Because the initiation codon for E6 is leaky and there are several splicing signals in the E6 ORF, various variants of E6 can be expressed. Those variants were reported to counteract the full-length E6, which might be important for the fine-tuning of E6 activities.

SA3358 is utilized in both the early and late phases of viral replication to produce HPV16 E1^E4 mRNA. A strong splicing enhancer was identified downstream of SA3358, and it accelerated the polyadenylation at AE and suppressed the late gene expression in the early phase (**Figure 5A**). In undifferentiated cells, SA3358 competed with the late splicing signals for the splicing factors (SRp20, hnRNPL etc.), which might contribute to the suppression of late gene expression (Rush et al., 2005; Jia et al., 2009).

The late mRNAs are transcribed from the late promoter, and a major splicing event occurs between SD880 and SA3358 in HPV16. For the production of L1 mRNA, additional splicing between SD3632 and SA5639 is required. The mRNAs for L1 and L2 are poly-cistronic, and the mechanism for bypassing the upstream ATG remains to be explained.

SD3632 in HPV16 is used exclusively for late mRNA production. SD3362 is located close to SA3358 and AE, and the usage of SD3362 was suppressed by a splicing suppressor in dividing cells. It was indicated that the polypyrimidine tract-binding protein (PTB) interfered with the splicing suppressor in differentiated

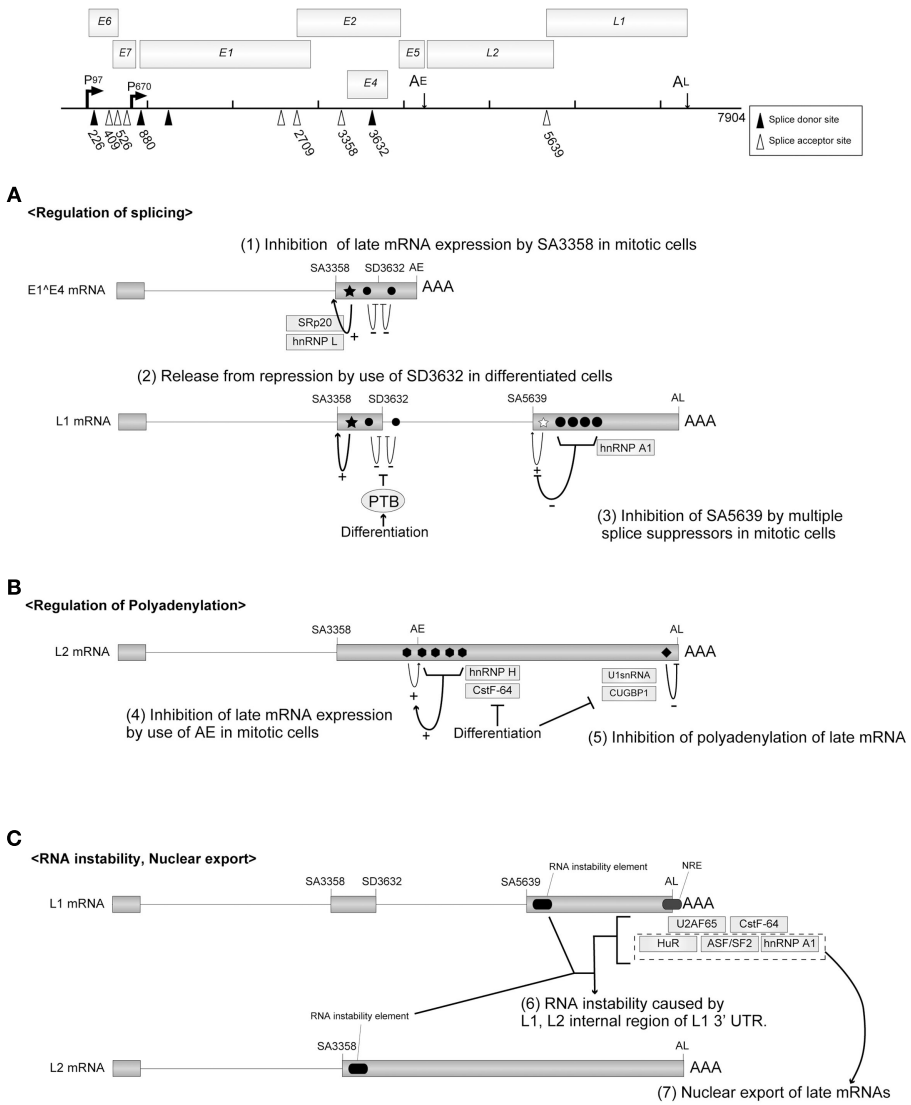

FIGURE 5 | Regulatory mechanisms for mRNA processing. The HPV16 genome with the promoters, the poly(A) signals, and the splicing signals are shown at the top of the figure. Open triangles indicate splicing acceptors and filled triangles indicate splicing donors. **(A)** HPV16 splicing regulatory elements are indicated as filled stars (splicing enhancers) or filled circles (splicing silencers). **(B)** HPV16 polyadenylation regulatory elements are indicated as filled hexagons (polyadenylation stimulatory elements) and a filled rectangle (polyadenylation inhibitory element). **(C)** The regulatory elements for the instability and nuclear export of HPV16 late mRNA are indicated. This figure is cited from a review (Schwartz, 2008).

cells, which potentiated late gene expression (**Figure 5A**; Somberg et al., 2008).

A late phase-specific SA5639 in HPV16 was reported to be regulated by the cis-acting elements found in the L1 coding region (Zhao et al., 2004). A splicing enhancer was identified in the 3′ region of SA5639. Although the enhancer was activated in dividing cells, its function was hindered by multiple splicing suppressors located in the L1 coding region. It was reported that hnRNP A1 is associated with those multiple splicing suppressors (**Figure 5A**; Zhao et al., 2007).

To produce the late mRNAs, it is essential to bypass the polyadenylation at AE. Enhanced utilization of AE could, therefore, inhibit the late gene expression. The 5′ 400 nt of the HPV16 L2 ORF was reported to enhance the polyadenylation at AE, where multiple-G-motifs are located (Oberg et al., 2005). The hnRNP H interacted with the G-motifs and accelerated the polyadenylation at AE, and the expression of hnRNP H decreased as the cell differentiation progressed. CstF-64 was also reported to bind the L2 coding region of HPV31 and enhance the polyadenylation at AE, and the expression of CstF-64 diminished during the cell differentiation process (**Figure 5B**; Terhune et al., 2001).

Within the 3′UTR of the late mRNA (late UTR), a motif highly homologous to the U1snRNA was identified. It was reported that U1-70K, a component of U1snRNP, bound to the U1snRNA motif of BPV1 and interfered with the polyadenylation (Furth et al., 1994). Although U1-70K binding was not detected with HPV16, CUG binding protein 1 (CUGBP1) was reported to interact with the late UTR element of HPV16 and inhibit the polyadenylation process (**Figure 5B**; Goraczniak and Gunderson, 2008).

CONTROL OF LATE mRNA STABILITY

There are RNA instability elements within the L1 and L2 coding mRNAs of HPV16, which function in undifferentiated cells (Mori et al., 2006), although the mechanism for RNA destabilization remains to be clarified (Sokolowski et al., 1998; Collier et al., 2002). There is a GU-rich negative regulatory element (NRE) in the late UTR of HPV16, which is a RNA instability element (Kennedy et al., 1991). Various factors, ASF/SF2, CstF064, U2AF65, hnRNPA1, and HuR, are reported to regulate the differentiation-dependent events of HPV replication through binding to the NRE (**Figure 5C**; Dietrich-Goetz et al., 1997; Koffa et al., 2000; Cheunim et al., 2008).

A highly U-rich region was located in the early UTR of HPV16 and reduced mRNA stability; a U-rich region is a signature feature of unstable mRNA (Jeon and Lambert, 1995; Barreau et al., 2005).

NUCLEAR EXPORT OF LATE mRNAs

The L1 mRNA of HPV16 was retained in the nucleus in undifferentiated W12 epithelial cells (Koffa et al., 2000), suggesting that the nuclear export of late mRNAs was inhibited in the dividing cells. Although the factor(s) that mediates the nuclear export of late mRNAs has not been identified, candidates include HuR, hnRNA, and ASF/SF2, which are proteins shuttling between the nucleus and cytoplasm (**Figure 5C**; Carlsson and Schwartz, 2000; Koffa et al., 2000; McPhillips et al., 2004; Zhao et al., 2004).

TRANSLATIONAL CONTROL OF LATE GENE EXPRESSION

As the inhibitory mechanism for late gene expression, the involvement of translational inhibition was also reported. Translation efficiency was suppressed with *in vitro* translated RNA containing the late UTR of HPV1, suggesting the late UTR had the potential to inhibit the translation. For the inhibition, poly(A)-binding protein (PABP) was considered to be responsible (Wiklund et al., 2002). It was also reported that poly(C) binding protein 1 and 2 (PCBP-1 and -2) and hnRNP K bound to the L2 coding region of HPV16 mRNA and inhibited the *in vitro* translation (Collier et al., 1998). The rare codon usages found in L1 and L2 might also contribute to the inhibition of late gene translation (Gu et al., 2004). In terminally differentiated cells, the altered expression ratios of tRNA species could compensate for the inhibitory effect of the rare codon usages (Fang et al., 2007).

REGULATION OF VIRAL DNA REPLICATION

E1 and E2 have essential roles in the HPV genome's replication (Kadaja et al., 2009). E2 is a DNA-binding protein that recognizes E2-binding sites (E2BSs) located in the LCR (**Figure 3**). E2 has transcriptional transactivator activity, as well as the capacity to bind to the viral DNA replication factor E1. E1 has DNA helicase and ATPase activities and weak DNA-binding capacity. Through its interaction with E2, E1 is recruited to the replication origin (ori), which is essential for the initiation of viral DNA replication (Chiang et al., 1992a,b). E2 also contributes to the segregation of viral DNA in the cell division process by tethering the viral DNA to the host chromosome through interaction with Brd4 and/or ChlR1, both of which can bind to chromatin (McBride et al., 2006). Accurate segregation of the viral genome is essential to maintain the HPV infection in the basal cells, in which the copy number of the viral genome is very low.

The replication potential of E1 is regulated by its interaction with cellular proteins. p56, one of the interferon stimulated genes (ISGs), directly interacts with E1 and translocates it to the cytoplasm, which might contribute to the interferon-mediated inhibition of HPV replication (Terenzi et al., 2008). The interaction of WD repeat protein p80 (WDR80) with E1 is reported to be required for the efficient maintenance of the viral genome in undifferentiated keratinocytes (Côté-Martin et al., 2008).

As mentioned, the expression levels of E1 and E2 increase in association with the differentiation of the epithelial cells, which could be responsible for the vegetative genome amplification.

Recently, it was reported that E6 and E7 activated the ATM DNA damage pathway in differentiation-dependent manner, by which Chk2 was activated. Chk2 potentiated caspase-3 and -7, and the caspases in turn cleaved the E1 protein, which might be required for viral DNA amplification in the differentiated cells (Moody et al., 2007; Moody and Laimins, 2009).

Nuclear accumulation of E1 blocks cell cycle progression in early S-phase and triggers the activation of a DNA damage response (DDR) and of the ATM pathway (Fradet-Toucotte et al., 2011), and the activation of DDR might facilitate HPV DNA replication (Sakakibara et al., 2011). The nuclear accumulation of E1 is regulated by phosphorylation of the nuclear export signal (NES) found in E1 via Cyclin E/A-Cdk2 (Fradet-Toucotte et al., 2010).

DNA replication of HPV utilizes the replication mode with a "θ-structure" in undifferentiated cells, and the mode changes to "rolling circle replication" in differentiated cells, which enables the rapid synthesis of multiple copies of viral DNA. The molecular mechanism supporting the DNA replication in the differentiated cells, however, has not been fully explained (Flores and Lambert, 1997).

INVOLVEMENT OF CELLULAR TRANSCRIPTION FACTORS IN VIRAL DNA REPLICATION

It was reported that the binding of hSkn-1a to its recognition site proximal to the ori region was required for the viral genome replication of HPV16 (Kukimoto et al., 2008). In other HPV types, Sp1 and TATA box binding protein (TBP) inhibit viral genome replication, in which competition between E2 and Sp1 or TBP may be involved (Demeret et al., 1995; Hartley and Alexander, 2002). These transcription factors might alter the chromatin structure, which could inhibit the access of E1 to the origin (Demeret et al., 1995). The inhibition of STAT-1 expression by E6 and E7 was also reported to be important for viral genome amplification (Hong et al., 2011).

VIRION PRODUCTION

A report described that HSP70 was activated in response to the keratinocyte differentiation and co-localized with HPV31 L1 in the differentiated layer of epithelium (Song et al., 2010). It was reported that the disulfide bond among the HPV16 L1 pentamer was formed in a differentiation-dependent manner and had an important role in virion stability (Conway et al., 2011), indicating that virion production was regulated not only by the quantity of the late gene products but also by a differentiation-dependent mechanism.

THE MODULATION OF CELL PROLIFERATION/ DIFFERENTIATION BY HPV GENE PRODUCTS

As described above, HPV replication is strictly regulated by the differentiation program of the host cells. Conversely the HPV infections modulate the proliferation/differentiation status of the host cells, indicating tight communication between the virus and the host cell, which is required for completion of the viral replication.

ACCELERATION OF CELL PROLIFERATION AND INHIBITION OF CELL DIFFERENTIATION

The acceleration of cell proliferation and inhibition of differentiation are induced by the expression of E6 and E7 (Longworth and Laimins, 2004; Moody and Laimins, 2010). E7 inhibits the functions of the pocket protein family, activates the E2F-dependent promoter, and induces S-phase-specific gene expression (Moody and Laimins, 2010). E7 maintains Cyclin E or Cyclin A–CDK2 activity in differentiated cells by targeting p21 and p27, important regulators for growth arrest in the differentiation process. E6 mediates ubiquitination of p53 in association with E6AP, causing the proteasomal degradation of p53 (Moody and Laimins, 2010), and disturbs p53-mediated growth arrest. The association between E6 and histone acetyltransferases (HATs) might be also involved in the inhibition of p53 function (Moody and Laimins, 2010). E6 was reported to target cellular PDZ proteins, which might contribute to the immortalization of the infected cells (Thomas et al., 2008). E6 was reported to activate telomerase activity by inducing the overexpression of c-Myc and by modulating the expression of NFX1-91, which also contributed to the immortalization process (Gewin et al., 2004).

The functions of E6 and E7 in the activation of the DNA replication machinery of the host cell are required to ensure the viral genome's replication in the differentiated cells (Thomas et al., 1999), resulting in the aberrant proliferation and the retarded differentiation of the host cells. With a transgenic mouse model expressing HPV16 E6 and/or E7 under the K14 promoter, dysplasia was observed at the stratified epidermis (Griep et al., 1993).

Although the normal differentiation of keratinocytes is not fully understood, reports describe the involvement of Notch, MAPK, NF-κB, p63, the AP2 family, C/EBP, IRF6, GRHL3, and KLF4 (Blanpain and Fuchs, 2009). There are also papers describing the contribution of c-Myc to the differentiation of epithelial stem cells, and differentiation-dependent demethylation at histone H3K27 (Blanpain and Fuchs, 2009). Recently, some of these factors were found to be associated with HPV gene products (Lathion et al., 2003; Chakrabarti et al., 2004; An et al., 2008; Melar-New and Laimins, 2010), and it is reasonable that these associations modify the cell differentiation program of the infected cells.

INHIBITION OF THE APOPTOTIC INDUCTION

The aberrant proliferation and/or DNA replication in the HPV-infected cells induce p53-dependent apoptotic cell death. The inactivation of pRb by E7 also potentiates the p53 activity. The p53-dependent apoptosis is counteracted by E6 activity (Garnett et al., 2006; Moody and Laimins, 2010). E6 induces the proteasomal degradation of p53. It has also been reported that E6 directly binds to p53 and inhibits its DNA-binding potential (Lechner and Laimins, 1994), and that E6 interacts with HDAC p300, ADA3, and/or CREB-binding protein (CBP), which destabilizes p53 (Patel et al., 1999; Zimmermann et al., 1999; Kumar et al., 2002). The HPV-infected cells also escaped from anoikis by the E6-mediated expression of FAK and the phosphorylation of paxillin, which activates FAK (McCormack et al., 1997). It was reported that interaction between E7 and p600 was involved in the inhibition of anoikis (Huh et al., 2005).

Several membrane-spanning death receptors have been identified, such as TNF receptor type 1 (TNFR1), FAS receptor, and TRAIL receptor. The high-risk type E6 was reported to interact with TNFR1 and suppress TNFα-induced apoptosis (Filippova et al., 2002). E6 is also known to interact with FADD and caspase8, which might inhibit the apoptosis mediated by FAS and TRAIL (Filippova et al., 2004; Garnett et al., 2006). In addition, E6 was reported to be associated with pro-apoptotic Bcl2 members, BAK and BAX, and to interfere with intrinsic apoptosis (Garnett et al., 2006). It was reported that E5 could inhibit FAS- and TRAIL-mediated apoptosis (Garnett et al., 2006). In addition, there was a paper that described the inhibitory effect of E7 on apoptosis, in which the upregulation of cellular inhibitor of apoptosis protein (c-IAP) by E7 was involved (Garnett et al., 2006).

THE MODULATION OF miRNA EXPRESSION THROUGH CELL DIFFERENTIATION

Recently, it was reported that HPV E6 and E7 induced the aberrant expression of tumor suppressive miRNAs (Zheng and Wang,

2011). E6 and E7 are known to target c-Myc, p53, and E2F, and these transcription factors are reported to be involved in the regulation of miRNA expression, so E6 and E7 could cause the uncoordinated expression of those miRNAs. E6 and E7 target p53 and pRb, respectively, and cause the unregulated expression of the miR-15/16 cluster, the miR-17-92 family, miR-21, miR-23b, miR-34a, and the miR-106b/93/25 cluster. Such aberrant expression was suspected to be involved in the development of cervical cancer. It was also reported that E6, E7, and E5 suppress the expression of miR-203 which participates in the differentiation of epithelial cells (McKenna et al., 2010; Greco et al., 2011). Through the suppression of miR-203, the expression level of p63 is upregulated in the differentiated cells, which might contribute to the genome amplification and the late gene expression in the upper layers of epithelium (Melar-New and Laimins, 2010).

EPIGENETIC ALTERATION INDUCED BY THE HPV INFECTION

There was a report describing the epigenetic alteration of cells mediated by HPV gene expression (Hsu et al., 2011; Hyland et al., 2011; McLaughlin-Drubin et al., 2011; Zheng and Wang, 2011). The epigenetic alteration induced by the HPV infection was considered to modify the miRNA expression pattern, which might change the cell differentiation program. Although there is a possibility that some epigenetic alteration occurs also in the normal cell differentiation process, there has been no report related to it.

CONCLUSION

Human papillomavirus suppresses its replication to a "maintenance level" or "latent infection mode" in the basal cells, and maintains the DNA synthesis potential of the infected cells detached from the basal membrane to maintain viral genome replication. In terminally differentiated cells, a tremendous level of genome amplification and late gene expression takes place. After completion of virion assembly, the virions are released externally with the cornified cells (the regulation of the differentiation-dependent lifecycle of HPV is summarized in **Figure 6**). One of the reasons for this unique lifecycle of HPV is escape from the immune-surveillance system (Bodily and Laimins, 2011). Because both L1 and L2 could have immunogenicity, their expressions should be suppressed until the infected cells move to the upper layer of the

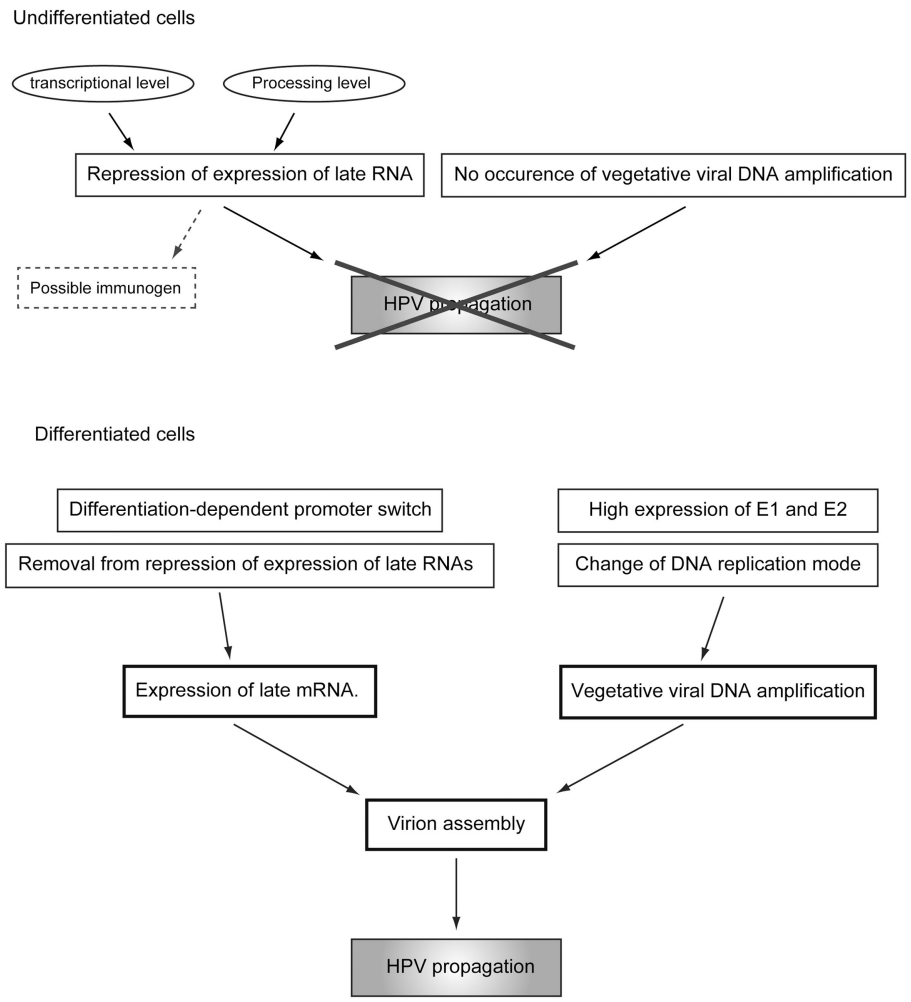

FIGURE 6 | Regulatory mechanisms of the differentiation-dependent lifecycle of HPV.

epithelium, where the host immune-surveillance system does not well function. The hyperproliferation induced by E6 and E7 is required for viral genome amplification and contributes to the augmentation of progeny virion production by expanding the pool of the infected cells.

Tight communication between the virus and the host cell is a unique character of HPV biology, and raises the possibility of using HPV as a probe to investigate the development of the stratified epithelium. In this review, we did not describe the details of the cancer progression induced by HPV infections. HPV-induced cancer is a good model for "multi-step carcinogenesis," and the study of HPV biology provides novel insights into cancer development.

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Poliovirus trafficking toward central nervous system via human poliovirus receptor-dependent and -independent pathway

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In humans, paralytic poliomyelitis results from the invasion of the central nervous system (CNS) by circulating poliovirus (PV) via the blood–brain barrier (BBB). After the virus enters the CNS, it replicates in neurons, especially in motor neurons, inducing the cell death that causes paralytic poliomyelitis. Along with this route of dissemination, neural pathway has been reported in humans, monkeys, and PV-sensitive human PV receptor (hPVR/CD155)-transgenic (Tg) mice. We demonstrated that a fast retrograde axonal transport process is required for PV dissemination through the sciatic nerve of hPVR-Tg mice and that intramuscularly inoculated PV causes paralysis in a hPVR-dependent manner. We also showed that hPVR-independent axonal transport of PV exists in hPVR-Tg and non-Tg mice, indicating that several different pathways for PV axonal transport exist in these mice. Circulating PV after intravenous inoculation in mice cross the BBB at a high rate in a hPVR-independent manner. We will implicate an involvement of a new possible receptor for PV to permeate the BBB based on our recent findings.

Keywords: axonal transport, poliovirus, poliovirus receptor, blood–brain barrier

INTRODUCTION

Poliovirus (PV), known to be the causative agent of poliomyelitis, is a human enterovirus that belongs to the Picornaviridae. After oral ingestion of PV in humans, the virus may suffer the low pH in stomach to decrease its viability (Ohka et al., 2007; **Figure 1**). Then, the virus multiplies in the alimentary mucosa (Bodian, 1955; Sabin, 1956), and moves into the bloodstream. The circulating virus invades the central nervous system (CNS) and replicates in motor neurons (MNs). Paralytic poliomyelitis occurs as a result from destruction of MNs by PV replication. In terms of routes for PV dissemination, there are three main routes; transmission from the alimentary mucosa to blood (**Figure 1A**; Bodian, 1955; Sabin, 1956), permeation through the blood–brain barrier (BBB) into brain (**Figure 1B**; Yang et al., 1997), and transmission via peripheral MNs from muscle into brain (**Figure 1C**; Howe and Bodian, 1942; Nathanson and Langmuir, 1963; Ren and Racaniello, 1992b; Ohka et al., 1998). The species specificity of this virus is governed by a specific cell surface molecule that serves as the PV receptor (PVR). Indeed, transgenic (Tg) mice carrying the human PVR (hPVR/CD155) gene show susceptibility to PV, although mice are generally not susceptible to the virus (Ren et al., 1990; Koike et al., 1991). Tg mice are susceptible to PV when the mice are infected via intravenous and intramuscular routes but not via oral routes (Ren et al., 1990; Koike et al., 1991, 1994a,b; Ren and Racaniello, 1992a,b; Horie et al., 1994; Zhang and Racaniello, 1997). The Tg mice became susceptible to oral infection when interferon (IFN)-α/β receptor gene is disrupted (Ohka et al., 2007). The two dissemination routes through BBB and MNs are also functional in PV-sensitive Tg mice, whereas the routes through alimentary mucosa is not functional in PV-sensitive Tg mice with normal IFN-α/β response.

PVR-DEPENDENT AND -INDEPENDENT RETROGRADE AXONAL TRANSPORT FROM MUSCLE TO CNS

Axonal transport is a cellular process responsible for movement of mitochondria, lipids, synaptic vesicles, proteins, and organelles to and from a neuron's cell body, through the cytoplasm of its axon. It is well known that some viruses, such as Rabies virus, hijacks the retrograde axonal transport, i.e., from synapse to cell body, to invade the CNS (Warrell and Warrell, 2004). Using a PV-sensitive Tg mouse line, we have shown that intramuscularly inoculated PV is taken up by endocytosis at synapses (**Figure 1C**; Ohka et al., 2004). PV is able to invade the CNS by traveling on this cargo. The vesicles containing intact PV particles are transported retrogradely along with the axon without initiating uncoating (Ohka et al., 1998). The uncoating event takes place at the cell body of the MN. We demonstrated that a fast retrograde axonal transport process is required for PV dissemination through the sciatic nerve of hPVR-Tg mice and that intramuscularly inoculated PV causes paralytic disease in a hPVR-dependent manner (Ohka et al., 1998). We also showed that hPVR-independent axonal transport of PV exists in hPVR-Tg and non-Tg mice, indicating that several different pathways for PV axonal transport exist in these mice (Ohka et al., 2009). The velocity of hPVR-independent axonal transport of PV (∼0.5 μm/s) is slower than hPVR-dependent axonal transport (>0.5 to ∼3.7 μm/s) *in vivo*. Using primary MNs isolated from

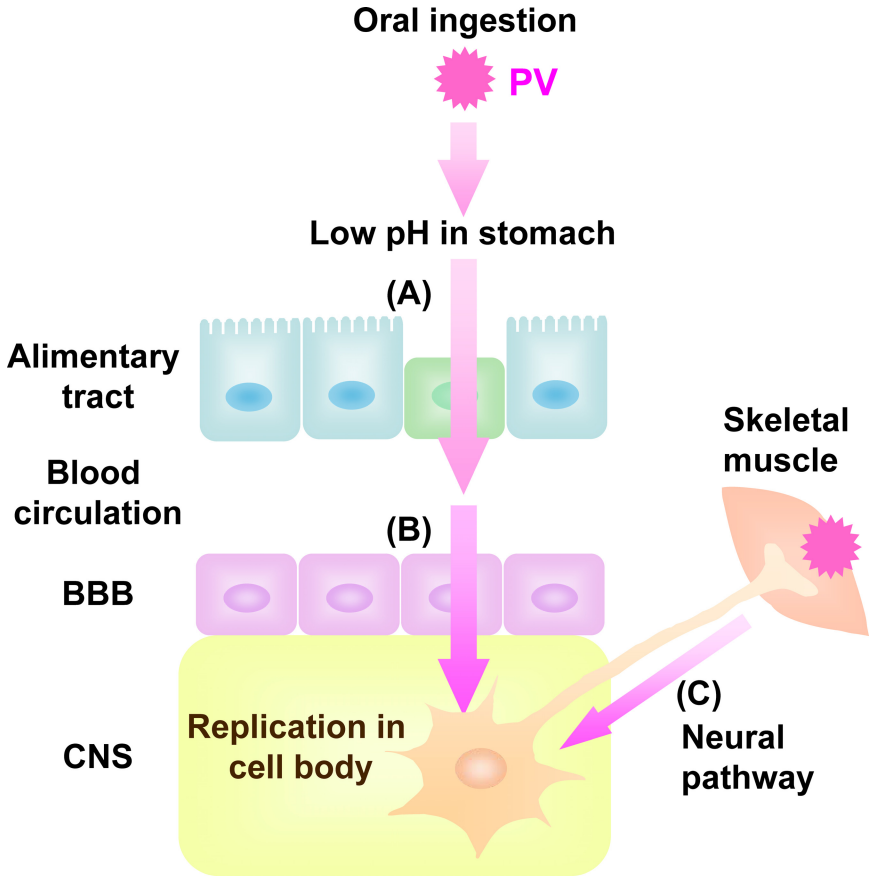

FIGURE 1 | Dissemination pathway for PV in human. Oral ingested PV invades into blood through alimentary tract **(A)** followed by viremia. The virus in the blood permeates BBB into CNS **(B)**. PV also invades into CNS directly by neural pathway through MNs from skeletal muscle to CNS **(C)**.

these mice or rats, we demonstrated that the axonal transport of PV requires several kinetically different motor machineries (Ohka et al., 2009). The fast speed component was ≥1.4 μm/s, whereas the slow component was centered between 0.4 and 1.0 μm/s. Using a mutant hPVR, that has a lower affinity to cytoplasmic dynein than intact hPVR, we revealed that the fast transport relies on a system involving cytoplasmic dynein (Ohka et al., 2004).

AXONAL TRANSPORT OF PV *IN VIVO* AND IN CULTURED MNs

Unexpectedly, the hPVR-independent incorporation of PV followed by the axonal transport was not observed in cultured MNs. Thus, PV transport machineries in cultured MNs and *in vivo* differ in their hPVR requirements (Ohka et al., 2009). **Figure 2** summarizes the PV transports depending on the cell types. In hPVR-Tg mice, most of the hPVR-containing vesicles are retrogradely transported with fast kinetics in an hPVR-dependent manner. PV is also transported in a hPVR-independent manner with slow kinetics by an unknown mechanism. Indeed, PV is transported with slow kinetics in non-Tg mice. In isolated MNs, only hPVR-dependent PV endocytosis is detected. No endocytosis of PV is detected in control cultured MNs lacking hPVR. These results suggest that the axonal trafficking of PV is carried out by several distinct pathways, and that MNs in culture and in the sciatic nerve *in vivo* are fundamentally different in the uptake and axonal transport of PV.

For elucidating the precise mechanisms of infection in MNs, it is important to establish a culture system closely mimicking *in vivo*. MNs in culture and in the sciatic nerve *in vivo* are different in the uptake and axonal transport of PV (Ohka et al., 2009). The differences might be due to the lack of neuromuscular junctions in MNs in culture. Furthermore, MNs are infected both at synapse side and cell body side in conventional culture system, whereas the natural infection occurs mainly at the synapse side *in vivo*. To reflect the conditions *in vivo*, we applied microfluidic culture platform made of polydimethylsiloxane (PDMS) that polarizes the growth of neural axons fluidically isolated environment (Taylor et al., 2005; Park et al., 2006), which enables infection with PV only from synapse side or cell body side. Using the system, we indicated that the infection from the synapse side intrinsically differ from the infection from the cell body side (author's unpublished data). This system may enable co-culture of the synapse side of MNs with muscular cells to form neuromuscular junctions, which more closely mimic *in vivo*, and will shed light on the precise analysis of early infection with infectious agents in MNs in culture.

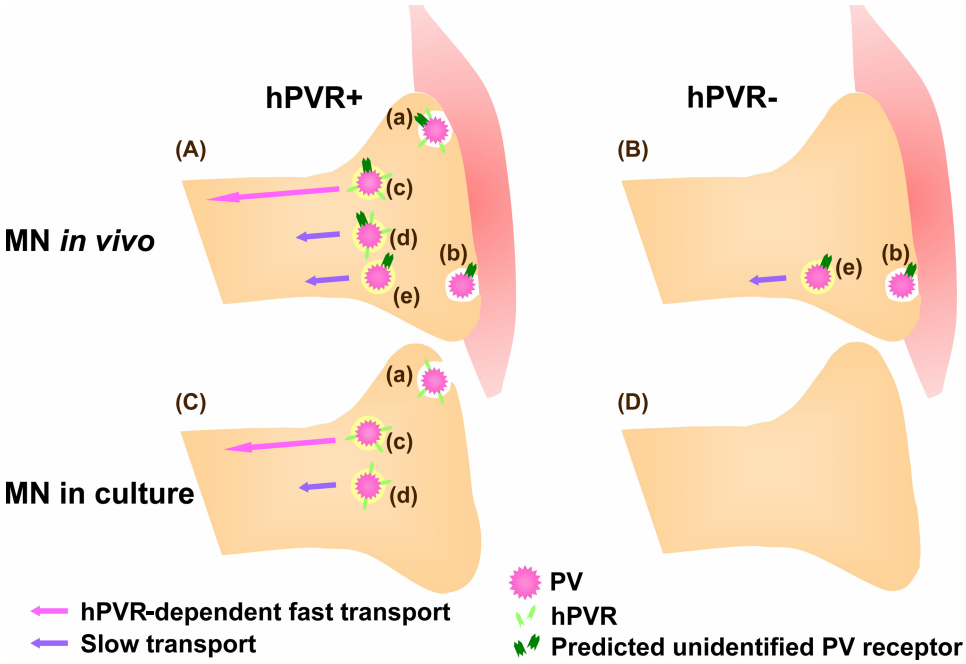

FIGURE 2 | Mechanisms for hPVR-dependent and -independent transport of PV in MNs. (A) In hPVR-Tg mice, PV is endocytosed after interacting to hPVR **(Aa)**. Most of the hPVR-containing vesicles are retrogradely transported in an hPVR-dependent manner with fast kinetics **(Ac)**. hPVR-independent endocytosis **(Ab)** and transport **(Ae)** of PV also occur. The hPVR-independent endocytosis is possibly mediated by an unidentified PV receptor expressed at neuromuscular junctions. Alternatively, hPVR-independent endocytosis may be promoted by synaptic activity *in vivo*. PV-containing vesicles with or without hPVR can be retrogradely transported in an hPVR-independent manner with slow kinetics **(Ad** and **Ae)**. **(B)** In non-Tg mice, PV is endocytosed and transported with slow kinetics in an hPVR-independent manner **(Be)**. The hPVR-independent endocytosis is possibly mediated by the unidentified PV receptor expressed at neuromuscular junctions **(Bb)**. **(C)** In cultured MNs, only hPVR-dependent PV endocytosis is detected **(Ca)**. The hPVR-dependent transport shows fast kinetics **(Cc)**. It might be possible that the slow component in cultured MNs **(Cd)** requires hPVR for endocytosis, whereas its transport may be hPVR-independent. **(D)** No endocytosis of PV is detected in cultured MNs lacking hPVR, possibly due to the absence of the unidentified PV receptor.

TRANSFERRIN RECEPTOR-DEPENDENT BBB PERMEATION

Vascular endothelial cells make up the walls that are sealed together at their edges by tight junctions. BBB does not allow the free transportation of materials including pathogens between the bloodstream and parenchyma of the CNS and blocks viral infection in the CNS. Evidences that support PV invasion of BBB by infecting endothelial cells *in vivo* have not been reported. PV is believed to invade the CNS through the BBB. PV permeation through BBB is hPVR-independent and fast as cationized rat serum albumin which is known to permeate the BBB at a high rate (Yang et al., 1997). Similarly, using transwell *in vitro* BBB model involving mouse brain capillary endothelial cells (MBEC4), we demonstrated that BBB permeation of PV is hPVR-independent and fast as that of transferrin (**Figure 3**, author's unpublished data). These evidences strongly suggest the presence of a new PV receptor for BBB permeation. To elucidate the new PV receptor which interacts with PV, we analyzed the PV-interacting protein by mass spectrometry. As a result, we identified mouse transferrin receptor 1 (TfR1), a membrane bound receptor for transferrin, as a binding protein to PV. Furthermore, peptide sequences of mouse TfR1 and PV involved in the binding were identified (author's unpublished data; the data will be published on another paper). The identified peptide of PV had a permeation activity in *in vitro* BBB model using MBEC4 cells cultured in transwell, and the permeation was fast as transferrin (**Figure 3**). Therefore, mouse TfR1 is a possible receptor of PV for the BBB permeation. As for the homology of TfR1 between mouse and human, both mouse TfR1 and human TfR1 conserve function of iron delivery and the binding site of TfR1 has higher sequence homology (89%) between human and mouse TfR1 than the full length TfR1 molecule (77%). The homology indicates that human TfR1 may function as a PV receptor for the BBB permeation in natural infection in human.

Surprisingly, the majority of the PV related materials after the BBB permeation were intact infectious particles in the brain of hPVR-Tg mice and non-Tg mice (Yang et al., 1997). Moreover, the permeated materials contained infectious particles in *in vitro* BBB model (author's unpublished data). These evidences indicate that PV retains infectivity during the BBB permeation, the transcytosis through capillary endothelial cell layers. At present, it cannot be excluded that TfR1 could actually be the long sought after molecule involved in the hPVR-independent axonal transport of PV *in vivo*. TfR1 is expressed in MNs in mouse spinal cord (Jeong et al., 2011), supporting the hypothesis that TfR1 exists in MNs of sciatic nerve and serves to facilitate hPVR-independent axonal transport. MNs extending neurite in culture express only a small amount of TfR1 (Nakamura et al., 2011), which is consistent with the lack of PV incorporation into MNs in culture (Ohka et al., 2009).

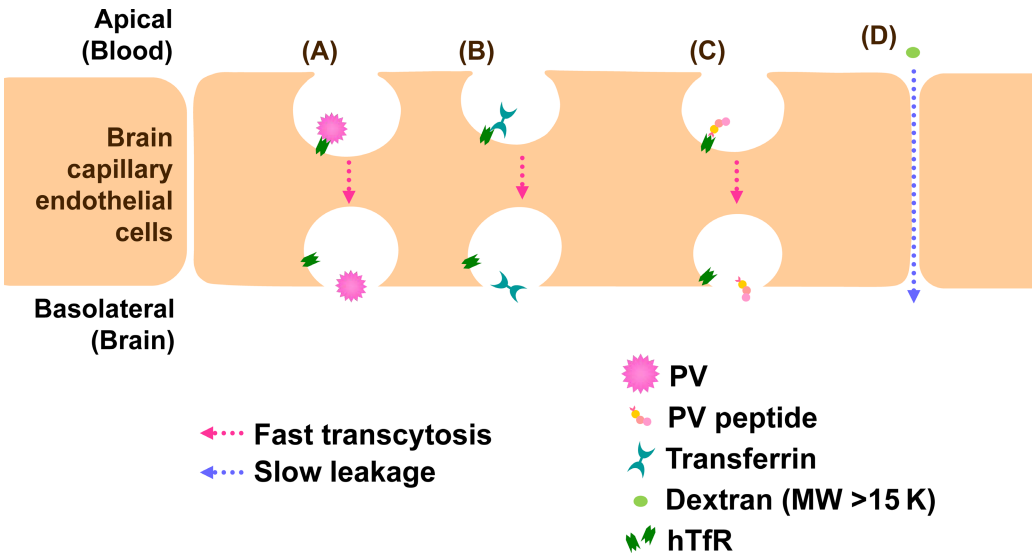

FIGURE 3 | Models for BBB permeation of PV. TfR1 attaches to PV **(A)**, transferrin **(B)**, or PV peptide **(C)** and transported through brain capillary endothelial cells into brain by a fast process. On the other hand, dextran (MW > 15 K) is not transcytosed by TfR1 and leaks between cell junctions by a slow process **(D)**.

DISSEMINATION THROUGH ALIMENTARY MUCOSA

The mechanism of the dissemination through alimentary mucosa is unclear (**Figure 1A**). The instability of the virus in the gastric environment decreases the efficiency of the oral infection in mice (Ohka et al., 2001). Furthermore, we have shown that IFN-α/β plays an essential role in preventing PV from replicating in the intestines using hPVR-Tg mice with or without IFN-α/β receptor expression (Ohka et al., 2001). Recently, Kuss et al. (2011) reported that intestinal bacteria promote PV replication and systemic pathogenesis using hPVR-Tg mice without IFN-α/β receptor expression, indicating that intestinal microbes may enhance PV intestinal infection even in human. In terms of the receptor localization in polarized epithelial cells, hPVRα, one of the two identified membrane bound forms of hPVR, shows basolateral localization due to its basolateral sorting signal, whereas hPVRδ, another membrane bound form of hPVR, non-polarized localization (Ohka et al., 2001). hPVRs mainly localizes to the basolateral in human colon carcinoma (Caco-2) epithelia cells (Ohka et al., 2001) and human Peyer's patches including human microfold cells (M cells), that are distinctive mucosal epithelial cells mediating immune surveillance (Iwasaki et al., 2002). Nevertheless, PV efficiently permeates alimentary mucosa from the apical side into bloodstream, suggesting the possibility that a small amount of apical hPVR or another PV receptor besides hPVR contributes to the transport. It should be noted that PV does not replicate in the alimentary tracts of hPVR-Tg mice that express high levels of hPVR in the intestinal epithelial cells after oral administration (Zhang and Racaniello, 1997) in contrast to its behavior in humans. Much work should be done to elucidate mechanisms of PV infection in and dissemination from the alimentary tract.

CONCLUSION

We demonstrated that axonal transport of PV includes hPVR-dependent and -independent pathways, implying the involvement of a new receptor for PV besides hPVR. We also demonstrated that PV permeates BBB at a high rate in an hPVR-independent manner, and we implicated a new possible receptor for PV to permeate the BBB. Thus, PV receptors including hPVR and new possible receptors play important roles for PV dissemination. It is important to elucidate new possible receptors in the future to clarify the precise mechanisms for PV dissemination pathways. Additionally, we revealed that the discrepancy exists between *in vivo* and in cultured MNs. It would be useful to establish adequate systems in culture for elucidating the transport mechanisms of PV. The discrepancy itself might be a clue for understanding the event *in vivo*.

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Tropism of pandemic 2009 H1N1 influenza A virus

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Substitutions at the receptor-binding site of the pandemic H1N1 2009 influenza A virus (H1N1pdm) hemagglutinin (HA) gene may be critical in determining whether a virus binds to human or avian receptors. Previous reports suggest that HA Gly^{222} and/or Arg^{223} allow viruses to bind preferentially to the α2,3-linked sialic acid found in avian species. We also demonstrated that serial passaging of influenza A virus in embryonated chicken eggs increased viral growth 32- to 64-fold, coincident with the increased prevalence of Gly^{222} or Arg^{223} in HA protein (Yasugi et al., 2012). In this study, we showed that the minor genotype of α2,3-linkage-tropic viruses in upper airways became dominant after passaging through chicken eggs. Viruses possessing HA containing N125D-Q223R, N125D-D187E-Q223R, K119N-D222G, and K119N-N129S-D222G, were detected in both clinical specimens and egg-passaged samples. These results might suggest that egg-adapted viruses, likely represented by α2,3-linkage-tropic virus, were also present in human upper airways as a minor population and transmitted in humans during the outbreak of H1N1pdm.

Keywords: influenza virus, H1N1pdm, next generation sequencer, genetic diversity, D222G

INTRODUCTION

Eight segments of a novel swine-derived H1N1 2009 influenza A virus (H1N1pdm) genome are derived from classical North American and Eurasian swine lineages, an avian lineage, and from human H3N2. The hemagglutinin (HA) gene is thought to be derived from classical North American swine (Cohen, 2009). The receptor-binding specificity of HA is responsible for the host range restriction of influenza A virus. Human and classical H1N1 swine influenza viruses bind preferentially to the glycosylated sialic acid (SA) linkage α2,6Gal, whereas most avian viruses have higher binding affinity for SAα2,3Gal (Nicholls et al., 2008). Receptor specificity is determined by the nature of the amino acids that form the receptor-binding pocket of HA. Gln at position 223 and Gly at position 225 found in avian virus isolates determine the specificity for SAα2,3Gal (Stevens et al., 2004), whereas Leu and Ser at these positions confer SAα2,6Gal specificity in H2 and H3 viruses (Gamblin et al., 2004). For H1 viruses, Asp (found in human viruses) or Glu (found in avian viruses) at position 187 determine preferential binding to α2,6 or α2,3 linkages, respectively (Gambaryan et al., 1999). The amino acids at positions 135, 191, and 222 also affect receptor-binding affinity and specificity (Rogers et al., 1983; Vines et al., 1998; Matrosovich et al., 2000; Chen et al., 2010).

Since the first appearance of H1N1pdm viruses, substitutions including D222G in HA protein and the K340N substitution in RNA polymerase basic 2 (PB2) have appeared sporadically (WHO Global Alert and Responses, 2009). We investigated the nucleotide diversity of the 2009 H1N1pdm HA gene during the first (May 2009) and second (Dec 2010) waves of the epidemic in Japan, with particular focus on the receptor-binding site (RBS). Sequencing analyses using conventional and next-generation high-throughput approaches showed that there were quasispecies in the HA genome, including D222G, in humans (Yasugi et al., 2012). In this study, we focused on the shift of genotype(s) of α2,3-linkage-tropic viruses in upper airways to become dominant after passaging through chicken eggs.

MATERIALS AND METHODS

CLINICAL SAMPLES

Nasal swab samples used in this study were obtained from mild H1N1pdm cases in Osaka, Japan at different time points. Three

and five samples (#1 to #3: first wave in May 2009 and #4 to #8: second wave in December 2010, respectively) were randomly selected (Yasugi et al., 2012). The analyzed samples were unlinked and anonymous at Osaka Prefectural Institute of Public Health. This study was approved by the ethics review committees of Osaka Prefectural Institute of Public Health and the Research Institute for Microbial Diseases (RIMD; Yasugi et al., 2012), Osaka University. Both ethics committees specifically waived the need for consent.

RNA EXTRACTION

Each nasal swab collected in Hanks solution was centrifuged at 20,000 × *g* for 10 min. The supernatants were suspended in TRIzol LS reagent (Invitrogen) for 60 min. Total RNA was extracted with a PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was eliminated with DNAase I (Invitrogen).

DIRECT SEQUENCING

Total RNA was subjected to one-step RT-PCR (SuperScript III/Platinum Taq One-step RT-PCR Kit; Invitrogen). Primer sets used to amplify each viral genome segment are shown in **Table A1** in Appendix. PCR products were purified using a MonoFas DNA purification kit (GL Sciences) and nucleotide sequences were obtained using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

VIRUS ISOLATION AND SERIAL PASSAGE IN EMBRYONATED EGGS AND MADIN–DARBY CANINE KIDNEY CELLS

Clinical specimens were injected into 9-day-old embryonated chicken eggs for virus isolation (Daidoji et al., 2008). After incubation at 37°C for 72 h, the allantoic fluids were collected and filtered (Passage 0: P0). The samples were then diluted 1- to 100-fold in phosphate buffered saline (PBS) and passaged either five (#3) or six times (#1, #2, and #4 to #8) in embryonated chicken eggs. Identical specimens derived from #1 to #2 were also inoculated into MDCK cells for virus isolation (Ueda et al., 2010) and the amplified viruses were serially passaged two or three times in MDCK cells.

HEMAGGLUTINATION TITRATION

Viral samples were serially diluted with PBS and added at a concentration of 0.5% to chicken red blood cells. After incubation at room temperature for 30 min, hemagglutination was observed.

HIGH-THROUGHPUT PYROSEQUENCING ANALYSIS

Total RNA was subjected to one-step RT-PCR using the primer set for HA-RBS (Yasugi et al., 2012). A total of 6 μg amplified DNA was subjected to high-throughput pyrosequencing analysis (Roche/454 GS-FLX Titanium system). A 70 × 75 PicoTiterPlate device (gasket for 16 regions) was divided into 16 regions and one region was used for the mixed samples. Data analysis was performed on each sequence read using computational tools as described previously (Nakamura et al., 2008, 2009).

RESULTS

The genotype of the egg-passaged viruses (P5 in #3 or P6 in #1 and #2) derived from the first wave was analyzed. Amplicons representing each segment of the P5 and P6 viral genomes were PCR amplified with gene-specific primers (**Table A1** in Appendix) and the nucleotide sequence of each PCR product was directly determined by traditional sequencing. No amino acid substitutions were detected in the PB2, RNA polymerase basic 1 (PB1 and PB1-F2), RNA polymerase acidic (PA), neuraminidase (NA), matrix proteins (M1 and M2), and non-structural proteins (NS1 and NS2) compared with A/Osaka/01/2009 (**Table 1**; Yasugi et al., 2012). Passaged 1- and 3-derived viruses exhibited one amino acid mutation in the nucleoprotein (NP), but not at the same site (**Table 1**), suggesting that the mutation was not directly correlated to egg adaptation. In contrast, several mutations (E66K, K119N, N125D, D222G, Q223R, and N370K) in the HA were detected and K119N, D222G, and Q223R were abundant substitutions in multiple specimen-derived viruses (**Table 1**). The sequencing data also showed that D222G and/or Q223R substitutions became dominant in the population of egg-passaged viruses, suggesting that the shift in HA genotype contributed to the adaptation of the passaged viruses to avian cells.

We performed high-throughput sequencing analysis by GS-FLX Titanium pyrosequencing. PCR products amplifying the same region (HA-RBS) as shown previously (Yasugi et al., 2012), which covers almost all the above mutations, except E66K and N370K, were prepared from egg-passaged viruses P1 and P5 or P6. The substitution rate of six critical amino acids (K119N, N125D, N129S, D187E, D222G, Q223R; Yasugi et al., 2012) and A215V is summarized in **Tables 2–4**. Viruses passaged five or six times in eggs (P5, P6) showed higher mutation rates than the parental viruses isolated from clinical specimens. HA sequences harboring N125D, D187E, and Q223R mutations found in the #1-P6 viruses (**Table 2**), K119N, N125D, N129S, D187E, D222G, and Q223R found in

Table 1 | Direct Sanger sequencing of RT-PCR products amplified from each viral genome segment in egg-passaged viruses.

Segment	#1 (P6)		#2 (P6)		#3 (P5)	
	nt	aa	nt	aa	nt	aa
PB2	0	0	0	0	0	0
PB1*	0	0	0	0	0	0
PA	0	0	0	0	0	0
HA*	G427A	E66 K	G408T	K119N	G408T	K119N
	A424G	N125D	A716G	D222G	A716G	D222G
	A719G	Q223R	A719G	Q223R	T1161A	N370K
NP	T159G	D53E	0	0	G447A	D375N
					G1123A	
NA	0	0	0	0	0	0
M	0	0	0	0	0	0
NS	0	0	0	0	0	0

P6 generation was studied for #1 and #2-derived viruses and the P5 generation was studied for the #3-derived viruses.

The results show nucleotide (nt) and amino acid (aa) substitutions compared with A/Osaka/01/2009.

**R634H found within PB1 and I142V found within HA were commonly detected in all egg-adapted viruses as well as the viruses in the clinical specimen (#2).*

Table 2 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P6), and MDCK-passaged samples derived from #1.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	4.04
	Mutation									R	91.5
									G		0.2
		N								R	0.4
				S						R	0.4
					S					R	0.4
						P				R	0.2
									G	R	2.42
		N							G	R	0.2
Egg (P6)	Wild type	K	N	N	T	S	D	A	D	Q	0
	Mutation		D								2.73
										R	2.12
			D				E				0.6
			D					V			0.3
			D						G		0.6
			D							R	75.8
							E			R	1.2
			D				E			R	9.09
			D					V		R	0.3
			D						G	R	5.76
			D			P			G	R	0.3
			D				E		G	R	1.2
MDCK	Wild type	K	N	N	T	S	D	A	D	Q	88.57
	Mutation	N									0.21
			D								0.21
				S							0.42
					S						0.21
							E				0.21
								V			5.61
									G		1.45
										R	1.45
							E			R	0.62
								V	G		0.42
			D				E			R	0.62

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

the #2-P6 viruses (**Table 3**), and K119N, N125D, N129S, A215V, and D222G mutations found in the #3-P5 viruses predominated (**Table 4**). The N125D-Q223R (76%) genotype derived from #1 and K119N-N129S-D222G (45.5%), and N125D-D187E-Q223R (21.8%) genotypes derived from #2 and K119N-A215V-D222G (39%), N125D-D222G (17%), and N125D-N129S-D222G (11%) genotypes derived from #3 were also detected as major populations (**Tables 2–4**). The shift in HA genotype through passaging in eggs is assessed in **Figure 1**. Of particular interest, sequences containing double and/or triple amino acid mutations, which were abundantly detected in egg-passaged viruses, were also found as a minor population in its nasal specimen. For example, HA harboring N125D-D187E-Q223R (**Table 2**; **Figure 1**), K119N-N129S-D222G and N125D-Q223R (**Table 3**; **Figure 1**), or K119N-D222G (**Table 4**) was detected in #1, #2, and #3 clinical samples (Yasugi et al., 2012), respectively. These results suggest that egg-adapted viruses, likely represented by α2,3-linkage-tropic virus, were also present in human upper airways as a minor population and transmitted in humans during the outbreak of H1N1pdm. Because 2,6Gal expression is abundant and 2,3Gal expression is minor in epithelial cells of the upper respiratory tracts (Shinya et al., 2006), it might make sense that α2,3-linkage-tropic viruses exist as a minor population in human upper airways.

In contrast to the first wave, both the G222 and R223 variants had almost disappeared (0.01–0.07 and 0.47–0.63%, respectively) in five nasal swabs (#4 to #8) obtained from individuals with a mild case of H1N1pdm in December 2010 (second wave; Yasugi et al., 2012). We then inoculated the five specimens (#4 to #8) into embryonated chicken eggs and serially passaged them six times in eggs. Hemagglutination was faintly detected in samples in the

Table 3 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P6), and MDCK-passaged samples derived from #2.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	2.94
	Mutation	N									0.29
			D								0.59
								V			2.35
									G		13.8
										R	65.6
		N								R	0.29
			D						G		9.41
			D							R	2.06
				S						R	0.29
						P				R	0.59
							E			R	0.29
								V		R	0.29
									G	R	1.18
Egg (P6)	Wild type	K	N	N	T	S	D	A	D	Q	0.5
	Mutation	N									1.98
			D								0.5
									G		0.5
										R	0.5
		N		S							3.96
		N							G		6.93
			D				E				0.5
			D						G		0.99
			D							R	7.92
				S					G		3.96
				S						R	0.5
		N		S				V			0.5
		N		S					G		45.5
			D				E		G		0.5
			D				E			R	21.8
		N		S			E		G		0.5
		N		S			E			R	0.99
			D				E		G	R	1.48
MDCK	Wild type	K	N	N	T	S	D	A	D	Q	98.84
	Mutation				S						0.19
						P					0.19
								V			0.39
									G		0.39

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

first passage (P0) but was detected in the third or fourth passage and showed 2^5–2^7 hemagglutination titers in the fifth or sixth passage (P5 or P6) in the four specimens except #8 (**Table 5**). PCR products amplifying the same region as #1 to #3 (first wave; Yasugi et al., 2012) were prepared and were examined using conventional direct PCR sequencing. The G222 (#4, #6) and R223 (#5 and #7) variants were detected even in P1 passages in the four specimens (**Table 6**), although both variants had very minor populations in nasal specimens. In addition, no other variants, except the above two amino acids, were detected (data not shown); thus, further high-throughput sequencing analysis was not performed in #4 to #8.

DISCUSSION

Previous work demonstrated that D222G and/or Q223R mutation led to the binding of α2,3-SA-resialylated chicken red blood cells (Chen et al., 2010). D222G and/or Q223R mutations might also be crucial for the tropism and/or pathogenesis of H1N1pdm. The function(s) of the other substitutions shown in **Tables 2–4** are largely unknown; however, significant similarities in the HA genotype between the viruses resident in upper airways (Yasugi et al., 2012) and egg-adapted viruses (**Tables 2–4**) suggest that the minor genotype of α2,3-linkage-tropic viruses in upper airways became dominant after passaging through chicken eggs. Viruses derived from #1 and

Table 4 | Frequency of multiple mutations in nine amino acids of the H1N1pdm HA in egg-passaged (P1 and P5) samples derived from #3.

		119	125	129	133	183	187	215	222	223	Total (%)
Egg (P1)	Wild type	K	N	N	T	S	D	A	D	Q	4
	Mutation	N									0.33
						P					35.7
									G		57.3
										R	0.67
		N				P					0.33
				S					G		0.33
						P			G		1.33
Egg (P5)	Wild type	K	N	N	T	S	D	A	D	Q	1.03
	Mutation	N									2.07
			D								2.07
									G		4.66
										R	0.52
		N	D								0.52
		N						V			5.7
		N							G		2.07
			D	S							1.55
			D						G		17.1
				S					G		0.52
								V	G		8.29
		N						V	G		38.9
			D	S				V			1.55
			D	S					G		10.9
			D					V	G		0.52
			D	S				V	G		2.07

Critical substitutions such as K119N, N125D, N129S, D187E, A215V, D222G, Q223R, and two additional substitutions T133S and S183P, as controls, are shown.

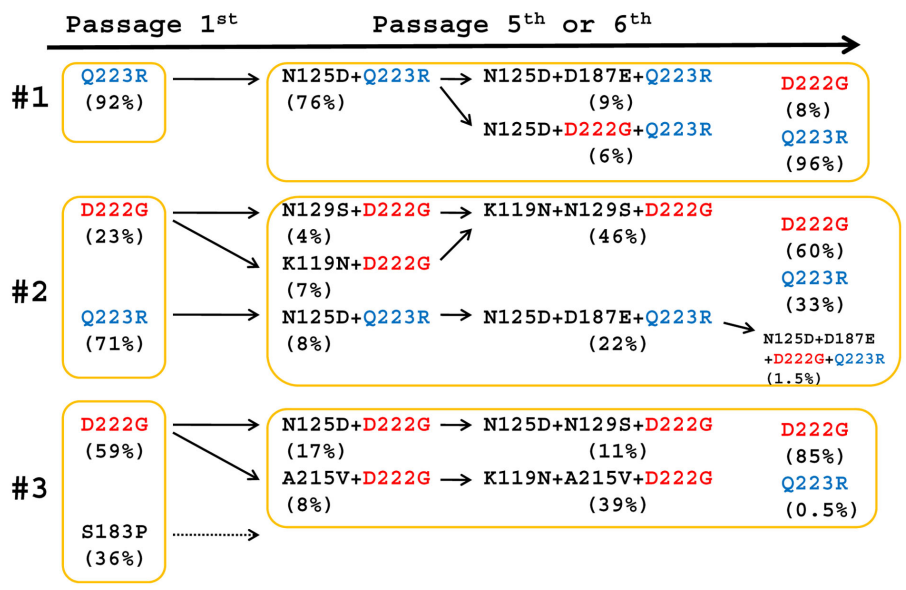

FIGURE 1 | Putative shift in HA genotype through the passage in eggs of H1N1pdm (first wave).

possessing HA containing N125D-Q223R and N125D-D187E-Q223R, viruses derived from #2 containing N125D-Q223R and K119N-N129S-D222G, and viruses derived from #3 containing K119N-D222G and N125D-S207N-D222G, were detected in both

Table 5 | HA titer of virus second wave H1N1pdm samples passaged in eggs.

Sample	HA titer (2 Log 10)						
	P0	P1	P2	P3	P4	P5	P6
#4	1	1	1	5	4	5	4
#5	1	6	7	7	6	7	7
#6	1	1	7	5	5	5	3
#7	1	7	7	5	6	6	5
#8	1	1	1	0	0	0	0

Table 6 | D222G and Q223R mutations on second wave H1N1pdm samples.

Sample	Passage					
	P0	P1	P3	P4	P5	P6
#4	No	D222G	D222G	No	D222G	No
#5	No	Q223R	Q223R	Q223R	Q223R	Q223R
#6	ND	D222G*	D222G	D222G	D222G	D222G
#7	No	Q223R	Q223R	Q223R	Q223R	Q223R

No, no mutation on 222 and 223 position.

ND, not done.

**Double peak = double peak as in GGT (wild type) and GAT (D to G) on position 222.*

clinical specimens and egg-passaged samples (**Figure 1**; Yasugi et al., 2012). These results suggest a direct linkage of α2,3-tropic viruses between the clinical samples and egg-adapted samples in each case.

However, such a direct linkage could not be observed in the second wave of H1N1pdm, because the G222 and R223 variants were almost undetected (0.01–0.07 and 0.47–0.63%, respectively) in five nasal swabs (#4 to #8; Yasugi et al., 2012). Thus, D222G or Q223R mutation may occur in inoculated eggs. Further investigations of clonal recombinant virus using reverse genetics are required to address this question. We also failed to rescue egg-adapted virus from #8 (**Table 5**). In addition, neither D222G nor Q223R genotype was detected in the sixth passage of #4 (**Table 6**), although egg-adapted viruses well grew (**Table 5**). These results might suggest that factor(s) other than mutation(s) in HA-RBS (Lu et al., 2005) are involved in egg adaptation.

We showed that MDCK-passaged virus isolates contain a similar ratio of α2,3-tropic and α2,6-tropic viruses compared to the original H1N1pdm (first wave) viruses identified in human specimens (**Tables 2** and **3**; Yasugi et al., 2012). Takemae et al. (2010) demonstrated that embryonated chicken egg-isolated classical H1 swine influenza viruses harbored substitutions including D187V/N and D222G in the HA, whereas MDCK isolates retained HA genes identical to those of the viruses present in the swine nasal swab samples. Passaging in MDCK cells may therefore be a better approach to establish genetic diversity and specific HA genotypes *in vivo* in human and swine influenza viruses. While egg adaptation is currently required to prepare vaccine candidates, alternative approaches involving the use of accredited anchorage-dependent and -independent preparations of the African Green monkey kidney (Vero), MDCK, and other cell lines have been pursued by several manufacturers in recent years (Audsley and Tannock, 2008). Our results, in this study, suggest the advantage of the cell-based influenza vaccine approach, which is able to maintain the genetic stability of clinical strains.

This study, together with our previous report (Yasugi et al., 2012), suggest that α2,3-SA-specific viruses, including G222 and R223, existed in humans as a minor population in the early phase (first wave) of the pandemic, and that D222 and Q223 became more dominant through human-to-human transmission (second wave) during the epidemic. Newly emergent influenza A viruses may have been dual specific but not exclusively α2,6-SA-specific during the early phase of the pandemic and adapted during multiple cycles of human-to-human transmission (Yasugi et al., 2012). Further investigation is required to determine the proportion of α2,3-tropic and α2,6-tropic viruses found in tissues and organs infected with other human-, avian-, and swine-derived influenza viruses. Deep sequencing approaches will provide a more comprehensive analysis of genetic diversity in egg- and MDCK-passaged viruses and original virus isolates *in vivo* and will help us to understand quasispecies of influenza viruses more precisely.

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APPENDIX

Table A1 | Primer pairs used to amplify segments of the H1N1pdm genome.

PB2-5′	F	TGTAAAACGACGGCCAGTATGGAGAGAATAAAAGAACTGAGAG
	R	GCTTGTCCTTTTGAAAGTGAACCCA
PB2-3′	F	TGCAAAAGTGCTTTTCCAGAACTGG
	R	CAGGAAACAGCTATGACCCTAATTGATGGCCATCCGAATTC
PB1-5′	F	TGTAAAACGACGGCCAGTATGGATGTCAATCCGACTCTAC
	R	ATTTTCATTCCACTTAGTGTTGTCC
PB1-middle	F	CAAAGATGCAGAGAGAGGCAAG
	R	CAGGTCCTGTAGAATCTGTCCAC
PB1-3′	F	GATTTTGCTCTCATAGTGAATGCAC
	R	CAGGAAACAGCTATGACCTTATTTTTGCCGTCTGAGTTCTTCAATGGTGG
PA-5′	F	TGTAAAACGACGGCCAGTATGGAAGACTTTGTGCGACAATG
	R	GTCCTCAAGAATGGTTCAATTTTGG
PA-middle	F	ATGGATTCGAGCCGAACGGCTGCATTG
	R	TATGTACACTCCCTTCATTATGTATTCAG
PA-3′	F	TTGATGAAATAGGAGAAGATGTTGC
	R	CAGGAAACAGCTATGACCCTACTTCAGTGCATGTGTGAG
HA-5′	F	TGTAAAACGACGGCCAGTATGAAGGCAATACTAGTA
	R	CGGGATATTCCTTAATCCTGTRGC
HA-3′	F	GTGCTATAAACACCAGCCTYCCA
	R	CAGGAAACAGCTATGACCTTAAATACATATTCTACACTGTAGAG
NP	F	TGTAAAACGACGGCCAGTATGGCGTCTCAAGGCACCAAACG
	R	CAGGAAACAGCTATGACCTCAACTGTCATACTCCTCTG
NA-5′	F	TGTAAAACGACGGCCAGTATGAATCCAAACCAAAAGATAATAACCATTG
	R	AGAATCAGGATAACAGGAGC
NA-3′	F	GAATGTGCATGTGTAAATGG
	R	CAGGAAACAGCTATGACCTTACTTGTCAATGGTAAATGGCAACTCAG
M	F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGTAGAT
	R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGTTT
NS	F	TATAAAACGACGGCCAGTAGCAAAAGCAGGGTGACAA
	R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTT

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Morbillivirus receptors and tropism: multiple pathways for infection

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Morbilliviruses, which include measles virus (MeV), canine distemper virus, and rinderpest virus, are among the most important pathogens in their respective hosts and cause severe syndromes. Morbilliviruses are enveloped viruses with two envelope proteins, one of which is hemagglutinin (H) protein, which plays a role in binding to cellular receptors. During morbillivirus infection, the virus initially targets lymphoid cells and replicates efficiently in the lymph nodes. The principal cellular receptor for morbillivirus is signaling lymphocyte activation molecule (SLAM, also called CD150), which is exclusively expressed on immune cells. This feature reflects the strong lymphoid cell tropism and viral spread in the infected body. Morbillivirus infection, however, affects various tissues in the body, including the lung, kidney, gastrointestinal tract, vascular endothelium, and brain. Thus, other receptors for morbilliviruses in addition to SLAM might exist. Recently, nectin-4 has been identified as a novel epithelial cell receptor for MeV. The expression of nectin-4 is localized to polarized epithelial cells, and this localization supports the notion of cell tropism since MeV also grows well in the epithelial cells of the respiratory tract. Although two major receptors for lymphoid and epithelial cells in natural infection have been identified, morbillivirus can still infect many other types of cells with low infectivity, suggesting the existence of inefficient but ubiquitously expressed receptors. We have identified other molecules that are implicated in morbillivirus infection of SLAM-negative cells by alternative mechanisms. These findings indicate that morbillivirus utilizes multiple pathways for establishment of infection. These studies will advance our understanding of morbillivirus tropism and pathogenesis.

Keywords: morbillivirus, measles virus, CD46, SLAM, nectin-4, cell tropism

INTRODUCTION

Morbilliviruses belong to the order Mononegavirales, family Paramyxoviridae, and include measles virus (MeV), rinderpest virus (RPV), and canine distemper virus (CDV). Morbilliviruses are highly contagious for their respective hosts and mediate similar consequences of pathogenesis, such as fever, cough and coryza, and respiratory and gastrointestinal diseases. In particular, induction of severe transient immunosuppression along with the gain of life-long immunity are the most notable features of morbillivirus infection (Griffin, 2007).

Measles virus is a leading cause of mortality in children worldwide. In particular, strong immunosuppression causes secondary infection and leads to high childhood mortality in the developing world. RPV affects several species of wild and domestic cloven-hoofed animals. The mortality rate can reach nearly 100% in highly susceptible cattle or buffalo herds; in fact, rinderpest had caused significant economic damage since records began. CDV is a cause of fatal disease in many species of carnivores. Recently, fatal CDV infection has been reported in other species such as large felids (Appel et al., 1994), javelinas (Appel et al., 1991), and freshwater and marine seals (Visser et al., 1990).

To limit these severe and fatal diseases of morbilliviruses, appropriate measures have been taken, including live attenuated and effective vaccines, which were developed more than 40 years ago and control the viruses well. In particular, international campaigns have been conducted to eradicate both MeV and RPV globally. As a result of vaccination efforts and culling of infected animals, eradication of rinderpest in all 198 countries and territories was declared by OIE (2011) and FAO (2011). Rinderpest became the second viral disease, after smallpox, to be eradicated through human efforts. In the case of measles, vaccination has contributed to reducing the mortality rate in infants, and deaths due to measles were reduced by 78% worldwide between 2000 and 2008 (from 733,000 to 164,000) after a global campaign for vaccination (WHO, 2011).

Morbilliviruses are enveloped virions that contain a non-segmented, negative-stranded RNA genome that encodes a single envelope-associated matrix protein (M), two glycoproteins (hemagglutinin H and fusion protein F), two RNA-polymerase-associated proteins (phosphoprotein P and large protein L), and a nucleocapsid protein (N) that encapsulates the viral RNA (**Figure 1**). The H gene encodes a key protein for morbillivirus and its animal hosts: the virus uses this protein to attach to cell receptors during the first step of infection (Griffin, 2007).

The search for the receptor for morbillivirus began in vaccine strains of MeV, and subsequently identified receptors for wild-type strains have revealed the closely related receptor usage and unique pathogenicity of the viruses.

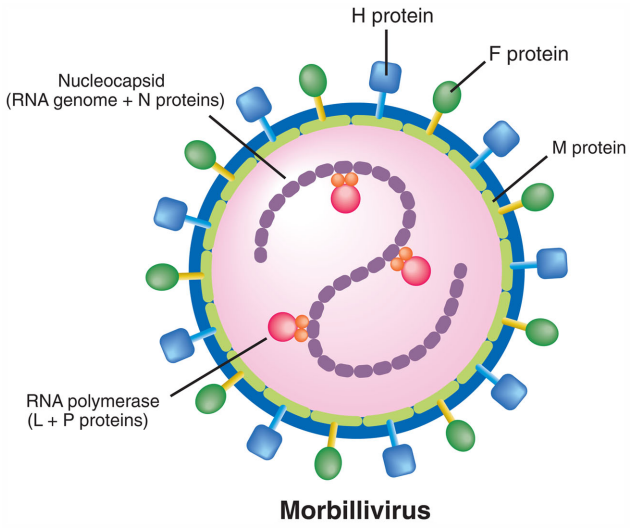

FIGURE 1 | Schematic diagram of the components of the morbillivirus. The viral particle contains the ribonucleoprotein complex consisting of the nucleocapsid (genomic RNA and N proteins) and viral RNA polymerase (P and L proteins), and the envelope consisting of the M, F, H proteins, and host cell-derived membrane.

In this review, we introduce the identified receptors for morbilliviruses, mainly MeV, and discuss cell tropism and pathogenicity in terms of receptor usage.

CD46

Measles virus was first isolated by Enders and Peebles (1954) from primary human kidney cells inoculated with the blood and throat washings of a child with measles. The virus strain (Edmonston) was passaged multiple times in primary human kidney and amnion cells and then adapted to eggs and multiply passaged in chick embryo cells to produce the original Edmonston B vaccine, which was licensed in 1963 (Griffin, 2007). Administration of the live attenuated vaccine results in transient immunosuppression, but induces both expression of the neutralizing antibody and cellular immune responses sufficient for protection.

Vero cells derived from the African green monkey kidney had been utilized to isolate MeV as a standard cell line because it is beneficial and safe. About 40 years after MeV was isolated, two groups reported in 1993 that CD46 acts as a cellular receptor for laboratory-adapted strains of MeV. Naniche et al. (1992) obtained a monoclonal antibody that inhibited cell fusion induced by recombinant vaccinia virus encoding the H and F proteins of the Halle strain of MeV. The antibody precipitated a cell-surface glycoprotein from human and simian cells but not from murine cells. N-terminal amino acid sequencing identified that the glycoprotein was human membrane cofactor protein (CD46), a member of the regulators of the complement activation gene cluster (Naniche et al., 1993). Transfection of non-permissive murine cells with a CD46 expression vector confirmed that the human CD46 molecule serves as a MeV receptor, allowing virus–cell binding, fusion, and viral replication. Dorig et al. (1993) showed independently that hamster cell lines expressing CD46 produced syncytia and virus proteins after infection with the Edmonston strain of MeV and that polyclonal antisera against CD46 inhibited virus binding and infection.

CD46 is a cell-surface, type I transmembrane 57–67 kD glycoprotein that belongs to the family of complement activation regulators and is ubiquitously expressed in all nucleated human cells. The most important function of CD46 is as an inhibitor of complement activation. It protects host cells from complement deposition by functioning as a cofactor for the factor-I-mediated proteolytic inactivation of C3b and C4b (Liszewski et al., 1991). In addition, CD46 has been implicated in the modulation of T-cell functions (Marie et al., 2002), generation of regulatory T-cells (Kemper et al., 2003), and control of interferon (IFN) production (Katayama et al., 2000). CD46 is also important during fertilization – it presumably promotes sperm–egg interaction (Riley-Vargas et al., 2004, 2005; Harris et al., 2006).

CD46 exists in multiple isoforms, which are generated by alternative splicing of a single gene. It has four short consensus repeats (SCR 1–4) comprising 60–64 aa each, an alternatively spliced serine/threonine/proline-rich region, a transmembrane region, and an alternatively spliced cytoplasmic tail.

Previous studies have located the MeV binding site on CD46 to the SCR1 and SCR2 domains of the receptor (Buchholz et al., 1997; Hsu et al., 1997; Casasnovas et al., 1999; Christiansen et al., 2000; **Figure 2**). Functional studies *in vitro* have suggested that signaling via CD46 is an important component of MeV pathogenesis. For example, the high degree of interaction between MeV-H and CD46 results in downregulation of CD46 from the surface of infected cells, rendering them more sensitive to C3b-mediated complement lysis (Schneider-Schaulies et al., 1995a,b; Schnorr et al., 1995). Interestingly, CD46-mediated immunosuppression in MeV infection has been reported. One mechanism involves inhibiting activation-induced expression of interleukin (IL)-12, which is essential for the generation of successful effector T-cell responses, by cross-linking CD46 on the surface of monocytes by MeV (Karp et al., 1996; Galbraith et al., 1998; Karp, 1999; Kurita-Taniguchi et al., 2000). Interaction of MeV-H and CD46 also induces IL-10, leading to inhibition of the contact hypersensitivity reaction (Marie et al., 2002). In contrast, MeV binding to CD46 induces IFN production, which further triggers the early antiviral immune response (Manchester et al., 2000; Naniche et al., 2000).

Amino acid residues interacting with CD46 in the H protein have been identified (F431, V451, Y481, P486, and I487; Masse et al., 2002; Santiago et al., 2002; Vongpunsawad et al., 2004). Among them, two amino acid residues (V451 and Y481) are crucial for determining the ability of MeV strains to cause hemadsorption, cell fusion, and CD46 downregulation.

ATTENUATION OF MeV PATHOGENICITY BY PASSAGE WITH VERO CELLS

Although many studies clarified the interaction between MeV-H and CD46 and the resultant cellular signaling events *in vitro*, it had also been revealed that these laboratory strains of MeV do not induce any typical symptoms in non-human primate species, which are susceptible to wild-type MeV. For example, rhesus and cynomolgus macaques have been described to cause

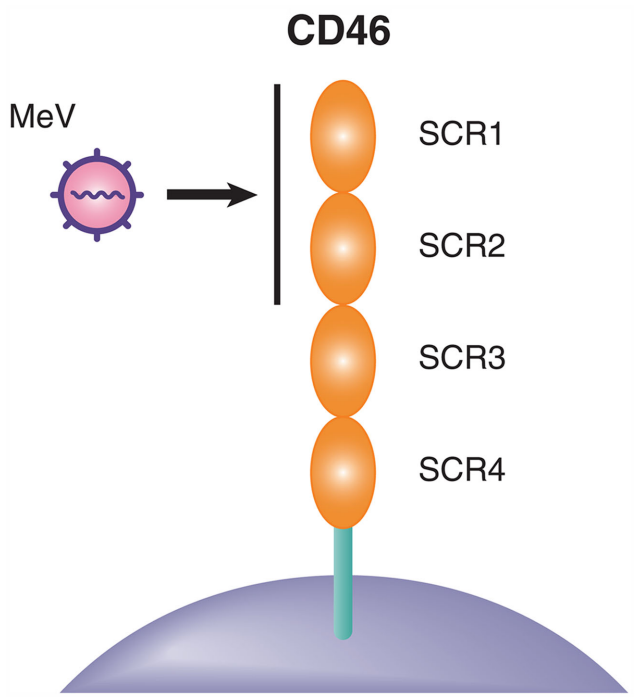

FIGURE 2 | Structure of CD46. CD46 has four SCRs at the amino terminus of its ectodomain. SCR1 and SCR2 interact with the laboratory strains of MeV, whereas SCR3 and SCR4 interact with complement proteins C3b and C4b.

outbreaks of measles in colonies; however laboratory strains of MeV do not induce disease in these animals (Kobune et al., 1990; van Binnendijk et al., 1994, 1995; McChesney et al., 1997). Furthermore, previous reports indicated that five strains of MeV that were adapted for growth in Vero cells showed little pathogenicity against experimentally infected macaques, whereas the Bilthoven strain of MeV, which grew in human cord blood cells, induced clinical symptoms of measles (Auwaerter et al., 1999).

Although Vero cells had been used for MeV isolation for a long time, the isolation was not highly efficient and usually required several blind passages. In contrast, Kobune et al. found that an Epstein–Barr-virus-transformed marmoset B-cell line, B95a, is 10,000-fold more sensitive to the MeV present in clinical specimens than Vero cells. Furthermore, MeVs isolated and propagated in B95a cells cause clinical signs in experimentally infected monkeys, which resemble those of human measles such as rashes and Koplik's spots, leukopenia, and marked histological lesions in the lymphoid tissues (Kobune et al., 1990). Subsequently, Kobune et al. (1990, 1996) reported that two strains of wild MeV from the same patient, one isolated in B95a cells and the other in Vero cells, had different virulence in monkeys. The former induced acute signs of MeV infection, whereas the latter did not induce any clinical signs of disease and caused milder histological lesions. These findings strongly indicated that MeV isolated in B95a cells maintains virulence similar to that in humans and that isolation in Vero cells leads to loss of virulence.

However, strains isolated in B95a cells or human B-cell lines were shown to grow only in a limited number of lymphoid cell lines (Kobune et al., 1990; Schneider-Schaulies et al., 1995b; Tatsuo et al., 2000a). Furthermore, the H protein of MeV isolated from B-cell lines neither induced downregulation of CD46 nor caused cell–cell fusion (upon coexpression of the F protein) in CD46-positive cell lines (Lecouturier et al., 1996; Bartz et al., 1998; Tanaka et al., 1998).

From these observations, it had been postulated that B-cell line-isolated strains do not use the ubiquitously expressed CD46 but utilize another molecule as a receptor (Lecouturier et al., 1996; Buckland and Wild, 1997; Bartz et al., 1998; Hsu et al., 1998; Tanaka et al., 1998; Tatsuo et al., 2000a).

SIGNALING LYMPHOCYTE ACTIVATION MOLECULE

Tatsuo et al. (2000b) performed a screening of a cDNA library of B95a cells, in which a non-susceptible human kidney cell line, 293T, was transfected with the cDNA library and then screened with a vesicular stomatitis virus pseudotype bearing the H protein of MeV isolated from B-cells and F protein from the Edmonston strain. As a result, a single cDNA clone capable of making transfected 293T cells susceptible to MeV-H protein bearing pseudotype was identified. The sequence of the clone was a homolog of signaling lymphocyte activation molecule (SLAM), and consequently, human SLAM was identified as a lymphoid cell receptor for wild-type MeV. Importantly, the Edmonston strain was found to utilize SLAM, in addition to CD46, as a receptor, indicating that SLAM acts as a receptor not only for B-cell line-isolated MeV strains but also for vaccine and laboratory-adapted strains (Tatsuo et al., 2000b).

Subsequent studies have demonstrated that MeV strains isolated and propagated by SLAM-positive cells show clinical signs of MeV in infected animals (van Binnendijk et al., 1994; McChesney et al., 1997; Zhu et al., 1997; Auwaerter et al., 1999; El Mubarak et al., 2007; Bankamp et al., 2008). Therefore, it has been verified that SLAM acts as the principal cellular receptor for MeV *in vivo*, and that use of CD46 may be the result of MeV adaptation *in vitro*. Furthermore, it has been demonstrated that all CDV and RPV strains use dog and cow SLAM as a receptor, respectively, and that SLAM is a common and principal receptor for morbillivirus (Tatsuo et al., 2001).

Signaling lymphocyte activation molecule is also known as CD150 and is expressed on thymocytes, activated lymphocytes, mature dendritic cells, macrophages, and platelets in humans and mice (Sidorenko and Clark, 1993; Cocks et al., 1995; Aversa et al., 1997). In humans, $CD14^+$ monocytes in tonsils and spleens express SLAM (Farina et al., 2004). SLAM is implicated in the regulation of T-cell activation by affecting T-cell antigen receptor signaling. In addition, SLAM has the ability to regulate the functions of several other immune cell types, including natural killer and dendritic cells. Hence, SLAM has a broad involvement in the modulation of innate and acquired immune responses (Veillette and Latour, 2003; Veillette et al., 2007; Schwartzberg et al., 2009).

Signaling lymphocyte activation molecule has two extracellular immunoglobulin superfamily domains, V and C2, and is associated with the adaptor molecules, SLAM-associated protein (SAP), or EWS/FliI-activated transcript 2 (EAT-2), in its cytoplasmic tail.

The extracellular domain of SLAM associates with another SLAM molecule present on adjacent cells. In $CD4^+$ T-cells, ligation of SLAM induces its binding to SAP, and combined with T-cell-receptor (TCR)-mediated signals, triggers downstream signaling for the production of T helper 2 (Th2) cytokines such as IL-4 and IL-13 (Veillette et al., 2007). Furthermore, SLAM controls production of IL-12, tumor necrosis factor α, and nitric oxide, presumably via EAT-2, by macrophages (Veillette et al., 2007).

The V domain of SLAM is necessary and sufficient for MeV receptor function and three amino acid residues, at positions 60, 61, and 63 of human SLAM, are crucial for its function (Ohno et al., 2003; **Figure 3**). Meanwhile, mutagenesis of the H protein based on its ability to induce SLAM-dependent cell–cell fusion has revealed that residues important for interaction with SLAM are I194, D505, D507, Y529, D530, T531, R533, H536, Y553, and P554 (Masse et al., 2004; Vongpunsawad et al., 2004; Navaratnarajah et al., 2008).

SLAM ACTS AS THE PRINCIPAL RECEPTOR FOR MORBILLIVIRUS *IN VIVO*

Signaling lymphocyte activation molecule-isolated strains express typical clinical symptoms in experimental animal models. Thus, the *in vivo* study of wild-type morbillivirus, in particular MeV and CDV, has proceeded in conjunction with the establishment of a novel method for generating recombinant virus, known as reverse genetics (Billeter et al., 2009).

To identify the host cells that support infection, a recombinant CDV that expressed green fluorescent protein (GFP) was produced by reverse genetics, based on a wild-type strain that is lethal to ferrets, and inoculated intranasally into animals (von Messling et al., 2004). CDV initially infected lymphocytes and massively replicated therein, thereby causing immunosuppression, systemic invasion, and host escape. In contrast, replication in epithelial cells was initially not detectable but substantial before host death.

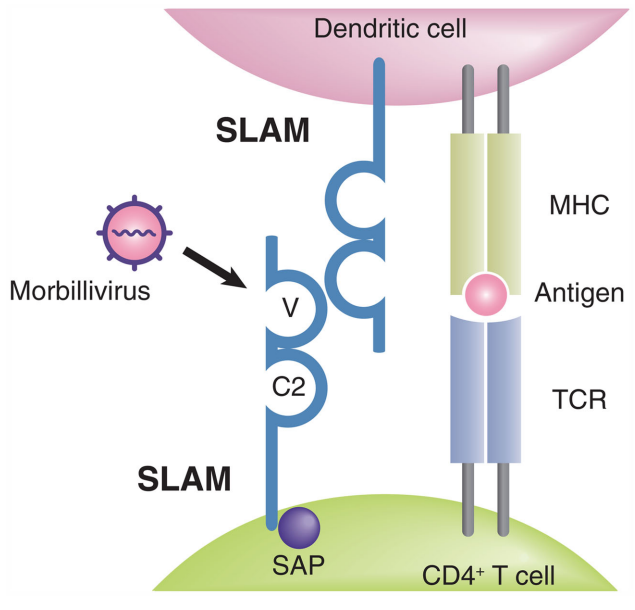

FIGURE 3 | Structure of SLAM. The SLAM extracellular domain is composed of a variable (V) and a constant (C2) Ig-like repeat. SLAM interacts with another SLAM on an adjacent cell. All morbilliviruses bind to the V domain of SLAM.

In a similar manner, GFP-expressing MeV was also generated and inoculated into macaques via the aerosol route, and the time course of propagation was monitored (de Swart et al., 2007). MeV entered the host at the alveolar level by infecting macrophages or dendritic cells, which carried the virus to bronchus-associated lymphoid tissue, followed by regional dissemination by viremia.

To further clarify the importance of SLAM for morbillivirus pathogenesis, recombinant viruses possessing H, which are incapable of recognizing SLAM but can enter epithelial cells (SLAM-blind), have been generated.

Signaling lymphocyte activation molecule-blind CDV infected primary ferret epithelial cells as efficiently as the parental wild-type CDV but was incapable of entering ferret peripheral blood mononuclear cells *in vitro*. Experimentally infected ferrets indicated that the SLAM-blind virus is completely avirulent in ferrets; infection with this virus caused only a small, short-lived decrease in the blood leukocyte count (von Messling et al., 2006).

Signaling lymphocyte activation molecule-blind MeV was also generated and inoculated intranasally into rhesus monkeys. As a result, the virus showed attenuated pathogenicity, inefficient infection of lymphocytes, and induced no clinical symptoms in these animals (Leonard et al., 2010).

Recently, our group has generated SLAM-blind RPV using a lapinized strain (RPV-L). RPV-L is highly virulent in rabbits and exhibits similar pathogenicity as virulent RPV in cattle. Thus, RPV-L-infected rabbits should represent a useful model for studying *in vivo* pathogenicity after RPV infection. SLAM-blind RPV-L induced few clinical signs, which is in agreement with studies with CDV and MeV, demonstrating that SLAM recognition is necessary for virulence. The virus was not detected in any of the lymphoid tissues, but was detected in lungs, suggesting that the SLAM-blind RPV in rabbits could infect epithelial but not lymphoid cells (unpublished data).

These results strongly indicated that SLAM-mediated cell entry is crucial for expression of full pathogenicity of morbillivirus.

A PUTATIVE RECEPTOR ON EPITHELIAL CELLS

Distribution and functions of SLAM provide a good explanation for the lymphotropism and immunosuppressive nature of morbillivirus. However, morbillivirus, in autopsied patients and some experimentally infected animals, has also been shown to infect the epithelial cells of the trachea, bronchial tubes, lungs, oral cavity, pharynx, esophagus, intestines, liver, and bladder (Griffin, 2007). These epithelial cells do not express SLAM, but the infected cells do shed virus, suggesting that entry into these SLAM-negative cells is mediated by other cellular receptors.

In vitro studies have shown that a number of SLAM-negative cell types of epithelial or neuronal origin result in cytopathic effects and virus release. In particular, several well-differentiated polarized epithelial cell lines showed high susceptibility to wild-type MeV (Takeda et al., 2007; Tahara et al., 2008). Further *in vitro* studies indicated that wild-type MeV enters human polarized airway epithelium basolaterally, whereas progeny viral particles are released exclusively from the apical surface of these cells (Tahara

et al., 2008; Ludlow et al., 2010). Moreover, it was shown that loss of tight junction proteins induced by the transcription repressor SNAIL blocked infection with MeV (Shirogane et al., 2010). These data strongly implied that polarized epithelial cells possess a putative epithelial receptor, EpR, and that the receptor appears to be expressed on the basolateral side of the cells that is associated with tight junctions.

From these studies, before identification of the components of EpR, the region of the H protein that interacts with the EpR was mapped to the H protein (I456, L464, L482, P497, Y541, and Y543; Leonard et al., 2008; Tahara et al., 2008).

Based on these data, an EpR-blind MeV maintaining SLAM-dependent cell entry was generated and inoculated intranasally into monkeys (Leonard et al., 2008). As a result, EpR-blind MeV-infected macaques developed signs of measles comparable to those of animals infected with wild-type virus, including skin rash and anorexia, indicating that the EpR-blind MeV remained virulent in the macaques. However, EpR-blind MeV could not be isolated from the tracheal aspirates of all of the monkeys, unlike wild-type MeV. This strongly suggested that MeV crosses the respiratory epithelium only when it leaves the host and that EpR-blind MeV does not shed in the airways.

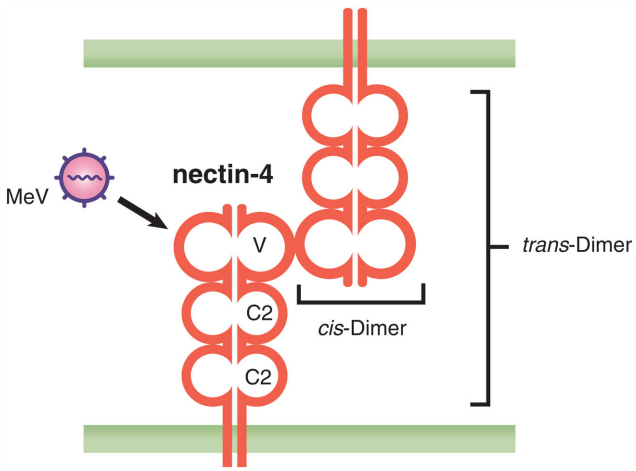

FIGURE 4 | Structure of nectins. The nectin family proteins contain three Ig-like loops (V and two C2-type domains) in their extracellular domain. Two nectin and nectin-like molecules of the same plasma membrane first form *cis*-dimers, and then this is followed by the formation of a *trans*-interaction between the Ig-like loops of *cis*-dimers located on opposing cells. MeV binds to the V domain of nectin-4, specifically.

NECTIN-4

In 2011, two independent groups reported identification of the EpR. Both groups utilized microarray data from susceptible versus non-susceptible cell lines and compared the membrane protein gene transcripts.

Noyce et al. (2011) described the susceptibility of many different tumor cell lines to MeV infection and selected susceptible and non-susceptible cell lines. They filtered the microarray data for membrane protein genes, and produced a short list of 11 candidate receptors. Of these, only human PVRL4 (nectin-4), a tumor cell marker found on breast, lung, and ovarian carcinomas, rendered cells susceptible to MeV infection. Transient knockdown of nectin-4 using siRNA abolished MeV infection in these cell lines. Furthermore, antibodies specific for human nectin-4 inhibited MeV infection. Mühlebach et al. (2011) performed microarray analysis of seven epithelial cell lines from human airways or bladder previously characterized as permissive (three lines) or non-permissive (four lines), and identified that nectin-4 renders CHO cells susceptible to MeV. It was demonstrated that the V domain of nectin-4 binds strongly to MeV-H (Mühlebach et al., 2011; **Figure 4**).

The nectin family is a cell adhesion molecule family comprising four members (nectin-1–4), and only nectin-4 functions as the EpR (Mühlebach et al., 2011; Noyce et al., 2011). Nectins contain immunoglobulin-like domains, similar to SLAM. The nectin family proteins have recently been shown to be essential contributors to the formation of cell–cell adhesions and are novel regulators of cellular activities, including cell polarization, differentiation, movement, proliferation, and survival (Takai et al., 2008; Ogita et al., 2010). Nectins are also involved in the establishment of apical–basal polarity at cell–cell adhesion sites and the formation of tight junctions in epithelial cells (Takai et al., 2008; Ogita et al., 2010).

To date, details of the interaction mechanism of the newly identified receptor, nectin-4, with MeV-H has not been elucidated. In particular, it is unclear whether nectin-4 produces intracellular signals upon engagement with MeV-H. Further studies are necessary to clarify the implications of the interaction of MeV-H and nectin-4 in MeV pathogenicity.

OVERVIEW OF MeV PROPAGATION IN THE INFECTED BODY

It has been postulated that the primary targets of MeV are SLAM-positive alveolar macrophages, dendritic cells, and lymphocytes of the immune system in the respiratory tract, rather than epithelial cells. This contention is supported by the finding that almost all $CD14^+$ monocytes in human tonsils express SLAM. MeV subsequently grows in SLAM-expressing lymphatic cells and spreads to lymph nodes throughout the body. After systemic infection, it is considered that the virus is transmitted from infected lymphocytes and dendritic cells to epithelial cells using nectin-4 on the basolateral side of epithelial cells, and virus particles are subsequently shed from the apical surface of these cells (**Figure 5**).

ALTERNATIVE RECEPTORS

From the above studies, the major transmission mode of morbillivirus, especially MeV, has been drawn. However, many histopathological studies have indicated that morbillivirus is also detected in endothelial and neuronal cells (Griffin, 2007), suggesting the existence of other routes for virus propagation to these cell types. In particular, MeV and CDV show strong neuronal tropism, and cause acute and persistent encephalitis (Griffin, 2007), nevertheless neural cells neither express SLAM nor nectin-4. These cells may have their own receptors or be infected by virus via an inefficient receptor.

Previous studies using recombinant morbilliviruses expressing GFP have demonstrated that cell entry independent of SLAM and CD46 (and probably nectin-4) occurs in a variety of cell lines with

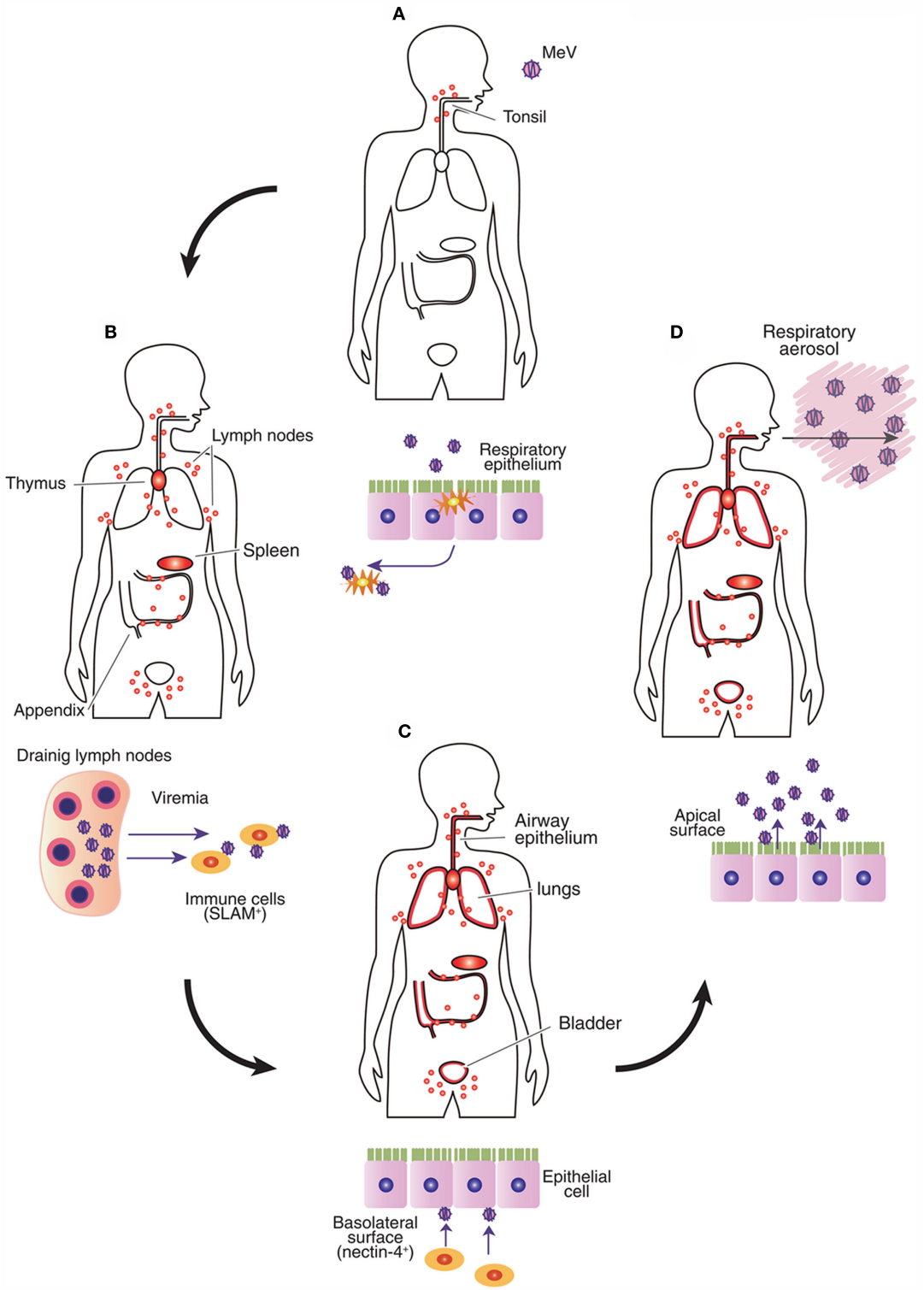

FIGURE 5 | Major transmission mode of MeV in infected body. (A) MeV enters through the respiratory route, and then infects the dendritic cells, macrophages, and lymphocytes in the respiratory epithelium. **(B)** MeV transports to draining lymph nodes. There, other immune cells, predominantly B and T-cells are infected, followed by a cell-associated viremia that distributes the infection to other organs. **(C)** MeV-infected immune cells transmit MeV to epithelial cells in various organs such as the airway, lung, and bladder from the basolateral side. **(D)** Progeny viruses are released from the host as respiratory aerosols.

low infectivity (Hashimoto et al., 2002; Fujita et al., 2007; Terao-Muto et al., 2008). This suggests the existence of inefficient but ubiquitously expressed receptors.

Previously, we have found that infection with several SLAM (and presumably nectin-4) negative cell lines with morbillivirus was inhibited by soluble heparin, and that virus bound to immobilized heparin. These results suggest that ubiquitously expressed heparin-like glycosaminoglycans are involved in morbillivirus infection (Fujita et al., 2007; Terao-Muto et al., 2008). More recently, we have also demonstrated a unique infection mechanism of MeV, in which viral particles incorporate cellular cyclophilin (Cyp)B on their surface and bind to cellular CD147, a receptor for CypA and B, independently of MeV-H (Watanabe et al., 2010). It is known that CypA incorporated into HIV-1 particles translocates to the surfaces of virions (Misumi et al., 2002), and that the interaction between CypA and CD147 enables HIV-1 to infect target cells via CD147, independently of the binding of gp120 and CD4 (Pushkarsky et al., 2001). Additionally, severe acute respiratory syndrome coronavirus (SARS-CoV) is proposed to use CD147 as a receptor in the same manner as HIV-1 (Chen et al., 2005). Unlike HIV-1 and SARS-CoV, MeV uses CypB instead of CypA for binding to CD147. This finding is the first among viruses belonging to the order Mononegavirales and shows a new infection mode of MeV, which is independent of H protein.

CONCLUSION

Investigations aimed at identifying the receptors for morbillivirus started in 1993 with CD46 for vaccine strains of MeV, followed by the lymphoid cell receptor, SLAM, in 2000, and the epithelial cell receptor, nectin-4, in 2011, for wild-type viruses. Along with the receptors, the cell tropism, transmission modes in the body, and unique pathogenicities of morbillivirus are being explained. However, many problems associated with morbillivirus remain to be clarified. In particular, the mechanism by which MeV spreads in the central nervous system during fatal subacute sclerosing panencephalitis is unknown. Further studies will lead to a better understanding of morbillivirus pathogenesis and to novel strategies for treatment and prevention.

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Filovirus tropism: cellular molecules for viral entry

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In human and non-human primates, filoviruses (Ebola and Marburg viruses) cause severe hemorrhagic fever. Recently, other animals such as pigs and some species of fruit bats have also been shown to be susceptible to these viruses. While having a preference for some cell types such as hepatocytes, endothelial cells, dendritic cells, monocytes, and macrophages, filoviruses are known to be pantropic in infection of primates. The envelope glycoprotein (GP) is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane. It has been demonstrated that filovirus GP interacts with multiple molecules for entry into host cells, whereas none of the cellular molecules so far identified as a receptor/co-receptor fully explains filovirus tissue tropism and host range. Available data suggest that the mucin-like region (MLR) on GP plays an important role in attachment to the preferred target cells, whose infection is likely involved in filovirus pathogenesis, whereas the MLR is not essential for the fundamental function of the GP in viral entry into cells *in vitro*. Further studies elucidating the mechanisms of cellular entry of filoviruses may shed light on the development of strategies for prophylaxis and treatment of Ebola and Marburg hemorrhagic fevers.

Keywords: filovirus, Ebola virus, Marburg virus, viral glycoprotein, receptor, tropism

INTRODUCTION

Ebola virus (EBOV) and Marburg virus (MARV), classified as biosafety level 4 agents, belong to the Family *Filoviridae*. Whereas MARV consists of a single species, Lake Victoria *Marburgvirus*, there are four distinct EBOV species, including *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), *Reston ebolavirus* (REBOV), and the proposed new species *Bundibugyo ebolavirus* (BEBOV) (Sanchez et al., 2007; Towner et al., 2008) (**Figure 1** left). Among these, ZEBOV, first identified in 1976, seems to be the most virulent, killing approximately up to 90% of infected individuals, whereas REBOV, which was initially isolated from cynomolgus monkeys imported from the Philippines into the USA in 1989, is less pathogenic in experimentally infected non-human primates (Fisher-Hoch and McCormick, 1999) and has never caused lethal infection in humans (Sanchez et al., 2007).

Ebola virus and Marburg virus are filamentous, enveloped, non-segmented, single-stranded, negative-sense RNA viruses (**Figure 2**). The viral genome encodes seven structural proteins, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). EBOV also expresses at least one secreted non-structural glycoprotein (sGP). **Figure 3** summarizes filovirus replication in cells. At the first step of replication, viral attachment through interaction between GP and some cellular molecules is followed by endocytosis, including macropinocytosis (Nanbo et al., 2010; Saeed et al., 2010). Subsequent fusion of the viral envelope with the host cell endosomal membrane releases the viral proteins (i.e., NP, VP35, VP30, and L) and RNA genome into the cytoplasm, the site of replication. Transcription of the negative-sense viral RNA by the viral polymerase complex (VP35 and L) yields mRNAs that are translated at cellular ribosomes. During replication, full-length positive-sense copies of the viral genome are synthesized. They subsequently serve as templates for replication of negative-sense viral RNA synthesis. At the plasma membrane, NP-encapsidated full-length viral RNAs and the other viral structural proteins are assembled with VP40 and GP and incorporated into enveloped virus particles that bud from the cell-surface (Noda et al., 2006; Bharat et al., 2011). Though filoviruses show broad tissue tropism, hepatocytes, endothelial cells, dendritic cells, monocytes, and macrophages are thought to be their preferred target cells, and infection of these cells is important for hemorrhagic manifestation and immune disorders (Geisbert and Hensley, 2004).

FILOVIRUS HOST RANGE

Filoviruses are known to cause severe hemorrhagic fever in human and non-human primates, but recent studies suggest that quadrupeds are also naturally susceptible to EBOV infection (**Figure 1**, right). In 2008–2009, REBOV infection was confirmed for the first time in pigs in the Philippines (Barrette et al., 2009). REBOV was occasionally isolated from the samples subjected to the diagnostic investigation of multiple outbreaks of a respiratory and abortion disease syndrome in swine, which were caused by porcine reproductive and respiratory syndrome virus, common in pigs in Asia. It is speculated that REBOV became detectable, most likely due to the coinfection with this porcine virus. Although pathogenicity of these swine REBOV strains to humans, non-human primates, or even pigs remains unclear, other EBOV species (i.e., ZEBOV) was shown to cause severe respiratory disease in experimentally infected pigs (Kobinger et al., 2011). During the 2001–2003 ZEBOV outbreaks in Gabon and the Democratic Republic of the Congo (DRC), when large numbers of

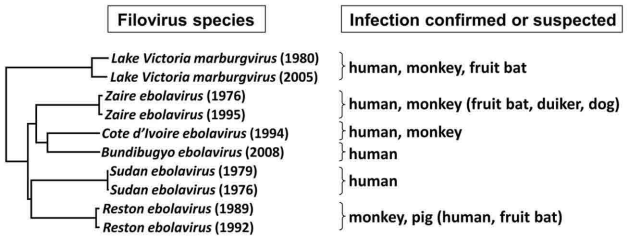

FIGURE 1 | Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed using the neighbor-joining method. For construction of this tree, ten complete GP amino acid sequences were used. Infectious viruses were isolated or viral genome and/or specific antibodies were detected (in parentheses) from the animals shown on the right.

gorillas and chimpanzees were infected, the viral genome was also detected in duikers, medium-sized Bovid related to antelopes and gazelles (Leroy et al., 2004). It was also reported that several dogs in the ZEBOV-epidemic area might have been highly exposed to the virus by eating infected dead animals, as suggested by high seroprevalence, but the putative infection seems to be asymptomatic (Allela et al., 2005).

Infectious MARV was recently isolated from Egyptian fruit bats (*Rousettus aegyptiacus*) in Uganda, indicating that this species is susceptible to MARV infection and potentially acts as the natural reservoir of the virus (Towner et al., 2009). Phylogenetic analysis showed that viruses in the bats were closely related to those isolated from victims of the 2007 MARV outbreak in Uganda, providing the first evidence for an epidemiological link between viruses in bats and hemorrhagic fever outbreak in humans. On the other hand, EBOV has not been isolated from any bat species. During the 2001–2003 EBOV outbreaks in Gabon and DRC, however, fruit bats (*Hypsignathus monstrosus*, *Epomops franqueti*, *and Myonycteris torquata*) captured in the outbreak area were found to have EBOV genomic RNA and virus-specific antibodies (Leroy et al., 2005), suggesting they are potential natural reservoirs for EBOV. However, it is still unclear whether these bats continuously maintain EBOV and/or MARV and act as a potential source of filovirus transmission to humans.

It has been shown that laboratory animals, including mice and guinea pigs, are susceptible to filovirus infection. However, these animals infected with filoviruses obtained from patients normally develop a non-lethal illness, though the viruses have the ability to replicate in the animals. Guinea pigs have been used as an animal model for filovirus infection since serial passage of MARV and EBOV in the animals results in a substantial increase in lethality (Bowen et al., 1980; Hevey et al., 1997; Volchkov et al., 2000; Subbotina et al., 2010). It was also demonstrated that passages of ZEBOV through young mice resulted in the selection of variants with pathogenicity associated with mutations in viral internal genes (e.g., NP and VP40) (Bray et al., 1998; Ebihara et al., 2006). This mouse-adapted ZEBOV is highly lethal to mice. Similarly, a mouse model for MARV infection has been established (Warfield et al., 2009). Interestingly, mutations found in the GP gene of these mouse- or guinea pig-adapted viruses were not the primary factor for efficient replication in mice and guinea pigs, suggesting the importance of some other mechanisms underlying in viral replication and/or immune evasion, as shown in the pathogenesis of influenza virus (Fukuyama and Kawaoka, 2011).

FILOVIRUS ENVELOPE GLYCOPROTEIN

The fourth gene from the 3′ end of the filovirus genome encodes the viral envelope GP (**Figure 2**), which is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane (Takada et al., 1997; Wool-Lewis and Bates, 1998) (**Figures 3** and **4**). GP is highly glycosylated with large amounts of N- and O-linked glycans, most of which are uniformly located in the middle one-third of the GP, designated the mucin-like region (MLR) (Yang et al., 2000; Manicassamy et al., 2007). The amino acid sequences of the MLR are highly variable among filovirus species (Sanchez et al., 1996, 1998). GP undergoes proteolytic cleavage by host proteases such as furin (Volchkov et al., 1998), which produces two subunits, GP1 and GP2, linked by a disulfide bond. The GP1 subunit mediates viral attachment, most likely through the MLR or the putative receptor binding region (RBR; Kuhn et al., 2006; Dube et al., 2009). The GP2 subunit has the heptad repeat regions required for assembling GP as a trimer. The hydrophobic fusion loop on GP2 is thought to catalyze fusion of the viral envelope and host cell membrane (Weissenhorn et al., 1998; Ito et al., 1999). Although the trigger to promote the conformational change leading to membrane fusion is not fully understood, it was recently suggested that endosomal proteolysis of EBOV and MARV GPs by cysteine proteases such as cathepsins B and L plays an important role in inducing membrane fusion (Chandran et al., 2005; Schornberg et al., 2006; Matsuno et al., 2010a). Since GP is the only viral surface GP, it is believed to have an important role in controlling the tropism and pathogenesis of filovirus infection (Takada and Kawaoka, 2001; Hoenen et al., 2006; Sanchez et al., 2007).

PSEUDOTYPE VIRUS SYSTEM TO SEARCH FOR FILOVIRUS RECEPTORS/CORECEPTORS

In the early years, studies of filoviruses were hampered by its extraordinary pathogenicity, which requires biosafety level 4 containment. To circumvent this problem, pseudotype virus systems for functional analysis of filovirus GPs have been established (Takada et al., 1997; Wool-Lewis and Bates, 1998). The systems rely on recombinant viruses (e.g., replication-competent or -incompetent vesicular stomatitis virus and retroviruses) that contain filovirus GP instead of their own GPs (**Figure 5**). Such pseudotype virus systems enable us to investigate cell tropism mediated by simple interaction between filovirus GP and its cellular ligands. Using such a system, it was shown that pseudotyped viruses infected primate cells more efficiently than any of the other mammalian or avian cells examined, in a manner consistent with the host range tropism of Ebola virus, and that cell-surface GPs with N-linked oligosaccharide chains might contribute to the entry of Ebola viruses, presumably acting as a specific receptor and/or cofactor for virus entry (Takada et al., 1997). Furthermore, filovirus receptor-deficient cell lines that have been used in expression cloning strategies searching for filovirus entry mediators were discovered in an early study (Wool-Lewis and Bates, 1998). Thus,

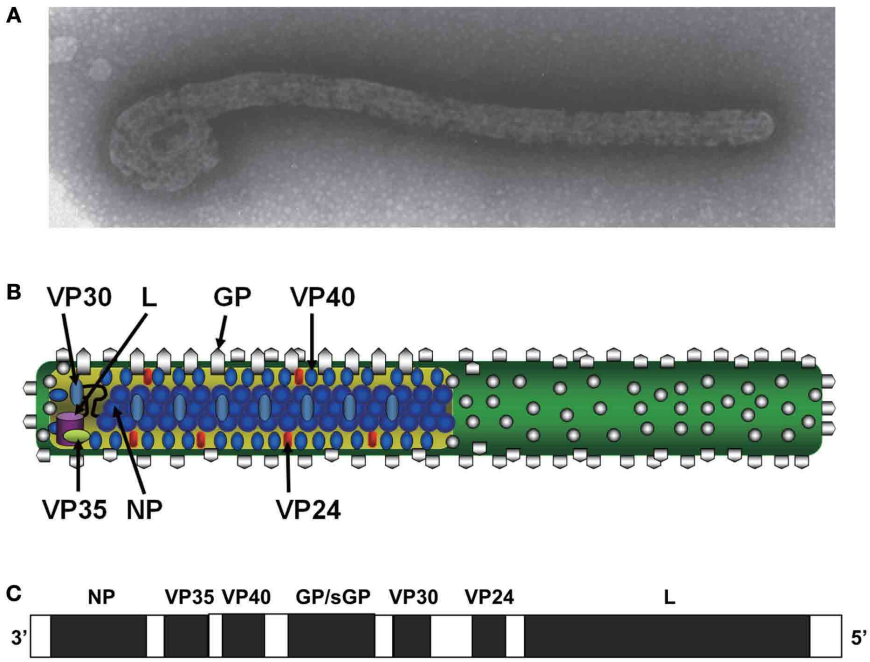

FIGURE 2 | Structure of Ebola virus particle and genome organization. Electron micrograph of Ebola virus particle **(A)**, its diagram **(B)**, and negative-sense genome organization **(C)** are shown. Viral protein names and functions are described in the text. Transcribing the glycoprotein (GP) gene produces a soluble GP (sGP). Transcriptional editing accompanied by frame shifting is required to produce full-length, membrane-anchored GP, which shares its first 295 amino acid residues with sGP.

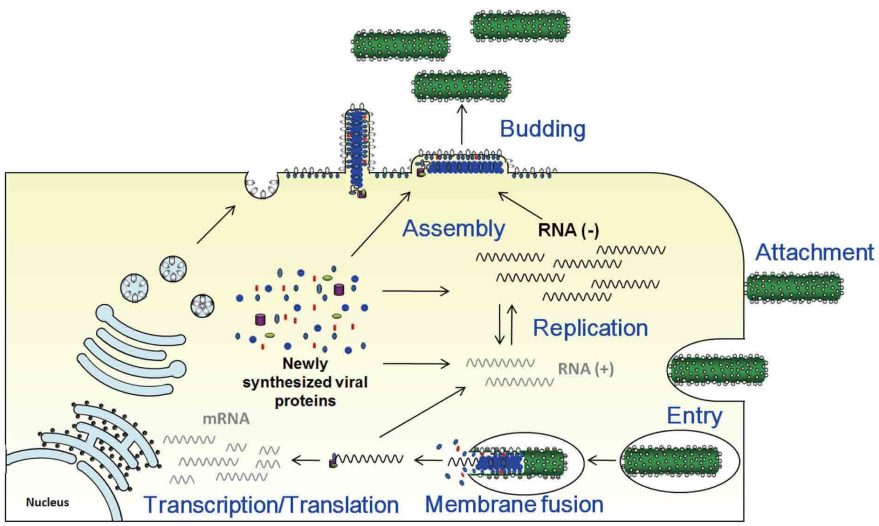

FIGURE 3 | Filovirus replication in a cell. Viral proteins involved in each step are described in the text.

pseudotype virus systems are an essential tool for recent filovirus receptor research.

CELLULAR MOLECULES IDENTIFIED AS UBIQUITOUS RECEPTORS FOR FILOVIRUS ENTRY

Although filoviruses can replicate in various tissues and cell types, the molecular mechanisms of their broad tropism remain poorly understood (**Figure 6**). By using an expression cloning strategy that has been used to identify several virus receptors, human folate receptor-α was first identified as a ubiquitous cellular cofactor that mediates infection by both MARV and ZEBOV (Chan et al., 2001). This molecule is a glycosyl-phosphatidylinositol-linked protein expressed on the cell-surface. However, a human immunodeficiency virus pseudotyped with EBOV GP could not

FIGURE 4 | Schematic diagram of filovirus glycoprotein. Both EBOV and MARV GPs contain signal peptides (SP), putative RBR, MLR, furin cleavage site, internal fusion loop (IFL), heptad repeat (HP), and transmembrane (TM) regions.

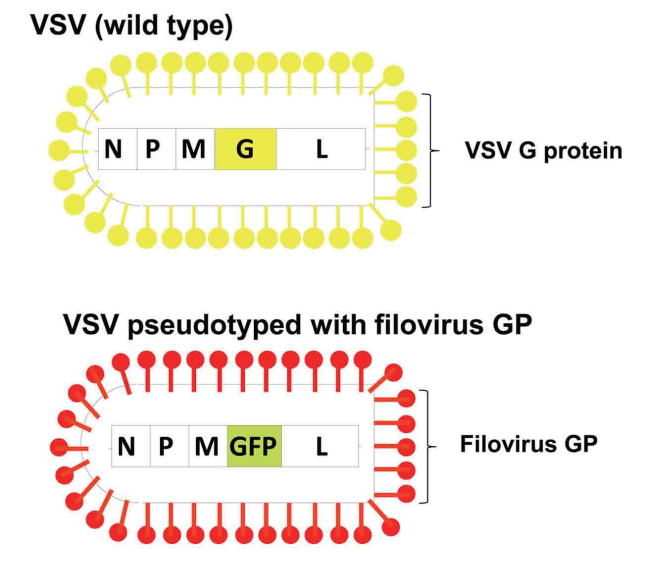

FIGURE 5 | Schematic diagram of vesicular stomatitis virus (VSV) pseudotyped with filovirus GP. The pseudotype virus relies on a recombinant virus that contains a reporter gene instead of the viral envelope protein gene responsible for receptor-binding and membrane fusion. Since filovirus glycoprotein is efficiently incorporated into VSV particles, a recombinant VSV that contains the green fluorescent protein (GFP) gene, instead of the G protein gene can be generated. This virus is not infectious unless the envelope protein responsible for receptor binding and membrane fusion is provided in *trans*.

infect T-cell lines stably expressing this protein, suggesting that folate receptor-α is not sufficient to mediate entry (i.e., some other molecules are required) (Simmons et al., 2003b; Sinn et al., 2003). A similar approach identified members of the Tyro3 receptor tyrosine kinase family (Axl, Dtk, and Mer) as molecules involved in cell entry of filoviruses (Shimojima et al., 2006). Expression of these family members in lymphoid cells, which are originally non-permissive to filoviruses, enhanced infection by pseudotype viruses bearing filovirus GPs on their envelopes. These molecules are widely distributed in many types of cells throughout the body, though not on lymphocytes and granulocytes (Linger et al., 2008). A more recent study demonstrated that reduction of Axl expression by RNAi treatment resulted in decreased ZEBOV entry via. macropinocytosis but had no effect on the clathrin-dependent or caveola/lipid raft-mediated endocytic mechanisms, suggesting that Axl enhances macropinocytosis (Hunt et al., 2011). However, direct interactions between these cellular molecules and the GP RBR remain to be demonstrated.

Recently, a bioinformatics approach, comparative genetics analysis, was used to screen the candidate genes involved in ZEBOV entry and T-cell immunoglobulin and mucin domain 1 (TIM-1) was identified as a candidate ZEBOV and MARV cellular receptor by correlation analysis between the gene expression profiles and permissiveness to viral infection (Kondratowicz et al., 2011). TIM-1 was shown to bind to the RBR of ZEBOV GP, and ectopic TIM-1 expression in poorly permissive cells enhanced EBOV infection. In addition, reduction of cell-surface expression of TIM-1 by RNAi decreased infection of highly permissive Vero cells, which are commonly used for filovirus propagation. However, the fact that not all cell types that are naturally permissive for filoviruses express the above-mentioned molecules implies that filoviruses may utilize multiple cellular proteins for infection of a wide variety of cells. More recent studies suggest that endo/lysosomal cholesterol transporter protein Niemann–Pick C1 (NPC1) is essential for filovirus infection, providing a model of EBOV infection in which cleavage of the GP1 subunit by endosomal cathepsin removes heavily glycosylated regions to expose the putative RBR, which is a ligand for NPC1 and mediates membrane fusion by the GP2 subunit (Carette et al., 2011; Côté et al., 2011). Available data indicate that the cellular tropism of filoviruses does not necessarily match the distribution of any cellular molecules so far identified. Importantly, it remains elusive whether these molecules act as functional receptors that mediate both viral attachment and membrane fusion or as so-called co-receptors whose interaction with viral GP is required only for membrane fusion.

MUCIN-LIKE REGION

Both MARV and EBOV GPs contain both N- and O-linked carbohydrate chains with different terminal sialylation patterns that seem to depend on the virus strains and cell lines used for their propagation. The MLR contains a number of potential N- and O-linked glycosylation sites as mentioned above. Though the MLR is found in all known filovirus GPs, its highly variable amino acid sequences and sugar chain structure suggest different GP properties among filovirus species. Interestingly, it is well documented that deletion of the MLR does not affect the fundamental function of GP in viral entry into cells *in vitro*, as indicated by the observation that pseudotyped viruses bearing GP lacking the MLR infect primate epithelial cells (e.g., Vero E6 cells) similarly or rather more efficiently than viruses with wild-type GP (Simmons et al., 2002; Takada et al., 2004; Matsuno et al., 2010a). According to the crystal structure of ZEBOV GP in its trimeric, prefusion conformation, the MLR may restrict access of the putative RBR to virus receptors (Lee et al., 2008). Thus, pseudotyped viruses bearing MLR-deletion mutant GP have often been used for approaches to identify filovirus-specific receptors (Shimojima et al., 2006; Kondratowicz et al., 2011). However, the MLR plays an important role in filovirus entry into preferred target cells such as endothelial cells, hepatocytes, and antigen-presenting cells, whose infection is likely involved in tropism and pathogenesis of filoviruses, as described below.

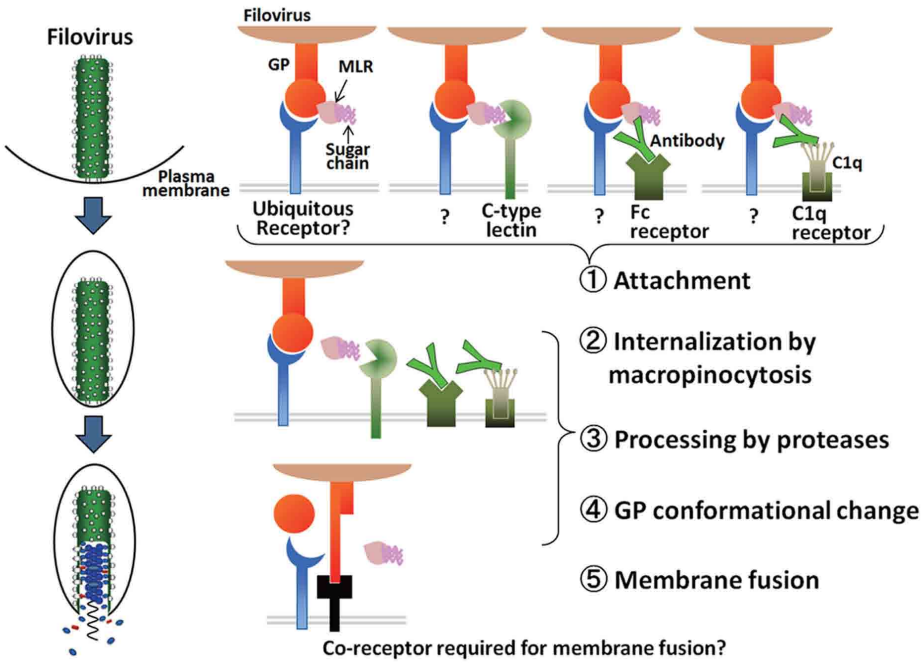

FIGURE 6 | Proposed models of filovirus entry into cells. Virus particles attach to the cell-surface through the interaction between GP and some cellular molecules (e.g., putative ubiquitous receptors, C-type lectins). Following virus uptake and trafficking to late endosomes, GP is cleaved by cellular proteases such as cathepsins to remove heavily glycosylated regions including the MLR and expose the RBR of GP1. Binding of cleaved GP1 to a coreceptor (e.g., NPC1) might be necessary for the GP conformational change leading to membrane fusion.

C-TYPE LECTINS AND THE MLR

C-type lectins are a family of Ca^{2+}-dependent carbohydrate-recognition proteins that play crucial roles in innate immunity. It has been demonstrated that membrane-anchored cellular C-type lectins facilitate filovirus infection *in vitro* by binding to glycans focused on the MLR (**Figure 6**). The asialoglycoprotein receptor, a C-type lectin found exclusively in hepatocytes, initially proposed as a receptor for Marburg virus (Becker et al., 1995), recognizes GPs displaying N-linked sugar chains with terminal galactose residues on the GP molecule and enhances filovirus infectivity. It was subsequently shown that carbohydrate chains on filovirus GP, especially on the MLR, are recognized by other cellular C-type lectins such as dendritic cell- and liver/lymph node-specific ICAM-3-grabbing non-integrin (DC/L-SIGN) (Alvarez et al., 2002; Lin et al., 2003; Simmons et al., 2003a; Marzi et al., 2004; Gramberg et al., 2008), human macrophage galactose-type C-type lectin (hMGL) (Takada et al., 2004; Matsuno et al., 2010a), and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) (Gramberg et al., 2005; Dominguez-Soto et al., 2007; Powlesland et al., 2008). Though these C-type lectins show different specificities, depending on the structures of target glycans, and thus MLR may not be the only binding site for the lectins, all have been reported to promote filovirus entry. It should be noted that C-type lectins enhance filovirus infectivity when expressed on the target cell-surface, but are unlikely to act as functional receptors mediating both attachment and membrane fusion (Simmons et al., 2003a; Marzi et al., 2007; Matsuno et al., 2010b). The fact that interaction between the GP MLR and C-type lectins is not essential for viral entry into cells lacking C-type lectins (e.g., Vero E6 cells) may also suggest that C-type lectins facilitate viral attachment but not infectious entry.

Hepatocytes, endothelial cells, dendritic cells, monocytes, and macrophages, all of which express C-type lectins, are thought to be the preferred target cells of filoviruses (Takada and Kawaoka, 2001; Geisbert and Hensley, 2004; Hoenen et al., 2006). Indeed, primary macrophage and dendritic cell cultures transduced for C-type lectin expression greatly increased their susceptibility to virus infection (Simmons et al., 2003a; Marzi et al., 2007). While C-type lectins do not directly mediate filovirus entry, their pattern of expression *in vivo* and their ability to enhance infection indicate that C-type lectins can play an important role in filovirus transmission and tissue tropism. Thus, increased infection of these cells might be directly involved in the pathogenesis of filoviruses. Accordingly, it was shown that soluble mannose-binding C-type lectin played a role in protection from lethal Ebola virus infection in a mouse model (Michelow et al., 2011). It should be noted that the ability to utilize the C-type lectins (i.e., DC-SIGN and hMGL) to promote cellular entry was correlated with the different pathogenicities among filoviruses (Takada et al., 2004; Marzi et al., 2006; Matsuno et al., 2010a). Interestingly, the MLR amino acid sequence does not seem to be the primary factor contributing to the difference (Marzi et al., 2006; Matsuno et al., 2010a; Usami et al., 2011). Although there might be some distinct mechanisms of entry between MARV and EBOV (Chan et al., 2000), the similarity of tissue tropism and pathological features of infection between these viruses suggests that C-type lectins are one of

the important molecules, likely as attachment factors, for filovirus entry into cells, and that they are directly involved in filovirus tropism at the cellular level.

ANTIBODY-DEPENDENT ENHANCEMENT AND EPITOPES ON THE MLR

In addition to the common receptor/co-receptor-dependent mechanism of cellular attachment and membrane fusion, some viruses utilize antiviral antibodies for their efficient entry into target cells (Takada and Kawaoka, 2003). This mechanism is known as antibody-dependent enhancement (ADE) of viral infection. Filoviruses utilize virus-specific antibodies for their entry into cells *in vitro* through interaction between anti-GP antibodies and the cellular Fc receptor (FcR) or complement component C1q and its ligand, which likely promotes viral attachment to cells (Takada et al., 2001, 2003a, 2007; Nakayama et al., 2011) (**Figure 6**). FcR are expressed exclusively on the cells of the immune system such as monocytes/macrophages, neutrophils, B-cells, and granulocytes (Fanger and Guyre, 1992), whereas C1q ligands have been identified in most mammalian cells (Eggleton et al., 1998; Nicholson-Weller and Klickstein, 1999), suggesting a ubiquitous mechanism for ADE of filovirus infection.

By using GP-specific monoclonal antibodies, several epitopes recognized by ADE antibodies were identified and these epitopes were mostly located in the MLR of the GP1 subunit (Takada et al., 2007; Nakayama et al., 2011). It should be noted that neutralizing antibodies appear to recognize different epitopes that are not located on the MLR (Takada et al., 2003b; Lee et al., 2008). As reflected by the high variability of the MLR amino acid sequences and limited overall cross-reactivity of anti-sera among filovirus species (i.e., ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, and MARV), ADE activities of the anti-sera to GP are virus-species-specific (Takada et al., 2001, 2007; Nakayama et al., 2010). Interestingly, potential viral pathogenicity is correlated with the ability to induce ADE antibodies, suggesting the possible contribution of ADE to different pathogenicity between filoviruses (Takada et al., 2001; Nakayama et al., 2011). More importantly, the demonstration of ADE of filovirus infection raises fundamental questions about the development of GP-based vaccines and the use of anti-GP antibodies for passive immunization.

Recently, GP has been used for viral vector-based or DNA vaccines that were shown to protect animals effectively. Replication-incompetent adenovirus expressing GP, a replication-competent vesicular stomatitis virus expressing GP, and a recombinant paramyxovirus expressing GP have been shown to protect non-human primates from lethal infections of filoviruses (Sullivan et al., 2000, 2003; Jones et al., 2005; Bukreyev et al., 2007; Feldmann et al., 2007). It should be noted that these vaccines potentially induce cytotoxic cellular response (i.e., CD8+ T lymphocytes) as well as antibody production, suggesting that activating cytotoxic T-cells is a key protective mechanism (Olinger et al., 2005; Sullivan et al., 2006; Reed and Mohamadzadeh, 2007). Since cytotoxic T-cell response cannot be fully induced by immunization with non-replicative protein antigens such as inactivated virus and subunit vaccines, viral vector-based, or DNA vaccines may be promising in preventing filovirus infection.

CONCLUSION

All enveloped viruses initiate infection by attaching to host cells followed by membrane fusion via interaction between viral surface proteins and receptor/co-receptor molecules on target cells, and this interaction is often a key determinant controlling viral tissue tropism and/or host range. As described above, it has been demonstrated that filoviruses utilize multiple molecules for their entry into cells. However, it remains elusive whether these molecules serve as functional receptors mediating both viral attachment and membrane fusion or play independent roles as either attachment receptors or fusion receptors. More importantly, none of the cellular molecules identified so far explains filovirus tissue tropism and host range reasonably. It might also be hypothesized that filoviruses do not use a single common receptor to infect a broad range of cells and, unlike many other viruses, may not need a "specific" receptor. Although the overall tropism and pathogenicity of filoviruses is controlled by multiple host cell factors (e.g., interactions with the host immune system), further studies aimed at identification of cellular molecules interacting with GP are needed to fully understand the mechanisms of cellular entry of filoviruses, and may shed light on the development of strategies for prophylaxis and treatment of Marburg and Ebola hemorrhagic fevers.

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Ebolavirus replication and tetherin/BST-2

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Ebolavirus (EBOV) is an enveloped, non-segmented, negative-stranded RNA virus, which consists of five species: *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*. EBOV causes a lethal hemorrhagic fever in both humans and non-human primates. The EBOV RNA genome encodes seven viral proteins: NP, VP35, VP40, GP, VP30, VP24, and L. VP40 is a matrix protein and is essential for virus assembly and release from host cells. Expression of VP40 in mammalian cells is sufficient to generate extracellular virus-like particles, which resemble authentic virions. Tetherin/BST-2, which was identified as an effective cellular factor that prevents human immunodeficiency virus-1 release in the absence of viral accessory protein Vpu, has been reported to inhibit ZEBOV VP40-induced VLP release. Tetherin/BST-2 appears to inhibit virus release by physically tethering viral particles to the cell surface via its N-terminal transmembrane domain and C-terminal glycosylphosphatidylinositol anchor. Replication of ZEBOV is not inhibited by tetherin/BST-2 expression, although tetherin/BST-2 was expected to inhibit EBOV release as well as VLP release. Recently, it was reported that viral glycoprotein of EBOV, GP, antagonizes the antiviral effect of tetherin/BST-2. However, the mechanism by which GP antagonizes the antiviral activity of tetherin/BST-2 and whether GP of the other EBOV species function as antagonists of tetherin/BST-2 remain unclear.

Keywords: ebolavirus, tetherin/BST-2, GP, antagonist, VLP

INTRODUCTION

Ebolavirus (EBOV) is a member of the family Filoviridae in the order Mononegavirales (MNV), and causes a lethal hemorrhagic fever in both humans and non-human primates (Peters, 2005). Five species of EBOV have been defined to date on the basis of genetic divergence: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus* (TFEBOV), *Reston ebolavirus* (REBOV), and *Bundibugyo ebolavirus* (BEBOV). ZEBOV, SEBOV, TFEBOV, and BEBOV cause clinical symptoms in humans and non-human primates, while REBOV causes disease only in non-human primates, and not in humans. At present, there are no licensed vaccines or effective therapies for EBOV infection. Recently, tetherin/BST-2 was identified as a cellular factor that inhibits the release of a wide variety of enveloped viruses, including retroviruses, herpesviruses, and Lassa virus, and the production of virus-like particles (VLPs) of filoviruses and Nipa virus (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009a; Radoshitzky et al., 2010). Tetherin/BST-2 may function as a host innate antiviral system against a wide variety of viruses, as tetherin/BST-2 is broadly induced by treatment with type I interferons (IFNs) in various cell types (Ishikawa et al., 1995; Blasius et al., 2006). This review will focus on EBOV replication and antiviral function of tetherin/BST-2.

EBOLAVIRUS AND ITS REPLICATION

Electron microscopic studies have indicated that EBOV is morphologically pleomorphic, with U-shaped, figure 6-shaped, or circular configurations, or as elongated filamentous forms of varying length (up to 14000 nm). The virions are usually 80 nm in diameter and 800–1000 nm in length and enveloped with a lipid bilayer (envelope) that is derived from the host cell, anchoring a glycoprotein that projects spikes 7–10 nm in length from its surface (Sanchez et al., 2007).

The genome is approximately 19 kb in length and encodes the viral proteins in the order NP–VP35–VP40–GP/sGP–VP30–VP24–L (**Figure 1**). The extragenic sequence at the 3′ end, which is called the leader, of EBOV is short, ranging from 50 to 70 bases in length, while the length of the 5′ end sequence, which is called the trailer, varies between species, ranging from 25 to 677 bases (25 bases for REBOV and 677 bases for ZEBOV). The extreme 3′ and 5′ end sequences are conserved and potentially form stem-loop structures (Geisbert and Jahrling, 1995; Sanchez et al., 2007). These sequences contain the encapsidation signals as well as the replication origin and transcription promoter.

The NP and VP30 proteins are the major and minor viral nucleoproteins, respectively, which are phosphorylated, and interact strongly with the genomic RNA molecule to form the viral nucleocapsid along with VP35 and L (Mühlberger et al., 1999). The L and VP35 proteins form the viral polymerase complex, which transcribes and replicates the viral genome. The L protein has the RNA-dependent RNA polymerase activity of the complex, and possesses motifs linked to RNA binding, phosphodiester bonding, and ribonucleotide triphosphate binding. VP35 is thought to play an essential role as a cofactor that affects the mode of viral transcription and replication. VP35 also functions as an antagonist against the type I IFN signaling pathway (Basler et al., 2000).

The GP gene contains a translational stop codon in the middle, thus preventing synthesis of full-length glycoprotein. Twenty

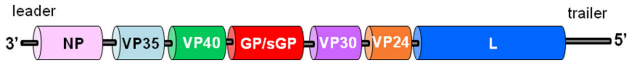

FIGURE 1 | Schematic representation of EBOV genome.

percent of the mRNA was shown to be edited, containing one additional non-template A in a stretch of seven consecutive A residues. The edited mRNA species encode full-length GP, whereas the primary gene product is a smaller secreted glycoprotein (sGP), which is produced in large amounts. The GP precursor (preGP), is synthesized as type I membrane protein in the endoplasmic reticulum (ER). preGP is cleaved by the cellular proprotein convertase furin into the N-terminal fragment GP1 and the C-terminal fragment GP2 which are linked by a disulfide bond forming the GP1, 2 complex in the *trans*-Golgi network (TGN; Volchkov et al., 1998). GP1 and GP2 function as a surface protein and a transmembrane protein, respectively. The trimeric spike of GP1, 2 is the only envelope glycoprotein of the virion and is assumed to be responsible for binding to cellular receptors and for fusion of the envelope with the cellular membrane in the course of viral entry into the host cell. GP has marked effects on viral pathogenesis and antigenicity. Recent report suggested that sGP can substitute for GP1 and present on virion as a structural protein, although sGP had been considered as a non-structural protein (Iwasa et al., 2011). The function of sGP is still unknown. sGP may contribute to disease progression, as it has been reported that large amounts of sGP are present in the blood of acutely infected patients (Volchkov et al., 1995; Sanchez et al., 1996).

The VP40 and VP24 proteins are viral matrix proteins and are associated with the virion envelope. VP40 is the most abundant protein in the virion and plays a key role in virus assembly and budding as viral matrix protein. Expression of VP40 in mammalian cells is sufficient to generate extracellular VLPs, which resemble authentic virions (Harty et al., 2000; Yasuda et al., 2003). Only small amounts of VP24 are present in the virion. VP24 has been reported to function as an antagonist of the type I IFN signaling pathway, along with VP35 (Reid et al., 2006).

TETHERIN/BST-2

Tetherin/BST-2 (also known as CD317 or HM1.24) has been identified as an effective cellular factor that prevents human immunodeficiency virus (HIV)-1 release in the absence of the viral accessory protein Vpu (Neil et al., 2008; Van Damme et al., 2008). Tetherin/BST-2 consists of four domains, i.e., an N-terminal cytoplasmic tail (CT), a single transmembrane domain, an extracellular domain, and a putative C-terminal glycosylphosphatidylinositol (GPI) anchor, and therefore both ends of this molecule are associated with the plasma membrane (**Figure 2A**). This molecule is localized to lipid rafts at the cell surface and membranes of the TGN and recycling compartments (Kupzig et al., 2003; Blasius et al., 2006; Rollason et al., 2007; Evans et al., 2010). In addition, tetherin/BST-2 has two putative N-linked glycosylation sites in the extracellular domain and forms a homodimer by intermolecular disulfide bonds (Ohtomo et al., 1999; Kupzig et al., 2003; **Figure 2A**).

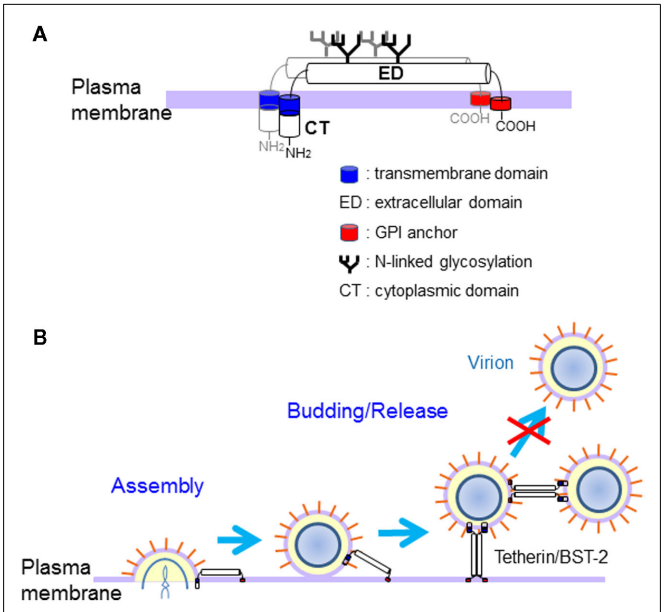

FIGURE 2 | Structure and antiviral action of Tetherin/BST-2. **(A)** Structural representation of tetherin/BST-2. Tetherin/BST-2 has two putative N-linked glycosylation sites in the extracellular domain and forms a homodimer by intermolecular disulfide bonds. Both ends of tetherin/BST-2 are associated with the plasma membrane via the N-terminal transmembrane domain and C-terminal GPI anchor. **(B)** Proposed model for the antiviral mechanism of tetherin/BST-2. Tetherin/BST-2 appears to inhibit the release of progeny viruses by directly tethering virions to cells, briefly by anchoring one end of the molecule on the cell membrane and the other end on the viral envelope. Progeny virions could be also directly tethered to each other by tetherin/BST-2.

Previous studies showed that tetherin/BST-2 is constitutively expressed in terminally differentiated B cells, bone marrow stromal cells, and plasmacytoid dendritic cells, and is also broadly induced by treatment with type I IFN in various cell types (Ishikawa et al., 1995; Blasius et al., 2006). In fact, the upstream region of tetherin/BST-2 contains a tandem repeat of IL-6 response elements, STAT3 binding site, and the IFN response elements IRF-1/2 and ISGF3 (Ohtomo et al., 1999). Therefore, tetherin/BST-2 may be involved in antiviral host defense as a mechanism of innate immunity. However, the physiological function of tetherin/BST-2 is not yet clear.

Recent analysis for *in vivo* expression of tetherin/BST-2 showed that tetherin/BST-2 was expressed to varying degrees in most organs and a number of specialized cell types, including hepatocytes, pneumocytes, ducts of major salivary glands, pancreas and kidney, Paneth cells, epithelia, Leydig cells, plasma cells, bone marrow stromal cells, monocytes, and vascular endothelium, without IFN stimulation (Erikson et al., 2011). Therefore, IFN may only partially regulate tetherin/BST-2 *in vivo*.

ANTIVIRAL ACTIVITIES OF TETHERIN/BST-2

We have shown that tetherin/BST-2 also efficiently inhibits the egress of VLPs of Marburgvirus and Lassa virus and retains VLPs on the cell surface (Sakuma et al., 2009a). Furthermore, tetherin/BST-2 has also been reported to inhibit the release of

retroviruses other than HIV-1, Kaposi's sarcoma-associated herpesvirus (KSHV), and the production of VLPs of EBOV and Nipa virus (Jouvenet et al., 2009; Kaletsky et al., 2009; Mansouri et al., 2009; Radoshitzky et al., 2010).

The N-linked glycosylation of tetherin/BST-2 is dispensable for the antiviral activity, while both N-terminal transmembrane domain and C-terminal GPI anchor are required for the activity (Neil et al., 2008; Andrew et al., 2009; Sakuma et al., 2009a). Interestingly, tetherin/BST-2 mutant with complete loss of dimerization activity (the cysteine mutant) still showed apparent inhibitory activity for the production of Lassa and Marburg VLPs, although its activity was reduced (Sakuma et al., 2009b). For HIV-1, the cysteine mutant showed much weaker antiviral activity (Andrew et al., 2009; Perez-Caballero et al., 2009). Nevertheless, these observations suggested that dimerization of tetherin/BST-2 is important but not essential for its antiviral activity. A recent study showed that tetherin/BST-2 inhibits HIV-1 release by directly tethering virions to cells, briefly by anchoring one end of the molecule on the cell membrane and the other end on the viral envelope (Perez-Caballero et al., 2009; **Figure 2B**). Progeny virions released from cells could also be directly tethered to each other by tetherin/BST-2. It is likely that tethering of virion by tetherin dimer is stronger than that by tetherin monomer because of stronger association with the membrane. Therefore, tetherin/BST-2 appears to inhibit release of a wide variety of enveloped viruses from host cells by a similar mechanism.

COUNTERACTION OF TETHERIN/BST-2 ANTIVIRAL ACTION BY GP

Human immunodeficiency virus-1 Vpu is known to antagonize the antiviral function of tetherin/BST-2 (Neil et al., 2008; Van Damme et al., 2008). Moreover, recent studies have shown that HIV-2 and simian immunodeficiency virus (SIV) Env, SIV Nef, KSHV K5, and EBOV GP also function as antagonists of tetherin/BST-2, suggesting that tetherin/BST-2 plays an important role in host defense against viral infection and these viruses have evolutionarily acquired viral-encoded antagonists to counteract the antiviral function of tetherin/BST-2 (Gupta et al., 2009b; Jia et al., 2009; Kaletsky et al., 2009; Le Tortorec and Neil, 2009; Mansouri et al., 2009; Zhang et al., 2009).

Kaletsky et al. (2009) demonstrated that EBOV GP interacts directly with tetherin/BST-2 and abrogates the inhibition of VP40-induced VLP release by tetherin/BST-2. In addition, analysis using infectious virus showed that the expression of tetherin/BST-2 had no effect on ZEBOV replication and spread (Radoshitzky et al., 2010).

One of the major functions of HIV-1 Vpu for counteraction of antiviral action of tetherin/BST-2 is downregulation of the surface expression of tetherin/BST-2. Several studies demonstrated that Vpu directs the degradation of human BST-2 via a β-TrCP-dependent mechanism. Vpu acts as an adapter molecule linking tetherin/BST-2 to the β-TrCP/SCF E3 ubiquitin ligase complex to induce the trafficking to late endosomes, lysosomes, and proteasomes, and subsequent lysosomal and/or proteasomal degradation of tetherin/BST-2. Vpu is thought to remove tetherin/BST-2 from the cell surface by these pathways (Douglas et al., 2009; Mitchell et al., 2009). Recent report suggests that β-TrCP-independent mechanism is also involved in the downregulation of cell surface expression of tetherin/BST-2 by Vpu, since mutations in the β-TrCP-binding motif of Vpu did not completely abrogate its antagonism of tetherin/BST-2. Schmidt et al. (2011) have reported that Vpu inhibited both the anterograde transport of newly synthesized tetherin/BST-2 and the recycling of tetherin/BST-2 to the cell surface and trapped trafficking tetherin/BST-2 molecules at the TGN. Vpu interacts with tetherin/BST-2 through species-specific determinants in their respective transmembrane domains (Gupta et al., 2009a; McNatt et al., 2009). HIV-1 Vpu specifically antagonizes the tetherin/BST-2s from human, chimpanzee, and gorilla, which are susceptible to HIV-1 infection, but not those from African green monkey, rhesus macaque, and mouse, which are not susceptible to this virus (McNatt et al., 2009; Kühl et al., 2011).

In contrast, Ebola GP counteracted tetherin/BST-2 from different primate species, including rhesus macaque and African green monkey (Kühl et al., 2011). Ebola GP does not seem to require a specific tetherin/BST-2 sequence for its activity (Lopez et al., 2010). It has been reported that tetherin/BST-2 interacts with the GP2 subunit of EBOV GP, although the antogonism of tetherin/BST-2 function by GP2 have not examined (Kühl et al., 2011). Vpu reduces cell surface expression of tetherin/BST-2, while Ebola GP appears to counteract tetherin/BST-2 without removing it from the cell surface, suggesting that both proteins employ different mechanisms to counteract tetherin/BST-2 (Lopez et al., 2010; Kühl et al., 2011). Ebola GP and tetherin/BST-2 colocalize in intracellular compartment, but not on the plasma membrane (Kühl et al., 2011). The sequestration of tetherin/BST-2 in the specific intracellular compartment may be one of the mechanisms of antagonism by Ebola GP. So far, the mechanism by which EBOV GP antagonizes tetherin/BST-2 remains unclear. Further investigations are required to understand the mechanism by which EBOV GP counteracts the antiviral function of tetherin/BST-2.

FUTURE PERSPECTIVES

Tetherin/BST-2 inhibits the production of a wide variety of enveloped viruses. On the other hand, several viruses have evolved viral-encoded antagonists to counteract antiviral action of tetherin/BST-2. EBOV also appears to have evolved GP as an antagonist of tetherin/BST-2. However, it has been reported that high-level expression of tetherin/BST-2 inhibits ZEBOV production even in the presence of GP (Kühl et al., 2011). Furthermore, it may be possible to identify tetherin/BST-2 mutants that are not counteracted by EBOV GP.

Therefore, regulation of the progeny EBOV release may be possible by *in vivo* induction or exogenous expression of tetherin/BST-2. Tetherin/BST-2 has great potential for the development of novel antiviral therapeutic strategies against EBOV infection.

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TRIM5α and species tropism of HIV/SIV

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Human immunodeficiency virus type 1 (HIV-1) infects humans and chimpanzees but not old world monkeys (OWMs) such as the rhesus monkey (Rh) and cynomolgus monkey (CM). HIV-1 efficiently enters cells of OWMs but encounters a block before reverse transcription. This narrow host range is attributed to a barrier in the host cell. In 2004, the screening of a Rh cDNA library identified tripartite motif 5α (TRIM5α) as a cellular antiviral factor. TRIM5α is one of splicing variants produced by *TRIM5* gene and TRIM5 proteins are members of the TRIM family containing RING, B-box 2, and coiled-coil domains. The RING domain is frequently found in E3 ubiquitin ligase and TRIM5α is degraded via the ubiquitin–proteasome-dependent pathway. Among TRIM5 splicing variants, TRIM5α alone has an additional C-terminal PRYSPRY (B30.2) domain. Previous studies have shown that sequence variation in variable regions of the PRYSPRY domain among different monkey species affects species-specific retrovirus infection, while amino acid sequence differences in the viral capsid protein determine viral sensitivity to restriction. TRIM5α recognizes the multimerized capsid proteins (viral core) of an incoming virus by its PRYSPRY domain and is thus believed to control retroviral infection. There are significant intraspecies variations in the Rh-*TRIM5* gene. It has also been reported that some Rh and CM individuals have retrotransposed cyclophilin A open reading frame in the *TRIM5* gene, which produces TRIM5–cyclophilin A fusion protein (TRIMCyp). TRIMCyp, which was originally identified as an anti-HIV-1 factor of New World owl monkeys, is an interesting example of the gain of a new function by retrotransposition. As different *TRIM5* genotypes of Rh showed different levels of simian immunodeficiency virus replication *in vivo*, the *TRIM5* genotyping is thought to be important in acquired immunodeficiency syndrome monkey models.

Keywords: TRIM5α, TRIMCyp, HIV-1, HIV-2, SIV, rhesus monkey, cynomolgus monkey

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of acquired immunodeficiency syndrome (AIDS). More than two million people are infected with HIV-1 annually around the world. Nevertheless, the host range of HIV-1 is extremely narrow, being limited to humans and chimpanzees (Gao et al., 1999). This narrow host range has hampered the establishment improved animal models of HIV-1 infection that are needed to facilitate the development of an efficacious vaccine against HIV-1 infection. In this review, we summarize current understanding regarding the species barrier of HIV-1 as discussed from the viewpoint of animal model development, focusing on tripartite motif 5α (TRIM5α), a restriction factor in monkeys.

LIFE CYCLE OF HIV-1

Human immunodeficiency virus type 1 belongs to the family Retroviridae, subfamily *Lentivirus*. It is an enveloped virus with a single-stranded RNA genome with positive polarity. HIV-1 enters $CD4^+$ T cells and macrophages through plasma membrane fusion. The virus RNA genome is subsequently reverse transcribed by viral-associated reverse transcriptase and resultant double-strand cDNA is transported to the nucleus. In the nucleus, viral-associated integrase (IN) inserts viral cDNA into the human chromosome. The transcription is enhanced by cellular activation and mRNA and full-length viral genome RNA are exported from the nucleus. Viral proteins assemble beneath the plasma membrane and virus particles bud from plasma membrane (**Figure 1**).

HOST FACTORS REQUIRED FOR HIV-1 REPLICATION IN HUMAN AND SPECIES-SPECIFIC BARRIER OF HIV-1 IN MICE

Many trials of small animal models for HIV-1 infection have failed due to lack of host factors in mice, which are necessary for efficient virus replication (**Figure 1**). CD4, the cellular receptor for HIV-1, was first identified as a host range barrier because mouse CD4 does not bind to HIV-1 envelope protein (Landau et al., 1988). Human CD4 transgenic mice, however, were not susceptible to HIV-1 infection (Lores et al., 1992). Chemokine receptors were identified as entry co-receptors (Alkhatib et al., 1996; Bleul et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996), but mice transgenic for human CD4 and either human CXCR4 (Sawada et al., 1998) or CCR5 (Browning et al., 1997) failed to show productive infection, even though murine CXCR4 is a functional co-receptor for CXCR4-tropic HIV-1 (Bieniasz et al., 1997).

Human immunodeficiency virus type 1 pre-integration complex (PIC) containing viral cDNA and viral IN are translocated into the nucleus. Two host cellular proteins have recently been

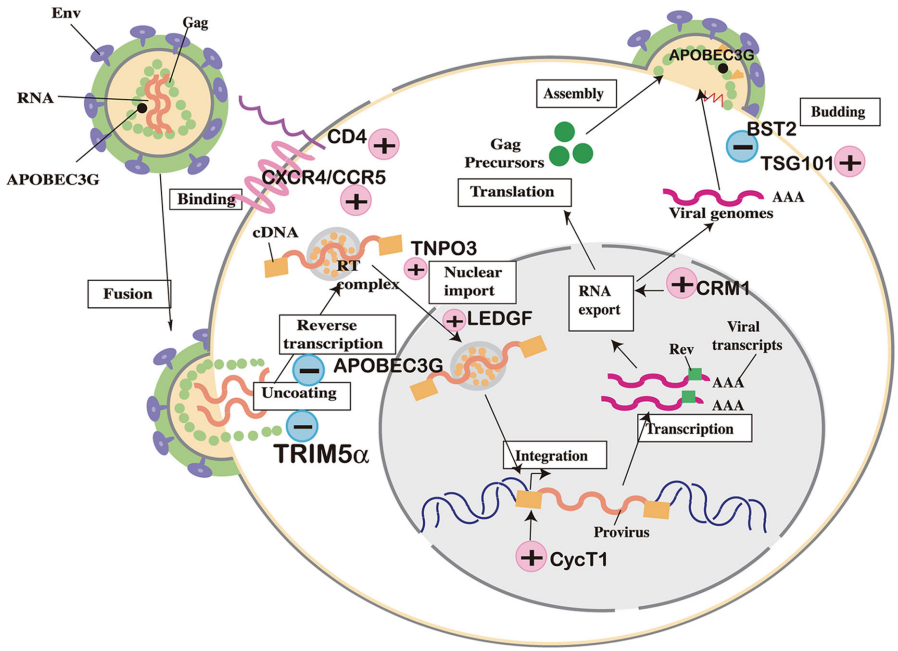

FIGURE 1 | Cellular factors involved in human immunodeficiency virus (HIV) replication cycle. (+) Positive factors required for viral replication. (–) Negative factors that suppress viral replication.

reported to mediate PIC import. The first is a lens epithelium-derived growth factor (LEDGF/p75; Cherepanov et al., 2003; Maertens et al., 2003), a protein implicated in the regulation of gene expression and cellular stress responses. LEDGF interacts with HIV-1 IN and is thought to guide PIC toward sites of active transcription for integration of viral cDNA into the human chromosome. The second is Transportin 3 (TNPO3/Transportin-SR2) identified by two independent screenings of host factors involved in HIV-1 replication (Brass et al., 2008; Christ et al., 2008). TNPO3 also binds to IN, but it is also thought to associate with viral capsid (CA) protein and supports nuclear translocation of PICs. It is currently unclear whether mouse orthologs of either or both of these factors are defective in nuclear transport and integration of HIV-1.

The integrated HIV-1 genome is then transcribed from its promoter in the long terminal repeat (LTR) by using NF-κB and Sp1 (Jones et al., 1986; Staal et al., 1990; Cullen, 1991). HIV-1 non-structural proteins, Tat, Rev, and Nef, are early gene products produced from multiply spliced mRNAs (Feinberg et al., 1986). Tat binds to the 5′ region of nascent HIV-1 transcripts and facilitates the elongation of transcribed RNA (Laspia et al., 1989; Feinberg et al., 1991). Mouse cells do not show Tat-dependent transcriptional activation of HIV-1. Cyclin T1 (CycT1) is responsible for this transcriptional barrier in mice (Newstein et al., 1990; Garber and Jones, 1999). CycT1 protein is a component of the CDK9/pTEFb transcription factor complex (Mancebo et al., 1997; Wei et al., 1998). Human but not mouse CycT1 binds to Tat and activates transcription from HIV-1 LTR (Garber et al., 1998). Nevertheless, triple-transduction of mouse cells with human CD4, CXCR4, and CycT1 was insufficient to induce productive viral infection (Bieniasz and Cullen, 2000). Additional barriers have been reported in the late stages of the viral life cycle (Mariani et al., 2000; Keppler et al., 2001; Koito et al., 2003a,b; Nagai-Fukataki et al., 2011). CRM1, a nuclear export factor that functions in association with Rev, has been suggested to be one of the late-phase factors important for the export of unspliced full-length viral genome from the nucleus (Zheng et al., 2003). Further studies are necessary to identify host cellular factors that are necessary for virus replication in humans but defective in mouse cells in order to establish small animal models of HIV-1 infection.

TRIM5α, ONE OF THE SPECIES-SPECIFIC BARRIERS TO HIV-1 IN MONKEYS

Amino acid sequences of CD4, CXCR4, CCR5, CycT1, and CRM1 in Old World monkeys (OWMs) are almost identical to those of the human orthologs, while New World monkeys, such as common marmosets and squirrel monkeys, have less functional CD4 and CCR5 receptors (LaBonte et al., 2002). Nevertheless, HIV-1 fails to replicate in activated $CD4^+$ T lymphocytes obtained from OWM, such as the rhesus monkey (Rh; *Macaca mulatta*; Shibata et al., 1995; Himathongkham and Luciw, 1996) and cynomolgus monkey (CM; *Macaca fascicularis*; Akari et al., 1996, 1999). Several studies have suggested that the blockade of HIV-1 replication in OWM cells occurs at a post-entry step (Shibata et al., 1995; Himathongkham and Luciw, 1996; Chackerian et al., 1997) and appears to result from a failure to initiate reverse transcription (Himathongkham and Luciw, 1996). Importantly, resistance against HIV-1 infection was shown to be dominant in heterokaryons between human and OWM cells, suggesting the

presence of inhibitory factor(s) against HIV-1 infection but not for simian immunodeficiency virus (SIV) in OWM cells (Munk et al., 2002).

In 2004, the screening of an Rh cDNA library identified TRIM5α as a factor that confers resistance to HIV-1 infection (Stremlau et al., 2004; **Figures 1** and **2**). Both Rh and CM TRIM5α restrict HIV-1 infection but fail to restrict SIV isolated from a macaque monkey (SIVmac; Stremlau et al., 2004; Nakayama and Shioda, 2010). In contrast, human TRIM5α is almost powerless to restrict the aforementioned viruses, but potently restricts N-tropic murine leukemia viruses (N-MLV) and equine infectious anemia virus (EIAV; Hatziioannou et al., 2004; **Figure 3**).

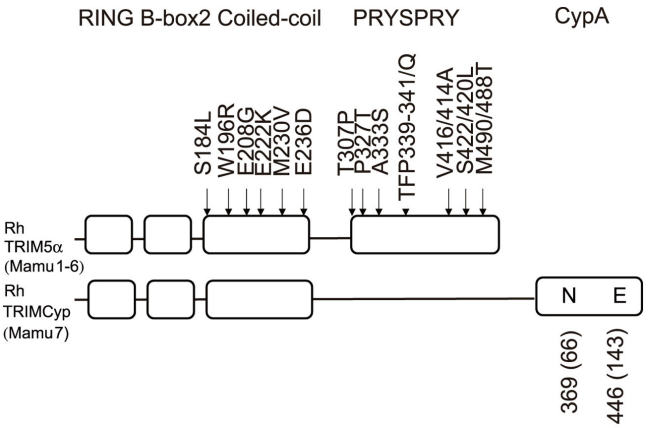

FIGURE 2 | Domains of rhesus monkey (Rh) TRIM5α and TRIMCyp proteins. The RING, B-box2, Coiled-coil, PRYSPRY, or CypA domains of Rh-TRIM5α and TRIMCyp are shown by squares. Polymorphisms are shown outside the squares. The numbers in parentheses show the amino acid positions counting from the initiation methionine codon of the CypA open reading frame.

	TRIM5α mediated viral restriction				
TRIM5α	HIV-1	SIVmac	SIVagm	N-MLV	B-MLV
Human	No	No	No	Yes	No
Rhesus monkey	Yes	No	Yes	Weak	No
Cynomolgus monkey	Yes	No	N.D.	N.D.	N.D.
AGM (tantalus)	Yes	Yes	No	Yes	No
AGM (pygerythrus)	Yes	No	No	Yes	No
Squirrel monkey	No	Yes	Weak	No	No
Owl monkey (TRIMCyp)	Yes	No	N.D.	No	No

FIGURE 3 | Species-specific restriction by TRIM5α. "Yes" denotes restriction. "Weak" denotes weak restriction. "No" denotes no restriction. "N. D." denotes no result has yet been published. SIVmac, simian immunodeficiency virus isolated from a macaque (Ohkura et al., 2006). SIVagm, simian immunodeficiency virus isolated from an African green monkey (Song et al., 2005b). N-MLV, N-tropic murine leukemia virus (Ohkura et al., 2006); B-MLV, B-tropic murine leukemia virus (Ohkura et al., 2006). AGM, African green monkey (Nakayama et al., 2005; Kim et al., 2011). Rhesus monkey (Stremlau et al., 2004; Ylinen et al., 2005; Ohkura et al., 2006), cynomolgus monkey (Nakayama et al., 2005), and owl monkey TRIMCyp (Nisole et al., 2004; Sayah et al., 2004) are also included.

TRIPARTITE MOTIF OF TRIM5α

TRIM5α is a member of the TRIM family of proteins, and consists of RING, B-box 2, coiled-coil, and PRYSPRY (B30.2) domains (Reymond et al., 2001; **Figure 2**). Proteins with the RING domains possess E3 ubiquitin ligase activity (Jackson et al., 2000); therefore, TRIM5α was thought to restrict HIV-1 by proteasome-dependent pathways. However, a proteasome inhibitor MG132 did not rescue HIV-1 infection from TRIM5α-mediated restriction, even though the levels of HIV-1 late reverse-transcription products were recovered (Anderson et al., 2006; Wu et al., 2006; Maegawa et al., 2010). TRIM5α is thus thought to use both proteasome-dependent and -independent pathways to restrict HIV-1. The distinct molecular mechanism of the proteasome-independent pathway has yet to be elucidated. It was shown that incubation of an artificially constructed HIV-1 core structure composed of the capsid–nucleocapsid (CA–NC) fusion protein with the chimeric protein containing the Rh-TRIM5α B-box 2, coiled-coil, and PRYSPRY domains and the RING domain of TRIM21 (TRIM5-21R) caused apparent breaks in the CA structure without any other cellular components (Langelier et al., 2008; Zhao et al., 2011). It is therefore likely that direct binding of Rh-TRIM5α proteins to incoming HIV-1 CA proteins causes CA disassembly, which is observed as proteasome-independent restriction.

The intact B-box 2 domain is also required for TRIM5α-mediated antiviral activity, as TRIM5α restrictive activity is diminished by several amino acid substitutions in the B-box 2 domain (Javanbakht et al., 2005). TRIM5α has been shown to form a dimer (Kar et al., 2008; Langelier et al., 2008), while the B-box 2 domain mediates higher-order self-association of Rh-TRIM5α oligomers (Li and Sodroski, 2008; Diaz-Griffero et al., 2009). The coiled-coil domain of TRIM5α is important for the formation of homo-oligomers (Mische et al., 2005), and the homo-oligomerization of TRIM5α is essential for antiviral activity (Javanbakht et al., 2006; Nakayama et al., 2006).

PRYSPRY DOMAIN OF TRIM5α, A DETERMINANT OF SPECIES-SPECIFIC RESTRICTION OF VIRUSES

The PRYSPRY domain is specific for the α-isoform among at least three splicing variants transcribed from the *TRIM5* gene. Soon after the identification of TRIM5α as a restriction factor of Rh, several studies found that differences in the amino acid sequences of the variable region 1 (V1) of TRIM5α PRYSPRY domain of different monkey species affect the species-specific restriction of retrovirus infection (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005; Ohkura et al., 2006; Perron et al., 2006; Kono et al., 2008, 2009). The PPYSPRY domain is thought to recognize viral cores, as TRIM5α lacking this domain does not show antiviral activity. Overexpression of truncated TRIM5α lacking the PRYSPRY domain shows a dominant negative effect on antiviral activity of full-length TRIM5α (Berthoux et al., 2005; Nakayama et al., 2006). Biochemical studies have shown that TRIM5α associates with CA in detergent-stripped N-MLV virions (Sebastian and Luban, 2005) or with an artificially constituted HIV-1 core structure composed of the CA–NC fusion protein in a PRYSPRY domain-dependent manner (Stremlau et al., 2006). Although the precise three-dimensional crystal structure of the

PRYSPRY domain has not been resolved, TRIM5-21R assembled and formed two-dimensional paracrystalline hexagonal arrays *in vitro* (Ganser-Pornillos et al., 2011). This assembly required RING and B-box 2 domains but was independent of the PRYSPRY domain. However, the hexagonal lattices of HIV-1 CA that mimic the surface of core act as template for stabilization of TRIM5-21R arrays in a PRYSPRY-dependent manner (Ganser-Pornillos et al., 2011). As the interaction between individual CA monomers and TRIM5α is very weak, CA recognition by TRIM5α is thought to be a synergistic combination of direct binding interactions with the PRYSPRY domain, higher-order assembly of TRIM5α, template-based assembly, and lattice complementarity.

VARIABLE SUSCEPTIBILITY OF SIMIAN IMMUNODEFICIENCY VIRUSES AMONG MONKEY SPECIES

Simian immunodeficiency virus isolated from sooty mangabey (SIVsm) and SIV isolated from African green monkey (SIVagm) replicate in their natural hosts (VandeWoude and Apetrei, 2006) and $CD4^+$ human cells. SIVmac evolved from SIVsm in captive macaques, and replicates efficiently in Rh (Shibata et al., 1995; Himathongkham and Luciw, 1996) and CM (Akari et al., 1996, 1999) as well as in human $CD4^+$ cells but not in African green monkey (AGM) cells. We found that a 37-amino acid residue region including a 20-amino acid duplication in the V1 of AGM TRIM5α determined species-specific restriction against SIVmac239 (Nakayama et al., 2005). However, AGM TRIM5α failed to restrict SIVagm, which naturally infects AGM, while Rh-TRIM5α can restrict SIVagm infection (Song et al., 2005b; **Figure 3**).

In contrast to HIV-1, AGM TRIM5α restricted SIVmac239 mainly in a proteasome-dependent manner, as SIVmac239 escaped completely from attacks by RING mutants of TRIM5α that could still moderately restrict HIV-1 infection. Kim et al. reported that AGM TRIM5α derived from *Chlorocebus tantalus* but not *Chlorocebus pygerythrus* subspecies of AGM restrict SIVmac239, while both potently restrict HIV-1 (**Figure 3**). Both AGM TRIM5α share the 20-amino acid duplication but a *C. pygerythrus*-specific leucine residue at the 34th position within the RING domain compromised the ability of *C. pygerythrus* AGM TRIM5α to restrict SIVmac239 infection (Kim et al., 2011). This result is consistent with the observation of RING-proteasome dependency of SIVmac239 restriction by TRIM5α.

Human immunodeficiency virus type 2 (HIV-2) is assumed to have originated from SIVsm as a result of zoonotic events involving monkeys and humans (Hahn et al., 2000). Previous studies have shown that HIV-2 strains vary widely in their ability to grow in cells of OWM, such as baboons, Rh, and CM (Castro et al., 1990, 1991; Locher et al., 1998, 2003; Fujita et al., 2003). By testing CM and Rh recombinant TRIM5α, three amino acid residues of TFP at the 339th to 341st positions of Rh-TRIM5α V1 were shown to be indispensable for restricting particular HIV-2 strains that are still resistant to CM TRIM5α bearing a single Q instead of TFP at the 339th to 341st positions (Kono et al., 2008; **Figure 4**). The TFP motif is also critical to restrict SIVsm (Kirmaier et al., 2010). Baboon and sooty mangabey (SM) TRIM5α bearing SFP at the 339th to 341st positions can potently restrict HIV-1, only weakly restrict HIV-2, and failed to restrict SIVmac239 (Newman et al., 2006; Kono et al., 2008, 2009).

VIRAL DETERMINANT OF SENSITIVITY TO MONKEY TRIM5α

Tripartite motif 5α is thought to recognize viral cores through its PRYSPRY domain. To determine the region in viral CA that interacts with TRIM5α, we focused on HIV-2, which closely resembles SIVmac (Hahn et al., 2000). Sequence analysis showed that the CM TRIM5α-sensitive viruses had proline (P) at the 119th or

		TRIM5α/TRIMCyp allels					
Viruses	Partial capsid sequences	Rh^{TFP}	Rh^{Q}	CM	Rh^{CypA}	$CM^{CypA(NE)}$	$CM^{CypA(DK)}$
HIV2 GH123	GP**LPA**GQLRDP**R**GSDIAGTTSTVEEQIQWMYR**P**	Yes	Yes	Yes	Yes	Yes	No
HIV2 UC1	GP**LPA**GQLRDP**R**GSDIAGTTSTVEEQIQWMYR**A**	Yes	**No**	**No**	Yes	Yes	No
HIV2 UC2	GP**LPA**GQLRDP**R**GSDIAGTTSTVDEQIQWMYR**Q**	Yes	**No**	**No**	Yes	Yes	No
SIVsmE543	GP**LPA**GQLREP**R**GSDIAGTTSTVEEQIQWMYR**Q**	Yes	**No**	**No**	Yes	Yes	N.D.
SIVsmE041	GP**IPA**GQLREP**R**GSDIAGTTSTVEEQIQWMYR**Q**	Yes	**No**	**No**	Yes	Yes	N.D.
SIVmac239	AP**QQ**-GQLREP**S**GSDIAGTTSSVDEQIQWMYR**Q**	**No**	**No**	**No**	**No**	No	No
SIVmac251	AP**QQ**-GQLREP**S**GSDIAGTTSSVDEQIQWMYR**Q**	**No**	**No**	**No**	**No**	No	No
HIV1 NL4-3	GP**IAP**GQMREP**R**GSDIAGTTSTLQEQIGWMT-**H**	Yes	Yes	Yes	No	No	Yes

FIGURE 4 | HIV-2/SIV capsid sequence variations and restriction patterns of rhesus (Rh) and cynomolgus monkey (CM) TRIM5α/TRIMCyp alleles. "Yes" denotes restriction. "Weak" denotes weak restriction. "No" denotes no restriction. "N. D." denotes no result has yet been published. The unique QQ sequence at the 89th–90th positions of SIVmac, which is critical for escape from Rh TRIMCyp, Rh^{CypA} (Kirmaier et al., 2010), is shown in red. Arginine 97 at the base of the loop between helices 4 and 5, which is important to escape from TFP alleles of Rh-TRIM5α, Rh^{TFP} (Kirmaier et al., 2010), is shown in blue. The glutamine and alanine residues at position 120 of GH123 or analogous positions of other HIV-2 strains, which is critical for resistance against Q alleles of Rh-TRIM5α, Rh^{Q} (Kirmaier et al., 2010) and CM TRM5α (Song et al., 2007; Kono et al., 2008), are shown in green. $CM^{CypA(NE)}$ and $CM^{CypA(DK)}$ denote the minor and major alleles of CM TRIMCyp, respectively.

120th position of CA, while the CM TRIM5α-resistant viruses had alanine (A), glutamine (Q), or glycine (G) at the same position (**Figure 4**). Replacing the proline of a CM TRIM5α-sensitive HIV-2 molecular clone with A, Q, or G changed the phenotype from sensitive to resistant and the mutant viruses replicated well in the presence of CM TRIM5α. The reverse was observed when the glutamine of a resistant SIVmac molecular clone was replaced with proline (Song et al., 2007; Miyamoto et al., 2011). The 119th or 120th position is located in a loop between α-helices 6 and 7 (L6/7; **Figure 5**).

In the case of Rh-TRIM5α, Ylinen et al. replaced a loop between α-helices 4 and 5 (L4/5) of SIVmac239 CA with that of HIV-2 in the SIVmac239 background and found that the resultant mutant virus showed impaired growth ability in Rh cells compared with the parental SIVmac239. However, the reciprocal virus with SIVmac239 CA L4/5 in the HIV-2 background did not gain resistance against Rh-TRIM5α, suggesting that Rh-TRIM5α interacts mainly with L4/5 but other portion(s) of HIV-2 CA are also involved (Ylinen et al., 2005). Lin and Emerman (2008) also reported that SIVagm with HIV-1 L4/5 and L6/7 was susceptible to Rh-TRIM5α restriction. In fact, we found that the 120th amino acid of HIV-2 CA, the determinant of CM TRIM5α sensitivity, also contributes to Rh-TRIM5α susceptibility (Kono et al., 2010). Furthermore, studies on chimeric viruses between Rh-TRIM5α-sensitive HIV-2 and Rh-TRIM5α-resistant SIVmac239 revealed that multiple regions including L4/5 in the N-terminal half of SIVmac239 CA contribute to evasion of SIVmac239 from Rh-TRIM5α (Kono et al., 2010; **Figure 5**).

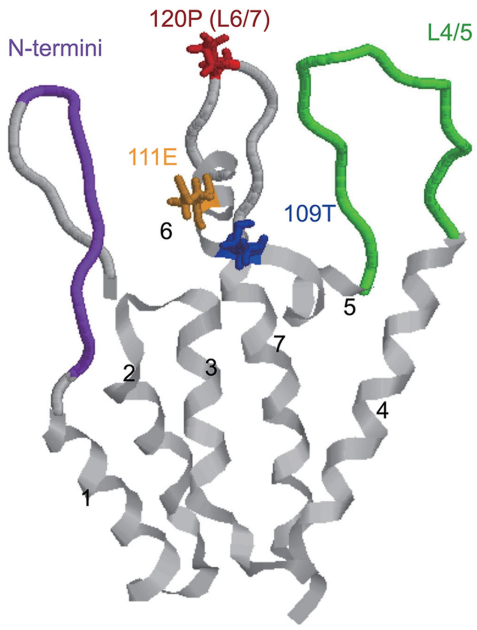

FIGURE 5 | Structure of the N-terminal half of HIV-2 capsid monomer. The ribbons represent the backbone of HIV-2 capsid proteins, and seven α-helices are labeled. The positions important in Rh-TRIM5α recognition are highlighted as N-termini (the 5th to 13th amino acid residues) in purple, the loop between α-helices 4 and 5 (L4/5) in green, the 109th T in blue, the 111th E in orange, and the 120th P in red.

To elucidate further details regarding the structure of CA recognized by TRIM5α, we generated mutant HIV-2 viruses each carrying 1 of 20 amino acid residues at position 120, and examined their susceptibilities to CM TRIM5α-mediated restriction. Amino acid residues with hydrophobic side chains or aromatic rings were associated with sensitivity to CM TRIM5α, while those with small side chains or amide groups conferred resistance (Miyamoto et al., 2011). Computer-assisted three-dimensional models showed that the mutations at the 120th position in L6/7 affected the conformation of the neighboring loop L4/5 by a hydrogen bond between aspartic acid 97 in L4/5 and arginine 119 in L6/7 (**Figure 6**).

Taken together, these observations suggested that TRIM5α recognized the overall outer surface of the N-terminal half of viral CA including L4/5 and L6/7. To determine further details regarding the interaction between CA and TRIM5α, biochemical and structural analyses of the PRYSPRY domain, especially the V1 loop bound with CA, are required. In contrast to SIV/HIV-2, the L4/5 loop of HIV-1 also binds cyclophilin A (CypA). It is not yet clear whether monkey TRIM5α does or does not recognize HIV-1 CA with endogenous CypA.

INTRASPECIES GENETIC VARIATION OF THE Rh-*TRIM5* GENE

The *TRIM5* gene varies considerably among primate species (Sawyer et al., 2005; Song et al., 2005a; Newman et al., 2006). It is not surprising that the PRYSPRY domain is highly variable as TRIM5α interacts with the retroviral core through this region, as described above, and the main pressure for positive selection may be endogenous retroviruses (Kaiser et al., 2007). Interestingly, there is a 339TFP341-to-Q polymorphism in Rh-TRIM5α (Newman et al., 2006; **Figure 2**), which reduces its anti-HIV-2 activity (Kono et al., 2008). Newman et al. (2006) grouped Rh-TRIM5α into six alleles (*Mamu-1* to *-6*) including rare alleles *Mamu-2* and *Mamu-6*. Wilson et al. (2008a) showed that *Mamu-1* and *-3* alleles restrict HIV-1, HIV-2, EIAV, and feline immunodeficiency virus (FIV), but not N-MLV, B-MLV, or SIVmac239, while *Mamu-4* and *-5* alleles restrict HIV-1, EIAV, and FIV but not HIV-2, N-MLV, B-MLV, or SIVmac239 using a TRIM5α-transduced cat cell line (CRFK).

Lim et al. independently reported 11 Rh-TRIM5α alleles in which alleles 1–5 contained 339TFP341. Remaining alleles 6–11 contained 339Q. They established B-lymphoblastoid cell lines (B-LCLs) from Rh and used these B-LCLs for infection with VSV-G pseudo-typed GFP-expressing viruses. They found more GFP-positive cells in B-LCLs with Rh-TRIM5α Q allele than in B-LCLs with Rh TFP allele infected with SIVmac239-, HIV-1-, and SIVsmE543-based GFP-expressing viruses. It should be noted that the anti-HIV-1 activity of the Rh-TRIM5α Q allele is significantly stronger than the anti-SIVmac239 and SIVsmE543 activities of the Rh-TRIM5α TFP allele (Lim et al., 2010b). Lim et al. (2010a,b) retrospectively analyzed plasma viral load of Rh after SIVmac251 challenge by intravenous route and found that Rh with the Q allele was associated with higher levels of plasma viral RNA at the time when the levels of viral RNA stabilized after a period of acute infection (0.6 log median difference at 70 days after infection), more rapid loss of central memory $CD4^+$ T cells, and higher rate of progression to AIDS. These results were consistent with their own *in vitro* observations described above.

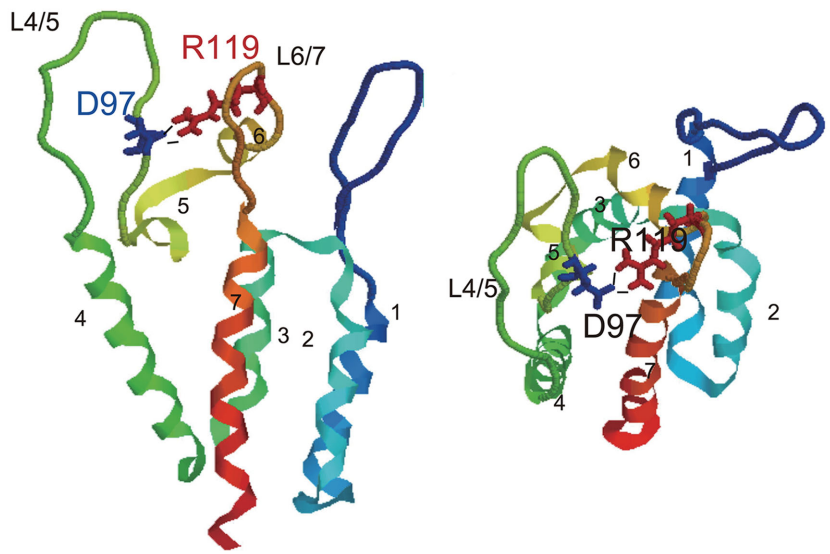

FIGURE 6 | The hydrogen bond between two external loops of HIV-2 capsid. The structures of the N-terminal domain of GH123 are shown, and seven color-coded α-helices are labeled. Blue and red wireframes denote side chains of glutamic acid at the 97th (D97) and arginine at the 119th (R119) positions, respectively. Black lines indicate hydrogen bonds between D97 and R119. Models are shown from two different angles.

On the other hand, Wilson et al. (2008a) failed to detect anti-SIVmac239 activity of both Rh-TRIM5α Q and TFP alleles. Similarly, Kirmaier et al. (2010) detected virtually no anti-SIVmac239 activity in both Rh-TRIM5α TFP and Q alleles (**Figure 4**), although numbers of infected cells in *Mamu-4* (Rh-TRIM5α Q allele) are slightly higher than those in *Mamu-1* (Rh-TRIM5α TFP allele). In contrast, Kirmaier et al. (2010) reported that the Rh-TRIM5α TFP allele restricted SIVsmE543 and SIVsmE041, although the Rh-TRIM5α Q allele did not show any anti-SIVsmE543 or anti-SIVsmE041 activity. SIVmac239 is a molecular clone of a highly adapted, emergent virus of Rh, generated in the 1980s by experimental passage of SIV-positive plasma through several monkeys (Daniel et al., 1985). In contrast, SIVsmE041 is a primary isolate from SM and SIVsmE543 was cloned after experimental passage of SIVsm through two Rh (Hirsch et al., 1997). SIVmac and SIVsm shared Q at the 118th position of CA, corresponding to the 120th position of GH123 (HIV-2), but SIVmac239 and SIVmac251 have an R-to-S change at position 97 at the base of L4/5 of CA that are critical for resistance against Rh-TRIM5α TFP allele (**Figure 4**).

In the case of SIVsmE543 *in vivo*, Rh-TRIM5α$^{TFP/TFP}$ homozygotes markedly diminished viral replication compared to Rh-TRIM5α$^{Q/Q}$ homozygotes at peak (2 log reduction) and 8 weeks (3 log reduction) after intravenous or intrarectal infection, consistent with the *in vitro* results (Kirmaier et al., 2010). It should be noted that the suppression of SIVsmE543 by Rh-TRIM5α TFP is more dramatic than that of SIVmac251. In low-dose repeated mucosal challenge experiments, two groups reported similar results using SIVsmE660, the CA sequence of which closely resembles that of SIVsmE543 (Reynolds et al., 2011; Yeh et al., 2011). Several studies evaluated MHC class I and *TRIM5* genotypes in SIV-infected Rh, and concluded that *TRIM5* genotype independently affected plasma viral load and survival rate after SIV infection (Lim et al., 2010a; Reynolds et al., 2011; Yeh et al., 2011). Taken together, these observations indicate that it is necessary to perform *TRIM5* genotyping of Rh when using SIVsm. It is also better to do so when using SIVmac239 and SIVmac251, although Fenizia et al. (2011) recently reported that there was no difference in SIVmac251 susceptibility among Rh with different TRIM5 genotypes in repeated rectal challenge.

TRIM5 AND CypA FUSION PROTEIN IN NEW WORLD MONKEY

Cells of the NWM, owl monkey (*Aotus trivirgatus*), are resistant to HIV-1 infection. Treatment of owl monkey cells with cyclosporin A, an inhibitor of CypA, allowed HIV-1 infection (Towers et al., 2003). In 2004, soon after the discovery of TRIM5α, analysis of the owl monkey *TRIM5* gene identified a long interspersed nuclear element (LINE)-1-mediated retrotransposition of CypA between exons 7 and 8, resulting in expression of a fusion protein designated as TRIMCyp (Nisole et al., 2004; Sayah et al., 2004). Owl monkey TRIMCyp contained the N-terminal half of TRIM5α, RING, B-box 2, and coiled-coil, but the PRYSPRY domain was replaced with CypA. As the CypA domain of owl monkey TRIMCyp binds to L4/5 of HIV-1 CA, owl monkey TRIMCyp showed similar antiviral activity to TRIM5α (**Figure 3**). The interaction between HIV-1 CA and CypA can be inhibited by cyclosporine A. This is a very interesting example of a gain-of-function by retrotransposition. The owl monkey has been shown to express only TRIMCyp, and not TRIM5α.

TRIMCyp IN OWMs

The expression of TRIMCyp was thought to be an anomaly unique to owl monkeys, but in 2008 another CypA insertion was found in several species of OWMs belonging to the Genus *Macaca*, Rh,

CM, and the pig-tailed monkey (PM; *Macaca nemestrina*; Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008b). It is reasonable to assume that the retrotransposition event occurred in the common ancestor of these three macaques. Insertion of the CypA gene was at the 3′ end of the *TRIM5* gene, which is different from the owl monkey, indicating that CypA retrotransposition into the *TRIM5* gene in OWMs occurred independently from that in owl monkeys. A G-to-T transversion linked with CypA insertion altering the canonical splicing acceptor of *TRIM5* exon 7 caused alternative splicing (Brennan et al., 2008). The resultant mRNA lacks exons 7 and 8, and the PRYSPRY domain is replaced with CypA. In PM, TRIM5α mRNA is absent. Instead, TRIM5 isoforms TRIM5θ and TRIM5η were detected. These isoforms are splicing variants of the TRIMCyp (Brennan et al., 2008). TRIM5θ is truncated at the N-terminus of the PRYSPRY domain and TRIM5η lacks nine amino acid residues encoded by exon 7 (Brennan et al., 2007). PM TRIMCyp restricted HIV-2 but not HIV-1 infection (Liao et al., 2007; Brennan et al., 2008).

In Rh, the allele frequency of TRIMCyp (*Mamu-7*) was 25% in an Indian population but TRIMCyp was completely absent from a Chinese population (Wilson et al., 2008b). Rh TRIMCyp restricted HIV-2 but not HIV-1 infection (Wilson et al., 2008b). In CM, Brennan et al. (2008) initially reported that the amino acid residue at position 357 of CM TRIMCyp, corresponding to position 54 counting from the methionine of CypA, was arginine (R), and CM TRIMCyp with R at this position failed to restrict HIV-1. Subsequently, Ylinen et al. (2010) reported another allele of CM TRIMCyp encoding histidine (H) at this position (*Mafa TRIMCyp2*) and Mafa TRIMCyp2 protein potently restricted HIV-1 but not HIV-2. Recently, Dietrich et al. (2011) examined 15 CMs from Indonesia, Indochina, Mauritius, and the Philippines carrying TRIMCyp, and did not find R at this position. We also examined 64 CMs from Malaysia, the Philippines, and Indonesia carrying TRIMCyp (34 heterozygotes and 30 homozygotes for TRIMCyp), and found that none of these 94 TRIMCyp genes carried R at this position (Saito et al., 2011a). On the other hand, both Dietrich et al. and our group found that TRIMCyp frequency in CM was apparently higher than that in Rh. TRIMCyp frequency tended to be higher in eastern Asia than in western Asia. Dietrich et al. and our group also found major and minor haplotypes of CM TRIMCyp with single nucleotide polymorphisms in the CypA domain. The major haplotype of CM TRIMCyp bears aspartic acid (D) and lysine (K) at positions 369 and 446, respectively (Brennan et al., 2008; Ylinen et al., 2010). The minor haplotype encodes asparagine (N) and glutamic acid (E) at positions 369 and 446, respectively (Dietrich et al., 2011; Saito et al., 2011a). N369 and E446 were also found in PM and Rh TRIMCyps, and the CypA portion of the NE haplotype of CM TRIMCyp has the same amino acid sequence as that of Rh TRIMCyp. The major CM haplotype of the TRIMCyp suppressed HIV-1 but not HIV-2, while the minor haplotype of TRIMCyp suppressed HIV-2 but not HIV-1 as PM and Rh TRIMCyp did (Saito et al., 2011a; **Figure 4**).

The original CypA sequence retrotransposed into the macaque *TRIM5* locus must have been the authentic macaque CypA. There are two or three amino acid differences between authentic CypA and the CypA portion of TRIMCyp in Rh, CM, and PM, and TRIMCyp with the authentic CypA sequence has been shown to restrict HIV-1 but to only weakly restrict HIV-2 (Virgen et al., 2008; Price et al., 2009). TRIMCyp from all three of these OWM species share H at the 372nd position, corresponding to the 69th position of CypA where the authentic macaque CypA has R. Rh and PM TRIMCyps and the minor haplotype of CM TRIMCyp share N at the 369th position (the 66th position in CypA), where the authentic CypA and major haplotypes of CM TRIMCyp (*Mafa TRIMCyp2*) have D. Structural analysis of CypA domain revealed that these mutations caused drastic changes in configuration of the active site loop (from the 64th amino acid residue to the 74th residue in CypA) in Rh TRIMCyp, leading to a decreased binding affinity to HIV-1 CA but an increased affinity to HIV-2 CA (Price et al., 2009). Therefore, these mutations enhanced antiviral activity of TRIMCyp against HIV-2 but diminished anti-HIV-1 activity (Price et al., 2009). In the case of the major haplotype of CM TRIMCyp, an additional E-to-K change at the 446th position (the 143rd position in CypA) decreased affinity to HIV-2 CA by its positive charge (Ylinen et al., 2010), and the D at the 369th position (the 66th position in CypA) supported its anti-HIV-1 activity.

How did these interspecies and intraspecies variations occur in TRIMCyp? It is reasonable to assume that the R-to-H mutation at the 372nd position (R69H) together with the D-to-N mutation at the 369th position (D66N), which enhanced antiviral activity of TRIMCyp against HIV-2 but diminished anti-HIV-1 activity (Price et al., 2009), arose early in a macaque common ancestor. After the separation of CM from other species, an additional E-to-K change at the 446th position (E143K) and the N-to-D reversion at the 369th position (N66D) may also have occurred in the major haplotype of CM TRIMCyp. Alternatively, polymorphisms at the 369th and 446th positions may have arisen early in a macaque common ancestor but only CM could transmit these polymorphisms until the present. As described above, CM TRIM5α has Q at amino acid position 339 (Nakayama et al., 2005), where Rh-TRIM5α has a Q-to-TFP polymorphism (Newman et al., 2006; **Figure 2**). This Q-to-TFP polymorphism in the PRYSPRY domain also altered the spectrum of anti-lentiviral activity of TRIM5α (Kono et al., 2008; Wilson et al., 2008a; Kirmaier et al., 2010; Lim et al., 2010b; **Figure 4**). Therefore, it is tempting to speculate that the selection pressure in CM drove amplification and diversification in TRIMCyp, while that in Rh drove diversification of the PRYSPRY domain of TRIM5α. It will be of interest to examine what retroviruses have driven the evolution of TRIMCyp and *TRIM5* genes (**Figure 7**).

With respect to SIV infection, Rh TRIMCyp failed to restrict SIVmac239 (Brennan et al., 2008; Wilson et al., 2008b; Kirmaier et al., 2010) but could restrict SIVsm (Kirmaier et al., 2010). The unique LPA-to-QQ change at positions 89–91 in L4/5 of SIVmac was critical for escape from Rh TRIMCyp (**Figure 4**). Rh heterozygous for the TFP allele of TRIM5α and TRIMCyp suppressed viral infection of both SIVsmE543 (Kirmaier et al., 2010) and SIVsm660 (Reynolds et al., 2011) more efficiently than Rh homozygous for either TRIMCyp or TRIM5α. It is possible that Rh heterozygous for the TFP allele of TRIM5α and TRIMCyp express two different molecules that bind distinct regions of CA and eliminate incoming virus more effectively than Rh with TRIM5 molecules targeting only one region of CA.

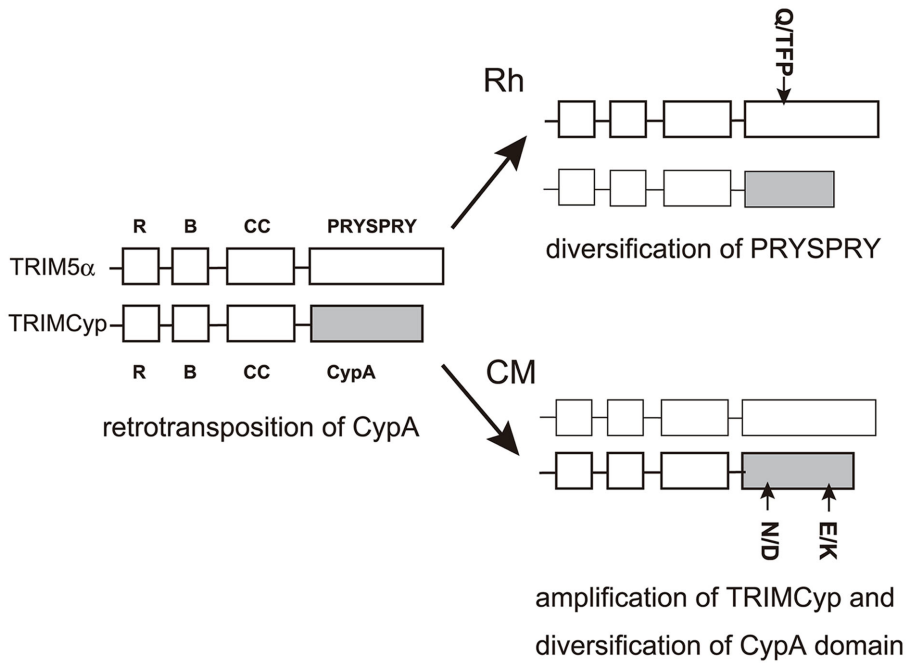

FIGURE 7 | Diversity of Rh and CM *TRIM5* genes. The RING, B-box2, Coiled-coil, and PRYSPRY domains of TRIM5α and TRIMCyp are shown by squares. CypA domains in TRIMCyp are shown as filled squares. Major alleles in Rh and CM are shown in bold lines. Polymorphisms are shown outside the squares.

OTHER RESTRICTION FACTORS AND DEVELOPMENT OF MONKEY-TROPIC HIV-1

To establish a monkey model for the study of HIV-1/AIDS, Kamada et al. (2006) developed an HIV-1 strain with minimal segments of SIVmac239. This virus (NL-ScaVR and DT5R) contains the L4/5 of CA and the entire *vif* segment of SIVmac239, and was designed to escape restriction mediated by ApoB mRNA editing catalytic subunit (APOBEC) 3G and CypA in OWM cells. APOBEC3G modifies the minus strand viral DNA during reverse transcription, resulting in impairment of viral replication (Sheehy et al., 2002; Harris et al., 2003; Mangeat et al., 2003), but this activity could be counteracted by the viral Vif protein (Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003). Although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against Rh APOBEC3G, which at least partly explains the restriction of HIV-1 replication in monkey cells. CypA binds directly to L4/5 of HIV-1 CA but not to SIVmac CA and augments HIV-1 infection in human cells but inhibits its replication in OWM cells (Kootstra et al., 2003; Berthoux et al., 2004; Nakayama et al., 2008). Although DT5R could replicate in PM primary $CD4^+$ T cells as well as in the CM T cell line HSC-F but not in Rh cells (Kamada et al., 2006), inoculation of this monkey-tropic HIV-1 (HIV-1mt) into PM did not cause $CD4^+$ T cell depletion or any clinical symptoms (Igarashi et al., 2007), probably due to inefficient viral growth in monkeys. In the case of CM, replacement of L6/7 of HIV-1 with that of SIVmac239 greatly enhanced viral replication in PBMC (Kuroishi et al., 2009, 2010) and in animals (Saito et al., 2011b). However, the virus could not escape completely from CM TRIM5α (Kuroishi et al., 2010). Another HIV-1mt carrying 202 amino acid residues of SIVmac239 CA and *vif*, generated by Hatziioannou et al. (2006), could replicate efficiently in Rh cells, confirming that the N-terminal half of CA is required to be that of SIVmac to escape from Rh-TRIM5α. Unfortunately, this virus has replaced nearly all of CA sequence with that of SIVmac239 and has lost important CTL epitopes of HIV-1, and thus further improvement is required to use Rh as an HIV-1 infection model. H87Q and/or V86M mutations induced by adaptation of HIV-1 to the cells expressing Rh-TRIM5α (Pacheco et al., 2010) would be useful. In contrast, lack of functional TRIM5α expression in PM enabled Hatziioannou et al. (2009) to construct an HIV-1mt strain that differs from HIV-1 only in the *vif* gene and can efficiently replicate in PM. This is the most promising HIV-1/monkey model at present, if PMs are available in sufficient numbers for research.

Other host factors capable of suppressing HIV-1 replication were recently identified (**Figure 1**). One is tetherin (also know as BST2 or CD317; Neil et al., 2008; Van Damme et al., 2008). BST2 is an interferon-inducible membrane protein that interferes with the detachment of virus particles from infected cells. HIV-1 overcomes this restriction by expressing an accessory protein, Vpu, which counteracts BST2. BST2 restriction is also counteracted by primate lentiviruses that do not express a Vpu protein. Anti-BST2 functions are provided by the Env protein in HIV-2 and SIVtan (Gupta et al., 2009) or the Nef protein in SIVsm/mac and SIVagm (Jia et al., 2009; Zhang et al., 2009). As chimeric virus containing the tat, rev, vpu, and env of the HXB2 strain of HIV-1 in the genetic background of SIVmac239 is pathogenic in Rh and PM (Joag et al., 1996), BST2 in monkeys can be canceled by HIV-1 Vpu. Another recently identified host factor is SAMHD1 as dendritic and myeloid-cell-specific

HIV-1 restriction factor counteracted by HIV-2/SIV Vpx (Laguette et al., 2011; Yeh et al., 2011). As HIV-1 lacks Vpx, it is necessary to elucidate whether monkey SAMHD1 restricts HIV-1.

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SIV replication in human cells

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Current human immunodeficiency virus type 1 pandemic is believed to originate from cross-species transmission of simian immunodeficiency virus (SIV) into human population. Such cross-species transmission, however, is not efficient in general, because viral replication is modulated by host cell factors, with the species-specificity of these factors affecting viral tropism. An understanding of those host cell factors that affect viral replication contributes to elucidation of the mechanism for determination of viral tropism. This review will focus an anti-viral effect of ApoB mRNA editing catalytic subunit, tripartite motif protein 5 alpha, and cyclophilins on SIV replication and provide insight into the mechanism of species-specific barriers against viral infection in human cells. It will then present our current understanding of the mechanism that may explain zoonotic transmission of retroviruses.

Keywords: HIV-1, SIV, APOBEC3G, TRIM5α, cyclophilin A, cyclophilin B

INTRODUCTION

There is significant evidence that the ongoing worldwide acquired immunodeficiency syndrome (AIDS) epidemic was caused by cross-species transmission of simian immunodeficiency viruses (SIVs) into the human population. Replication of primate lentiviruses in their natural hosts is generally non-pathogenic; however, cross-species transmission of these viruses can result in highly pathogenic phenotypes. How and when this transmission occurred is still debated but it is now generally accepted that HIV-2 originated from a sooty mangabeys strain of SIV (SIVsm; Hirsch et al., 1989; Chen et al., 1996) while HIV-1 appears to have originated from a chimpanzee strain of SIV (SIVcpz; Gao et al., 1999). Zoonotic transmission of SIVs, however, is not common and is controlled by host factors that generally prohibit SIV replication in human hosts and many human-derived cell lines.

Viral replication is modulated by host cell factors, with the species-specificity of these factors affecting viral tropism. Some of these host factors can restrict viral replication and the anti-viral systems mediated by such host restriction factors, termed intrinsic immunity, play an important role in determining species-specific barriers against viral infection. For instance, Fv-1 in mice is known to restrict replication of a murine leukemia virus (Rein et al., 1976; Gautsch et al., 1978; Towers et al., 2000) and tripartite interaction motif 5α (TRIM5α) recently has been found to be responsible for restricting HIV-1 but not SIV infection in Old World monkey (OWM) cells (Hatziioannou et al., 2004b; Keckesova et al., 2004; Stremlau et al., 2004; Yap et al., 2004; Song et al., 2005; Ylinen et al., 2005). Restriction of retroviral replication by these host cell factors takes place after viral entry, but before the integration step, and the viral determinants for this type of restriction have been mapped to the capsid (CA) protein (Gautsch et al., 1978; Kozak and Chakraborti, 1996; Towers et al., 2000; Goff, 2004; Stremlau et al., 2006). Two recent studies showed that the cellular protein SAMHD1 is myeloid-lineage cell-specific HIV-1 restriction factor counteracted by Vpx proteins from HIV-2 and SIVsm (Hrecka et al., 2011; Laguette et al., 2011). Restriction of lentivirus infection by SAMHD1 is likely to take place at the reverse transcription step. Another anti-retroviral protein, tetherin (also referred to as BST-2, CD317, or HM1.24) inhibits retrovirus release and is antagonized by HIV-1 Vpu protein, Nef protein of many SIVs, or Env protein of HIV-2 (Neil et al., 2008; Le Tortorec and Neil, 2009; Zhang et al., 2009). Understanding how host cell factors affect viral replication, positively or negatively, would contribute to elucidating the molecular mechanism that determines viral tropism. Here, we discuss an anti-viral effect of ApoB mRNA editing catalytic subunit (APOBEC), TRIM5α, and cyclophilins (Cyps) on SIV replication.

APOBEC: ENZYMATIC RESTRICTION FACTOR THAT TARGET RETROVIRUSES

Replication of HIV-1 in primary CD4+ T cells, monocyte, and some immortalized T cell lines depends on the presence of the HIV-1 accessory gene product, Vif (standing for virus infectivity factor; Fisher et al., 1987; Strebel et al., 1987), and it works in a host cell-specific manner. Vif is required for enhanced HIV-1 replication in some cell types called non-permissive cells. In contrast, HIV-1 replication is Vif-independent in permissive cells (Akari et al., 1992; Fan and Peden, 1992; Gabuzda et al., 1992; Blanc et al., 1993; Sakai et al., 1993; von Schwedler et al., 1993; Borman et al., 1995). Recently, some cytidine deaminases were identified as a new class of host restriction factors that target retroviruses such as HIV-1 or SIV (Harris and Liddament, 2004; Cullen, 2006). APOBEC3G (Apo3G), a member of the APOBEC family of cytidine deaminases, is the first identified enzymatic restriction factor and the determinant that makes cells permissive or non-permissive. Apo3G is also a host factor that restricts replication of human and simian lentiviruses in their respective target cells. Unlike TRIM5α or Fv-1, Apo3G does not exert its anti-viral activity by targeting the viral CA protein, but it has to be incorporated

into a newly synthesized virion during a production step, and then inhibits virus replication by targeting single-stranded viral cDNA during a subsequent infection step. HIV-1 counteracts Apo3G with Vif expression. During the production of progeny virions, Vif binds to Apo3G and induces Apo3G's proteasomal degradation, resulting in the decreased steady-state levels of human Apo3G (hApo3G; Yu et al., 2003).

There are several anti-retroviral mechanisms of Apo3G against HIV-1 infection. First, Apo3G-containing virus can accumulate in a large number of substitutions that register as cytidine (C) to deoxyuridine (dU) in a virus minus-strand during reverse transcription, resulting guanine (G) to adenine (A) mutations in a viral plus-strand, known as "G-to-A hypermutation" (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003; Yu et al., 2004b). Second, Apo3G can inhibit tRNA annealing or tRNA processing during reverse transcription (Guo et al., 2006, 2007; Mbisa et al., 2007). Third, Apo3G inhibits DNA strand transfer or integration (Li et al., 2007; Luo et al., 2007; Mbisa et al., 2007). Although Apo3G has the most potent anti-HIV-1 activity among the APOBEC family of proteins, another member of the family, APOBEC3F (Apo3F) was shown to inhibit HIV-1 infection in the absence of Vif (Bishop et al., 2004a; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004), whereas APOBEC3B (Apo3B) can inhibit HIV-1 infection in both the presence and absence of Vif (Bishop et al., 2004a; Doehle et al., 2005; Rose et al., 2005).

Although we can imagine the broad range of anti-retroviral activity of APOBEC family because APOBEC proteins from non-human species can also inhibit HIV-1 infection (Mariani et al., 2003; Bishop et al., 2004a,b; Wiegand et al., 2004; Cullen, 2006), the Vif-Apo3G interaction is thought to be species-specific (Simon et al., 1998; Mariani et al., 2003). Accordingly, hApo3G is insensitive to SIVagm Vif while African green monkey Apo3G (agmApo3G) is insensitive to HIV-1 Vif and the determinant of this species-specificity depends on amino acid 128 of hApo3G and agmApo3G (Mariani et al., 2003; Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004).

However, such species-specificity is not strictly controlled, for example, a report from the laboratory of Klaus Strebel demonstrated that SIVagm Vif supported replication of SIVagm virus in the hApo3G-positive human A3.01 T cell line (Takeuchi et al., 2005). Replication of *vif*-defective SIVagm in A3.01 cells was severely restricted, resulted in an accumulation of cytidine deaminase-induced G-to-A mutations in SIVagm genome (Takeuchi et al., 2005).

Moreover, two independent groups showed that the different APOBEC3 family members function to neutralize specific lentiviruses (Yu et al., 2004a; Dang et al., 2006). One report from the lab of Dr. Nathaniel R. Landau showed that APOBEC3B and APOBEC3C were potent inhibitors of SIV (Yu et al., 2004a). Both enzymes were efficiently encapsidated by HIV-1 and SIV. Another report from the lab of Dr. Yong-Hui Zheng demonstrated that APOBEC3DE blocked the replication of both HIV-1 and SIV but not that of MLV (Dang et al., 2006) and APOBEC3H inhibited the replication of HIV-1 by a cytidine deamination-independent mechanism (Dang et al., 2008). These findings raise the possibility that the various APOBEC3 family members protect against different lentiviruses and point to a possible role in the zoonotic transmission of SIV.

TRIM5α: FV-1-TYPE HOST FACTOR RESTRICTING HIV-1 IN PRIMATE CELLS

The host protein which dictates Ref1 activity was identified as an α-isoform of rhesus macaque TRIM5 protein by the laboratory of Dr. Joseph Sodroski (Stremlau et al., 2004). TRIM5 is a member of the TRIM family of proteins, and has RING, B-box 2, and coiled-coil as common and conserved domains among the family and B30.2 (PRYSPRY) domain on its C-terminal region (Nisole et al., 2005). Subsequently, the human and non-human primate homologs of TRIM5α were shown to restrict retroviruses, such as N-MLV, and equine infectious anemia virus (Hatziioannou et al., 2004b; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004; Song et al., 2005; Ylinen et al., 2005; Si et al., 2006). Rhesus monkey TRIM5α (rhTRIM5α) has strong anti-HIV-1 activity but only modestly restricts SIV isolated from a macaque monkey (SIVmac) and does not block MLV infection, whereas its human homolog does not restrict HIV-1 infection.

TRIM5α recognizes incoming viral cores, but not a monomeric CA protein, thorough its B30.2 (PRYSPRY) domain. B-box 2 and coiled-coil domains are required for TRIM5α multimerization, and both coiled-coil and B30.2 (PRYSPRY) domains are essential for viral core binding (Reymond et al., 2001; Stremlau et al., 2006). TRIM5α captures HIV-1 core at a very early step(s) after infection, immediately after the release of the core into cytoplasm. To restrict HIV-1 infection and to recognize viral core, TRIM5α must oligomerize through its B-box 2 and coiled-coil domains (Mische et al., 2005; Li and Sodroski, 2008). Its RING domain has E3 ubiquitin ligase activity. It self-ubiquitination occurs TRIM5α is quickly degraded (Diaz-Griffero et al., 2006). This rapid degradation of TRIM5α is not required for post-entry restriction since replacement of TRIM5α RING domain with the corresponding domain of TRIM21, which has lower self-ubiquitination activity and a longer half-life than TRIM5α did not alter the anti-viral activity (Kar et al., 2008). Recently, the laboratory of Dr. Mark Yeager discussed a novel architecture made with dimers of TRIM5-21R. TRIM5α-21R forms a dimer through its B-box 2 and coiled-coil domains, and these dimers form six-sided rings on CA lattices to promote rapid core disassembly (Ganser-Pornillos et al., 2011). Overexpression of TRIM5α leads to the formation of cytoplasmic bodies and is believed to be required for its anti-viral activity (Stremlau et al., 2006; Campbell et al., 2008). During TRIM5α-mediated post-entry restriction, disassembly of viral cores is induced too quickly and the accumulation of viral RT-products is reduced (Stremlau et al., 2006). On the other hand, MG132 treatment inhibited quick-disassembly, yet HIV-1 infectivity was still restricted. Two reports showed that TRIM5α could block not only viral cDNA accumulation but also the nuclear import of viral cDNA (Berthoux et al., 2004; Wu et al., 2006). Thus, TRIM5α-mediated post-entry restriction is thought to have at least two phases: (i) TRIM5α induces rapid disassembly of viral core in a proteasome-dependent manner and (ii) TRIM5α degrades HIV-1 cDNAs in a proteasome-independent manner. The determinant of specificity and magnitude of the post-entry

restriction lies on B30.2 (PRYSPRY) domain. Previous report showed that TRIM5α alleles did not cluster by species between rhesus macaques and sooty mangabeys and none of the alleles from either species restricted SIV, suggesting that there is little effect of rhTRIM5α on transmission of SIVsm within species (Newman et al., 2006). Recently, Pacheco et al. (2010) reported that New World monkey (NWM) TRIM5α restricts foamy virus infection. Another consideration is the clinical significance of TRIM5α against AIDS in human. Moreover, several reports showed that the efficacy of TRIM5α-mediated suppression of HIV-1 replication might interfere with disease progression of AIDS in humans (van Manen et al., 2008; Cagliani et al., 2010; Takeuchi et al., 2012). Thus, TRIM5α-mediated restriction may be a multi-step process in retrovirus replication with the relationship between other host factor(s).

Recently, the lab of Dr. Yasuhiro Ikeda reported that rhesus macaque TRIM5α also inhibits HIV-1 production by inducing the degradation of a viral precursor Gag protein (Sakuma et al., 2007). To restrict HIV-1 production, amino acid residues in B-box 2 and coiled-coil domains dictated the specificity of the restriction. In the late restriction, the accumulation of HIV-1 RNA was not affected but the accumulation of precursor Gag was inhibited in an ubiquitin–proteasome-independent manner. This TRIM5α-mediated late-restriction is still controversial (Zhang et al., 2008), yet it is conceivable that TRIM5α restricts HIV-1 infection and production in two distinct mechanisms. Although TRIM5α restricts HIV-1 infection in a broad range of cells, its late restriction involved transient overexpression (Sakuma et al., 2007).

Here is another notable class of the TRIM family called TRIM-Cyp isolated from NWM. A report from the laboratory of Dr. Jeremy Luban demonstrated that owl monkey cells express TRIM-Cyp that restricts HIV-1 infection (Sayah et al., 2004). Although TRIM-Cyp has a cyclophilin A (CypA) sequence in its C-terminal region instead of B30.2 (PRYSPRY) domain that dictates the specificity and the magnitude of post-entry restriction in OWM TRIM5α-mediated post-entry restriction, it recognizes incoming core structure and restricts HIV-1 infection (Stremlau et al., 2006). Recently, TRIM-Cyp mRNA was also detected in a rhesus macaque cell, and overexpressed rhesus TRIM-Cyp restricts HIV-1 infection and production (Newman et al., 2006; Brennan et al., 2008; Wilson et al., 2008; Dietrich et al., 2010).

Unlike other restriction factors, there is no known accessory gene product of HIV-1 to antagonize TRIM5α-mediated restrictions. Indeed, human TRIM5α has only a modest restriction activity against HIV-1 infection. TRIM5 proteins from several NWM species restrict infection by SIVmac and SIVagm (Song et al., 2005). This suggests that TRIM5α could be a key molecule of the species-species barrier.

CYCLOPHILINS: HOST FACTORS INVOLVED IN RETROVIRUS REPLICATION

Cyclophilins are ubiquitous proteins and first identified as the target of cyclosporine A (CsA), an immunosuppressive reagent (Takahashi et al., 1989). CypA has proline-isomerase activity that catalyzes the *cis–trans* isomerization of proline residue (Fischer et al., 1989). The binding of CsA to CypA inhibits this isomerase activity (Takahashi et al., 1989). In retrovirus replication, CypA was found to bind HIV-1 CA in the yeast two-hybrid system (Luban et al., 1993). The sequence Ala88-Gly89-Pro90-Ile91 of CA protein is the major fragment bound to the active site of CypA (Franke et al., 1994; Gamble et al., 1996; Zhao et al., 1997). Interestingly, The peptidyl-prolyl bond between Gly89 and Pro90 of the CA fragment has a *trans* conformation, in contrast to the *cis* conformation observed in other known CypA–peptide complexes (Zhao et al., 1997; Bosco et al., 2002), and Gly89 preceding Pro90 has an unfavorable backbone formation usually only adopted by glycine, suggesting that special Gly89-Pro90 sequence but not other Gly-Pro motif is required for the binding of CA protein to CypA. Therefore, CypA might be likely to act as a molecular chaperone but not a *cis–trans* isomerase (Zhao et al., 1997). However, one report showed that CypA does not only bind CA protein but also catalyzes efficiently *cis–trans* isomerization of Gly89-Pro90 peptidyl-prolyl bond (Bosco et al., 2002). The relationship between the Gly89-Pro90 bond and catalysis of *cis–trans* isomerization by CypA remains unclear.

It has been well established that CypA promotes an early step of HIV-1 infection in human cells (Franke et al., 1994; Thali et al., 1994; Braaten et al., 1996a,c; Franke and Luban, 1996; Braaten and Luban, 2001; Sokolskaja et al., 2004; Hatziioannou et al., 2005). CypA is efficiently encapsidated into HIV-1 produced from infected cells through interaction with the CA domains of the Gag polyprotein and disruption of CypA incorporation into virions by CsA or HIV-1 Gag mutants caused a decrease in replication efficiency (Franke et al., 1994; Thali et al., 1994; Ott et al., 1995; Braaten et al., 1996a; Bukovsky et al., 1997; Ackerson et al., 1998; Braaten and Luban, 2001). It is still unclear how CypA is efficiently packaged into HIV-1 virion, but several reports showed that both dimerization of CA and multimerization of CypA are required for efficient interaction (Colgan et al., 1996; Javanbakht et al., 2007). Although CA-CypA interaction is required for infectivity, the important point is that CypA interacts with incoming HIV-1 cores in newly infected target cells rather than during HIV-1 budding from the virion producer cells, indicating that target cell CypA promotes HIV-1 infectivity (Kootstra et al., 2003; Towers et al., 2003; Sokolskaja et al., 2004).

CypA-dependent virus replication is only limited to retroviruses which encode CA that binds CypA. In fact, only those retroviruses are dependent upon CypA for replication (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994; Braaten et al., 1996c; Franke and Luban, 1996). These observations suggested that CA–CypA interaction might contribute tropism determinants for retroviruses. HIV-1 infection in non-human primate cells is blocked prior to reverse transcription after virus entry (Shibata et al., 1995; Himathongkham and Luciw, 1996; Hofmann et al., 1999; Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002; Hatziioannou et al., 2003; Towers et al., 2003). This restriction is thought to be the same step in the retrovirus life cycle where CypA works (Braaten et al., 1996b). Indeed, analysis of CypA-binding region of CA with chimeric viruses of HIV-1 and SIV showed the viral determinant for species-specificity (Shibata et al., 1991, 1995; Dorfman and Gottlinger, 1996; Bukovsky et al., 1997;

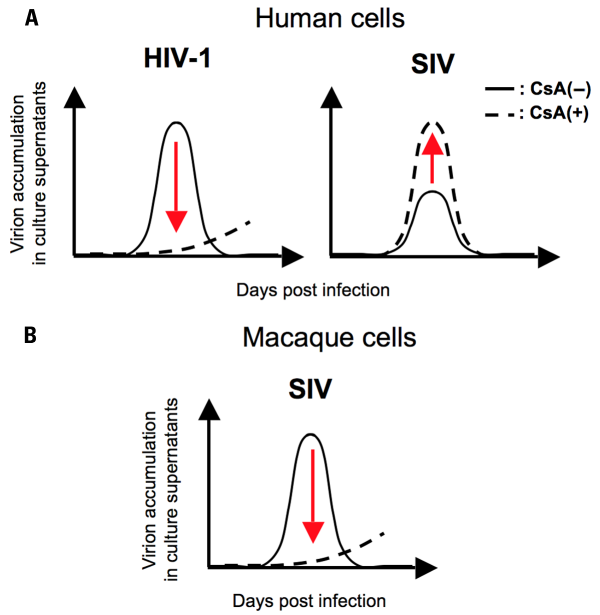

FIGURE 1 | A schema for the effect of CsA on HIV/SIV replication in human/macaque cells. (A) CsA treatment impairs the replication of HIV-1 (left panel) but enhances SIV replication (right panel) in human cells. **(B)** CsA treatment inhibits SIV replication in macaque cells. The solid line indicates virion accumulation of culture supernatant in the absence of CsA and the broken line indicates that of culture supernatant in the presence of CsA.

Cowan et al., 2002; Kootstra et al., 2003; Owens et al., 2003, 2004; Towers et al., 2003; Berthoux et al., 2004; Hatziioannou et al., 2004a, 2006; Ikeda et al., 2004; Sayah et al., 2004; Stremlau et al., 2004; Kamada et al., 2006).

Human CypA is required for efficient HIV-1 infection but not SIV. There is no known role for CypA in SIV infection in human cells. Recently, the first report from the laboratory of Klaus Strebel showed that human CypA acts as restriction factor against the infection of two SIVs (SIVmac and SIVagm) in human cells, and Vif protein of two SIVs counteracts a CypA-imposed inhibition against the infection of two SIV strains with exclusion of CypA from SIV virion (Takeuchi et al., 2007). This phenomenon is different from the function of SIVagm Vif against hApo3G previously reported from the same laboratory (Takeuchi et al., 2005) because they used human cells lacking detectable deaminase activity.

Moreover, a recent report showed a species-specific effect of CsA, a peptidyl-prolyl *cis–trans* isomerase (PPIase) inhibitor, on SIV replication, implying a possible contribution of Cyps to the determination of SIV tropism (**Figure 1**; Takeuchi et al., 2012). They demonstrated a host species-specific effect of CypA on SIV replication: CypA affects the replication of two SIVs (SIVmac and SIVagm) negatively in human cells but positively in macaque cells (**Figure 1**). Further analysis indicated that the infection of two SIVs was not significantly affected by CypA but inhibited by cyclophilin B (CypB), another PPIase, in human cells (**Figure 2A**; Takeuchi et al., 2012). In contrast, CypA is likely to have positive

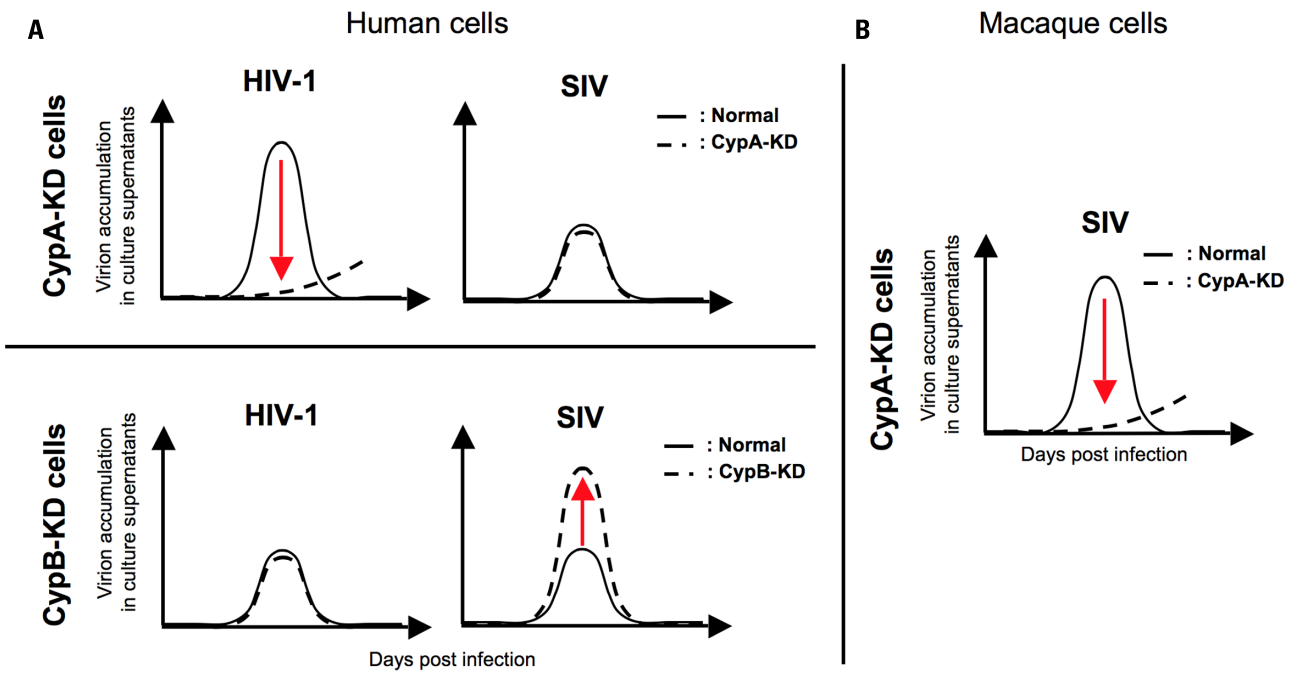

FIGURE 2 | A schema for the effect of cyclophilin A and cyclophilin B on HIV/SIV replication in human/macaque cells. (A) CypA knock-down (CypA-KD) impairs the replication of HIV-1 (upper left panel). In contrast, SIV replication is not reduced but rather enhanced by CypA knock-down (upper right panel). CypB knock-down (CypB-KD) shows no significant effect on HIV-1 replication (lower left panel) but enhances the replication of SIV (lower right panel). **(B)** CypA-KD inhibits SIV infection. The solid line indicates virion accumulation of culture supernatant produced from normal cells and the broken line indicates that of culture supernatant produced from CypA or CypB knock-down cells.

effects on the infection of two SIVs in macaque cells (**Figure 2B**; Takeuchi et al., 2012). These results suggest that Cyps might have a host species-specific effect of Cyps on SIV replication and provide insight into the mechanism of species-specific barriers against viral infection.

CONCLUDING REMARKS

Viral replication is modulated by host cell factors. Many of these factors function in a species-specific manner. On the other hand, there exist host factors that restrict viral replication. The antiviral system mediated by some of these restriction factors, termed intrinsic immunity, which is distinguished from the conventional innate and adaptive immunity has been indicated to play an important role in making species-specific barriers against viral infection. As discussed in this review, we describe the current progress in understanding of such restriction factors against retroviral replication, especially focusing on TRIM5α and APOBEC whose anti-retroviral effects have recently been recognized. Additionally, we mentioned a host species-specific effect of Cyps including CypA and CypB on SIV replication. Such restriction factors would play an important role in determining species-specific barriers against viral infection.

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Species tropism of HIV-1 modulated by viral accessory proteins

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Human immunodeficiency virus type 1 (HIV-1) is tropic and pathogenic only for humans, and does not replicate in macaque monkeys routinely used for experimental infections. This specially narrow host range (species tropism) has impeded much the progress of HIV-1/acquired immunodeficiency syndrome (AIDS) basic research. Extensive studies on the underlying mechanism have revealed that Vif, one of viral accessory proteins, is critical for the HIV-1 species tropism in addition to Gag-capsid protein. Another auxiliary protein Vpu also has been demonstrated to affect this HIV-1 property. In this review, we focus on functional interactions of these HIV-1 proteins and species specific-restriction factors. In addition, we describe an evolutional viewpoint that is relevant to the species tropism of HIV-1 controlled by the accessory proteins.

Keywords: HIV-1, species tropism, accessory protein, Vif, Vpu

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is strictly adapted to humans, and cause disease-inducing persistent infection only in humans (Nomaguchi et al., 2008). This property is unique among primate immunodeficiency viruses, and represent one of the most evident and important viral characteristics to understand the biology/molecular biology of HIV-1. Of numerous primate immunodeficiency viruses so far identified (Kirchhoff, 2009; Sharp and Hahn, 2011), HIV-1 with an extremely limited host range exhibits exceptionally high replication ability, transmissibility, and pathogenicity in sensitive host humans. For basic HIV-1 researchers, it would be final goal to realize the basis/mechanism underlying these properties by experimental approaches.

Primate immunodeficiency viruses can be divided into three groups based on their genome structure in the central regions (Kirchhoff, 2009; Fujita et al., 2010; Sharp and Hahn, 2011). While viruses of HIV-1 type contain *vpr* and *vpu* genes, viruses of HIV-2 type carry *vpx* and *vpr* genes in tandem (**Figure 1**). The other simian immunodeficiency viruses (SIVs), the prototype virus, have only the *vpr* gene in the corresponding genomic region. HIV-1 is believed to emerge from the prototype virus via SIVmon/mus/gsn (isolated from the mona, mustached, and greater spot-nosed monkeys), SIVcpz (isolated from the chimpanzees), and SIVgor (isolated from the gorilla) through mutational and recombinational events. SIVmon/mus/gsn is known to recombine with SIVrcm (isolated from the red-capped mangabey monkey) to generate SIVcpz (for genome structures, see, **Figure 1**). SIVcpz served as parental virus for HIV-1 (M and N) and SIVgor (and finally for HIV-1 P).

The biological and molecular biological bases for species tropism of HIV-1 should reside in the above outlined evolutional processes. In particular, the so-called accessory proteins encoded by extra genes are important. Each virus group has a unique set of the accessory proteins in terms of their combinations and of their activities. Therefore, studies on viral accessory proteins are also meaningful for understanding viral evolution by cross-species transmission.

VIRAL AND CELLULAR DETERMINANTS FOR HIV-1 SPECIES TROPISM

Our early studies have already suggested the possible viral determinants and viral replication stage involved in the HIV-1 species tropism described above (Shibata et al., 1991, 1995; Shibata and Adachi, 1992). By the use of numerous chimeric molecular clones between HIV-1 and dual-tropic (tropic for human and monkey cells) SIVmac (isolated from the macaque monkey), we have claimed in essence, together with a work on the cyclophilin A (CypA; Dorfman and Gottlinger, 1996), that Gag-capsid (CA) and a viral protein(s) encoded by the central genomic region of HIV-1 are the determinants. We also have showed that HIV-1 is replication-incompetent in monkey cells because a certain replication step(s) before/during reverse transcription, other than the viral entry into cells, does not proceed normally. Subsequent extensive studies by us and others have clearly indicated that the interactions of Gag-CA/CypA, Gag-CA/tripartite motif (TRIM) proteins, and Vif/apolipoprotein B mRNA-editing enzyme-catalytic (APOBEC) proteins are major determinants for the HIV-1 species tropism (Nomaguchi et al., 2008, 2011; Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012) as summarized in **Table 1**. Gag-CA, CypA, and TRIM proteins have been described in detail in two articles in the Research Topic of this journal (Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012).

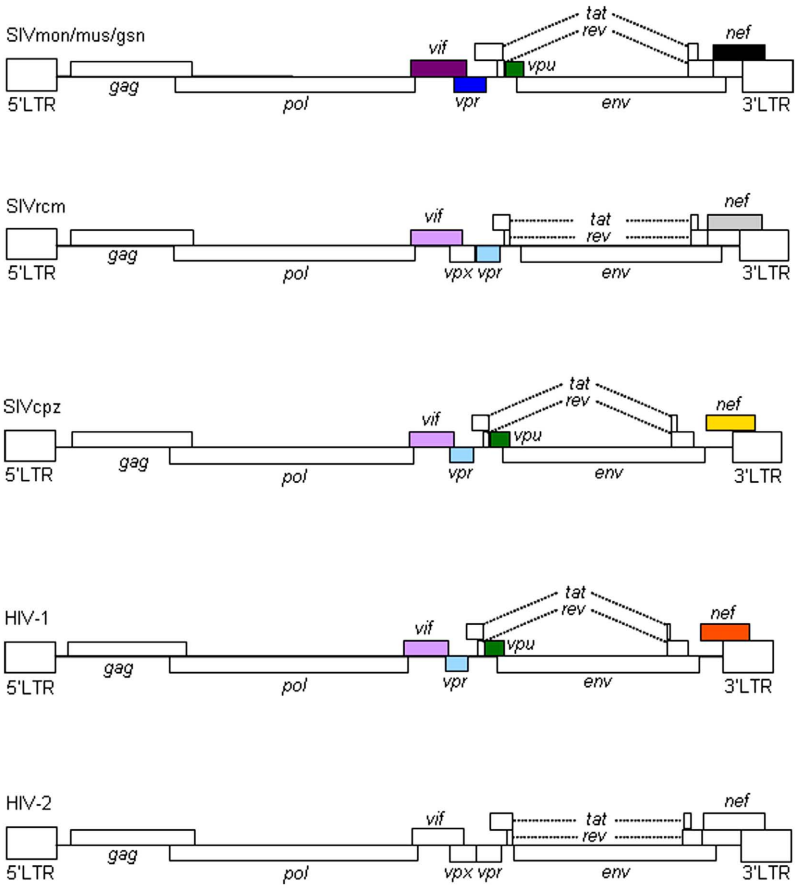

FIGURE 1 | Genome organization of primate immunodeficiency viruses. Various proviral genomes are schematically shown. As indicated by colored boxes, the *vpr* and *vpu* genes of SIVcpz/HIV-1 came from those of SIVrcm and SIVmon/mus/gsn, respectively. Also, the *vif* genes of SIVcpz/HIV-1 originated from that of SIVrcm. In addition, as shown by colored boxes, HIV-1 *nef* gene is similar to but distinct from SIVcpz *nef* gene. HIV-1 *nef* gene is different from those of SIVmon/mus/gsn, SIVrcm, and HIV-2 as indicated. For virus designations, see text.

Table 1 | Major viral and cellular determinants for HIV-1 species tropism.

Virus	Cell	Viral replication step affected
Gag-CA	CypA	
Gag-CA	TRIM5α	Uncoating (early phase)
Gag-CA	TRIMCyp	Uncoating (early phase)
Vif	APOBEC3G	Reverse transcription (early phase)
	APOBEC3F	Reverse transcription (early phase)
Vpu	Tetherin/BST-2	Virion release (late phase)

For details, see references (Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012) for Gag-CA, and Figures 3 and 4 for Vif/Vpu.

ACCESSORY PROTEINS OF PRIMATE IMMUNODEFICIENCY VIRUSES

All primate immunodeficiency viruses encode a number of extra proteins (Vif, Vpx, Vpr, Vpu, and Nef) in addition to regulatory (Tat and Rev) and structural (Gag, Pol, and Env) proteins (**Figure 1**). Structural proteins are common to all retroviruses, but the regulatory and accessory proteins are unique to the complex primate lentiviruses and not found in the other simple mammalian retroviruses. Regulatory Tat and Rev proteins are trans-activators for transcription and for the expression of late viral proteins, respectively. While the regulatory and structural proteins are essential for viral replication, the extra proteins, unfairly generically called "accessory," are dispensable under certain circumstances. However, in some cells, some of them are essential and the others are quite critical/important for optimal viral replication as illustrated for ΔVif and ΔVpu viruses (viruses that lack Vif or Vpu) in **Figure 2**. Another point to be mentioned here is relating to Vpr/Vpx proteins. Although Vpr and Vpx are genetically very similar (Khamsri et al., 2006), some primate immunodeficiency viruses bear two of them as described above (Fujita et al., 2010). Furthermore, the other viruses have Vpr only. What about the functional relationship of the two proteins? At present, the function of Vpr/Vpx is least well understood relative to that of the other accessory proteins (Malim and Emerman, 2008; Fujita et al., 2010). **Table 2** summarizes the important information regarding these accessory proteins so

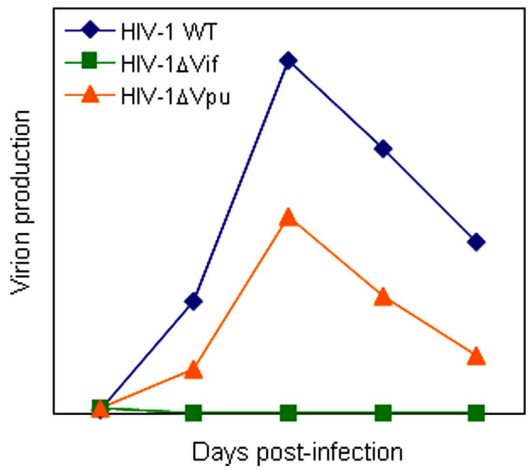

FIGURE 2 | A schema of replication kinetics by HIV-1 wild-type and mutant viruses. Viral growth properties in cells are illustrated based on numerous infection experiments in our laboratory. WT, wild-type.

Table 2 | Accessory proteins of primate immunodeficiency viruses.

Viral Proteins	Major functions for viral replication reported so far
Vif	Neutralize APOBEC3G/F. Essential for viral replication in natural target cells.
Vpx	Degrade SAMHD1/APOBEC3A. Critical for viral replication in natural target cells.
Vpr	Important for viral replication in macrophages (HIV-1).
Vpu	Down-regulate Tetherin/BST-2. Important for viral replication in CD4-positive cells.
Nef	Down-regulate cell surface molecules (CD4, MHC-I etc.).

far reported. In total, it is fairly reasonable to believe that the accessory proteins are regulators to optimize viral replication and persistence *in vivo* thereby enhancing viral transmission between individuals.

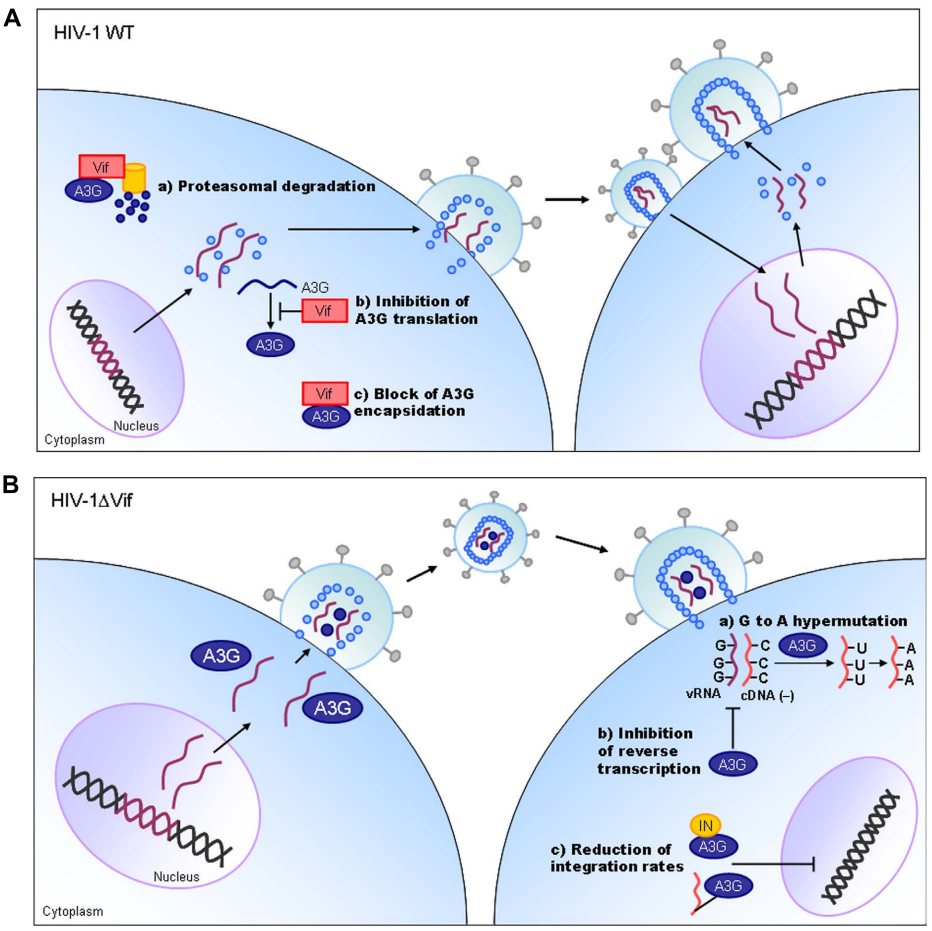

FIGURE 3 | HIV-1 replication and APOBEC3G. On the basis of results reported so far, the action mechanism of Vif is depicted. Replication process for wild-type (WT) and ΔVif mutant viruses are schematically shown on the basis of previously reported review articles (Holmes et al., 2007; Huthoff and Towers, 2008; Strebel et al., 2009). A3G, APOBEC3G; IN, viral integrase protein.

Vif AND Vpu PROTEINS

Vif protein is essential for viral replication in natural target cells such as CD4-positve lymphocytes and macrophages. Recent identification of its cellular object for attack (Sheehy et al., 2002) has clearly revealed the biological and biochemical bases for the growth property of ΔVif virus in natural target cells. This finding (identification of a family of APOBEC3 proteins, cellular cytidine deaminases, as potent inhibitors of HIV-1 replication in primary cells) has also contributed much to establish the concept of "the restriction factor" to well understand virus–cell interaction (Malim and Emerman, 2008; Sato et al., 2012). Of the APOBEC3 family proteins, APOBEC3G and APOBEC3F (Kitamura et al., 2011) strongly inhibit viral replication in the absence of Vif (**Figure 3**). Although HIV-1 Vif can abrogate the activities of human APOBEC3, it cannot do so against monkey APOBEC3. In contrast, SIVmac Vif can neutralize the anti-viral activity of APOBEC3 of both origins. Finally, it has been demonstrated that Vif and APOBEC3 are the major determinants for the HIV-1 species tropism by constructing macaque-tropic HIV-1 (HIV-1mt) and monitoring the HIV-1mt growth property in various genetic contexts of macaques (Hatziioannou et al., 2006, 2009; Kamada et al., 2006; Igarashi et al., 2007; Thippeshappa et al., 2011).

Vpu protein, unique to viruses of the HIV-1 group (**Figure 1**), modulates viral replication in human CD4-positive cell lines and primary cells. Mutant HIV-1 without Vpu (ΔVpu virus) grows poorly relative to wild-type virus. Recently, a cellular protein named Tetherin (also called BST-2) has been identified as a restriction factor against HIV-1 and is antagonized by Vpu (Neil et al., 2008; Van Damme et al., 2008). Vpu down-regulates the Tetherin from cell surface, and thereby promotes extracellular production of progeny virions (Malim and Emerman, 2008; Arias et al., 2011; Sato et al., 2012). The baseline mechanism for this action of Vpu is well studied as shown in **Figure 4**. Here, it must be attentive that the anti-Tetherin activity of Vpu is host species-specific as observed for Vif. HIV-1 Vpu acts against human but not (or poorly) macaque Tetherins (Sauter et al., 2009, 2010). Although the biological effect of Vpu is much milder than that of Vif as judged by the growth kinetics of mutant viruses (**Figure 2**), Vpu may be critical for interspecies transmission

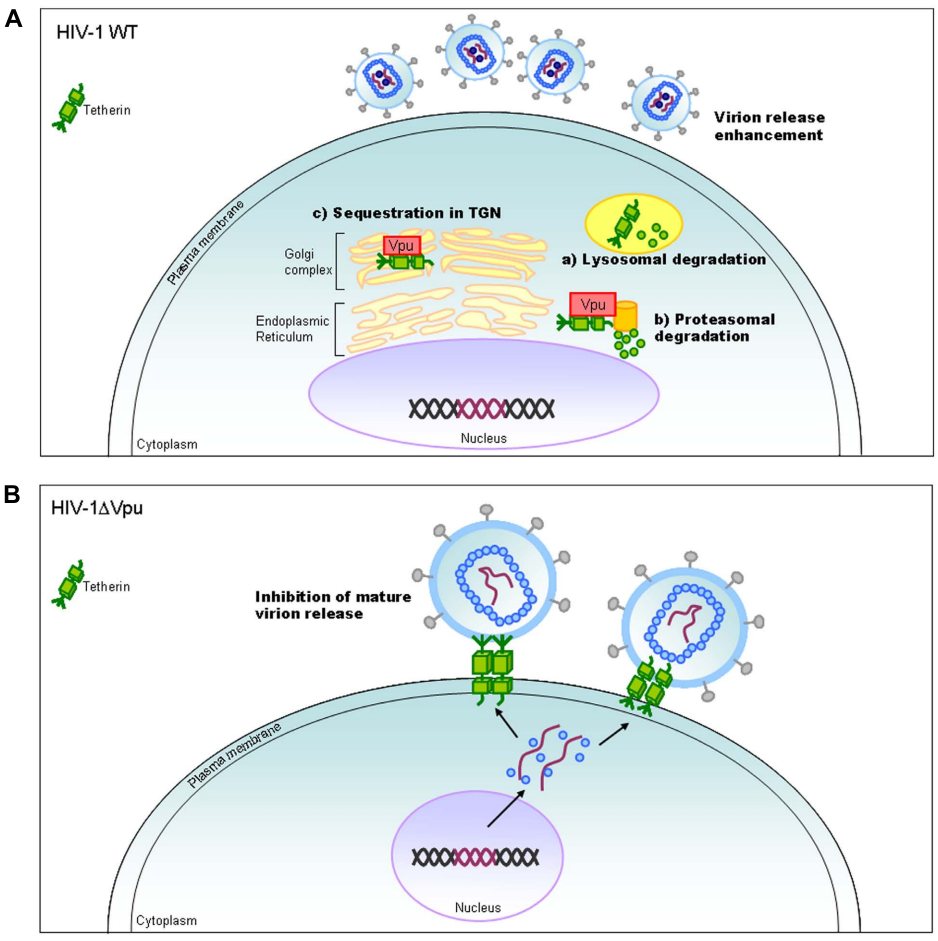

FIGURE 4 | HIV-1 replication and Tetherin. On the basis of results reported so far, the action mechanism of Vpu is depicted. Replication process for wild-type (WT) and ΔVpu mutant viruses are schematically shown on the basis of previously reported review articles (Tokarev et al., 2009; Douglas et al., 2010; Evans et al., 2010). TGN, trans-Golgi network.

through mutation/adaptation/recombinations (Kirchhoff, 2009; Sauter et al., 2009, 2010; Sharp and Hahn, 2011). Thus, Vpu and Tetherin affect the HIV-1 species tropism, but the effect may be relatively small.

In sum, Vif and Vpu counteract the major restriction factors APOBEC3 proteins and Tetherin/BST-2, respectively, and represent viral determinants for the host range of HIV-1 (**Tables 1 and 2**). It is intriguing to note that these factors would have shaped HIV-1 and made it unique among various primate immunodeficiency viruses (**Figure 1**).

Vpx AND Vpr PROTEINS

Vpx and Vpr proteins are necessary for efficient viral replication (Malim and Emerman, 2008; Fujita et al., 2010). In macrophages, ΔVpx replication is not detectable and this defect has been shown to be present at post-entry and before/during the reverse transcription process (Fujita et al., 2008, 2010; Srivastava et al., 2008). Also in some lymphocyte cell lines and in primary lymphocytes, Vpx protein is critical for viral replication (Ueno et al., 2003; Fujita et al., 2010; Doi et al., 2011). Because ΔVpr virus is somewhat replication-defective in some cells (for both HIV-1 and HIV-2), it is not unreasonable to assume that Vpr may play a role in the viral growth cycle. As such, Vpx and Vpr are important for *in vivo* viral replication and finally for viral pathogenicity (Fujita et al., 2010).

Very recently, SAMHD1 and APOBEC3A have been reported to be myeloid cell-specific restriction factors against HIV-1 counteracted by Vpx (Berger et al., 2011; Hrecka et al., 2011; Laguette et al., 2011). Whether these proteins are associated with the HIV-1 species tropism described in this review article, and whether they can explain the *in vitro* and *in vivo* situation of HIV-2/SIVmac mutant viruses mentioned above remain to be determined (Fujita et al., 2010; Nomaguchi et al., 2011).

CONCLUSION

In this review, we have described the major determinants for the species tropism of HIV-1. Structural Gag-CA and accessory Vif and Vpu proteins are clearly involved in this host range of HIV-1 as viral factors (**Table 1**). Cellular proteins that interact with these and contribute to this tropism are definitely the restriction factors (**Table 1**). In total, interplays between the viral and cellular responsible factors decide this unique and limited tropism of HIV-1. Whether there are the other factors that affect the HIV-1 species tropism is awaiting further investigations. In this regard, the biology of Vpx deserves attention. Because Vpx is present in SIVmac but not in HIV-1 (**Figure 1**), it may inactivate a cellular anti-viral protein(s) which is not recognized by HIV-1 proteins.

In both basic and applicable points of view, the narrow host range of HIV-1 is burdensome obstacle to overcome. Assuming that HIV-1mt can grow and cause disease similarly with SIVmac in macaques, we would be able to better perform model studies to precisely analyze viral replication and pathogenicity *in vivo*, and to establish the effective anti-HIV-1/AIDS strategies. To the best of our knowledge, there are no such HIV-1mt clones so far (Hatziioannou et al., 2006, 2009; Kamada et al., 2006; Igarashi et al., 2007; Kuroishi et al., 2009; Saito et al., 2011; Thippeshappa et al., 2011). We may further improve the ability of HIV-1mt by today's powerful methodology if we knew all the cellular determinants for the species tropism of HIV-1. Studies in this direction are in progress in our laboratory.

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Development and applications of VSV vectors based on cell tropism

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Viral vectors have been available in various fields such as medical and biological research or gene therapy applications. Targeting vectors pseudotyped with distinct viral envelope proteins that influence cell tropism and transfection efficiency are useful tools not only for examining entry mechanisms or cell tropisms but also for vaccine vector development. Vesicular stomatitis virus (VSV) is an excellent candidate for development as a pseudotype vector. A recombinant VSV lacking its own envelope (G) gene has been used to produce a pseudotype or recombinant VSV possessing the envelope proteins of heterologous viruses. These viruses possess a reporter gene instead of a VSV G gene in their genome, and therefore it is easy to evaluate their infectivity in the study of viral entry, including identification of viral receptors. Furthermore, advantage can be taken of a property of the pseudotype VSV, which is competence for single-round infection, in handling many different viruses that are either difficult to amplify in cultured cells or animals or that require specialized containment facilities. Here we describe procedures for producing pseudotype or recombinant VSVs and a few of the more prominent examples from envelope viruses, such as hepatitis C virus, Japanese encephalitis virus, baculovirus, and hemorrhagic fever viruses.

Keywords: vesicular stomatitis virus, pseudotype, recombinant, entry mechanism

INTRODUCTION

Viruses are obligate parasites of living organisms, and their replication is absolutely dependent on the host cell's machinery. The entry of enveloped viruses requires host cell binding and membrane fusion that is mediated by envelope proteins located on the surface of the virion.

Some viruses utilize a single molecule as a receptor for entry into the host cell, while many viruses require co-receptor(s) localized near the receptor for complete entry. Identification of viral entry receptors that are composed of membrane proteins, lipids, or carbohydrates is important for examining the life cycle of a virus and for further developing entry inhibitors. However, receptors or co-receptors of several viruses have been difficult to identify because of the lack of reliable cell culture systems, an insufficient amount of native viral particles, or difficulty in handling because of the requirement for biosafety level (BSL)-3 or -4 containment. Therefore, several surrogate systems have been developed to study the initial step of infection. One of the most primitive assays is a binding assay. Purified soluble envelope proteins, viral-like particles which are produced in insect cells by baculoviral vectors, and authentic viral particles obtained from patients have been used to study the mechanisms of viral attachment and to identify binding receptor molecules. However, these binding assays cannot be used to analyze further steps of infection such as fusion and penetration. A cell fusion assay was established to examine the membrane fusion activity of viral envelope proteins. This assay is sensitive and can easily determine cell fusion using reporter genes. Pseudotype virus systems based on vesicular stomatitis virus (VSV), influenza virus, retroviruses, and lentiviruses have also been established to examine entry mechanisms and to identify putative entry receptors for targeted viruses (**Table 1**). Pseudotype viruses have also been applied in neutralization tests for antibodies and vaccine development (**Table 1**). As for the application of a pseudotype virus system for VSV, a recombinant virus system with a heterologous viral envelope gene together with a reporter gene encoded into its own genome instead of the G gene has also been developed.

In this paper, we describe the properties of pseudotype or recombinant VSVs and their application to some enveloped viruses we have studied, such as the hepatitis C virus (HCV), Japanese encephalitis virus (JEV), baculovirus, and hemorrhagic fever viruses.

PSEUDOTYPE AND RECOMBINANT VSV

Vesicular stomatitis virus is a non-segmented, negative-stranded RNA virus that belongs to the family *Rhabdoviridae*, genus *Vesiculovirus*. VSV infects a broad range of animals, including cattle, horses, and swine. The genome of the virus codes for five major proteins, glycoprotein (G), matrix protein (M), nucleoprotein (N), large protein (L), and phosphoprotein (P). The G protein mediates both viral binding and host cell fusion with the endosomal membrane following endocytosis. The L and P proteins are subunits of the viral RNA-dependent RNA polymerase.

The simple structure and rapid high-titer growth of VSV in mammalian and many other cells has made it a useful tool in the fields of cellular and molecular biology and virology, and this was further strengthened with the establishment of the reverse

genetics system for VSV. Recombinant VSV in which native envelope G protein is replaced with a foreign reporter gene such as a fluorescent reporter protein, luciferase, or secreted alkaline phosphatase (SEAP) can normally bud from producing cells even in the absence of G protein, and heterologous viral envelope proteins are incorporated into the virion. Previous studies demonstrated that VSV forms a "pseudotype" when a cell is co-infected with VSV and other enveloped viruses (Huang et al., 1974; Witte and Baltimore, 1977). A pseudotype virus is defined as a viral particle harboring other types of viral envelopes or host cellular proteins with or without its own envelope. By virtue of these characteristics of VSV, pseudotype virus systems, in which VSV G proteins are completely replaced with other types of viral envelope proteins, have been established (**Figure 1**). Up to the present, numerous types of pseudotype viruses have been constructed with heterologous viral envelope proteins and used in studies examining the entry of viruses, for identification of novel viral receptors, for development of neutralization tests, and as vaccine vectors (**Table 1**). In particular, availability of pseudotype viruses has been useful in the study of several high-risk viruses that require high-level containment facilities, i.e., in the handling of BSL-3 or -4 viruses. Infectivity of these pseudotype viruses can be easily and quantitatively evaluated by measurement of the reporter gene activities. A recombinant virus system, which encodes a heterologous viral envelope gene instead of an envelope gene in its own genome, has also been made available by establishment of reverse genetics (**Figure 2**). This recombinant virus is replication-competent both *in vitro* and *in vivo* and can contribute to the study of targeted viruses that inefficiently replicate in experimental systems. Although the pseudotype virus is limited to single-step infection and therefore provides a poor model for actual infection processes, the recombinant virus is a far more authentic and powerful tool for investigating targeted viral infection. Currently, this system is applicable to the VSV or other several viruses and not to retroviruses or lentiviruses. Recombinant VSV can be produced in various cells without regard to transfection efficiency; on the other hand, recovery of pseudotype VSV as well as pseudotype retroviruses or lentiviruses is restricted to 293T or some other type of cells that exhibit a high competency of transfection. Recombinant VSV could also lead to the induction of cellular and humoral host immunity (Schnell et al., 1996).

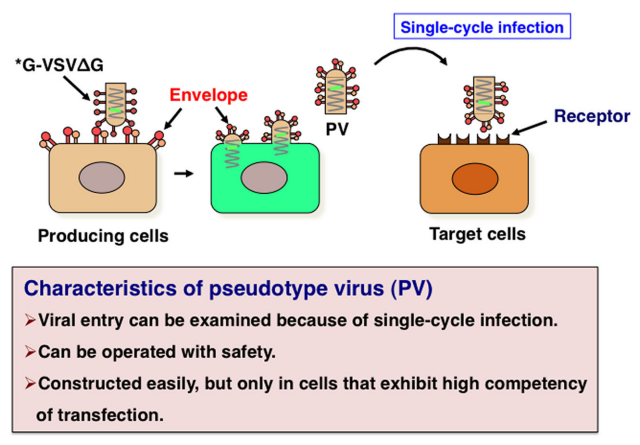

FIGURE 1 | Schematic representation of the production of pseudotype VSV. Producer cells were transfected with an expression plasmid encoding foreign envelope genes and then infected with a VSV G-complemented pseudotype virus (*G-VSVΔG). The pseudotype virus released from the producer cells infected target cells but was not able to produce infectious progeny viruses.

CONSTRUCTION OF PSEUDOTYPE AND RECOMBINANT VSV

Seeded or recombinant VSVs in which the G gene is replaced by a foreign reporter gene such as a fluorescent reporter protein (green fluorescent protein, GFP; red fluorescent protein, RFP; and so on), luciferase, or SEAP or each viral envelope gene were generated as described below. Either 293T or BHK cells were grown to 90% confluence on 35-mm tissue culture plates. The cells were infected with a recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (vTF7-3) at a multiplicity of infection (MOI) of 5. After incubation at room temperature for 1 h, the cells were transfected with helper plasmids, pBS-N, pBS-P, pBS-L, and pBS-G, and template plasmids, pVSVΔG–GFP (RFP), pVSVΔG–Luci, pVSVΔG–SEAP, or pVSVΔG–Env using a cationic liposome reagent. After 4 h, the supernatants were

Table 1 | Application studies of pseudotype and recombinant VSV.

Target viruses	Reference
CHARACTERIZATION OF GLYCOPROTEIN, ENTRY TROPISM, ETC.	
Ebola virus	Takada et al. (1997)
HCV	Lagging et al. (1998)
	Matsuura et al. (2001)
	Tani et al. (2007)
HIV-1	Boritz et al. (1999)
Measles virus	Tatsuo et al. (2000)
HTLV-1	Okuma et al. (2001)
RSV	Kahn et al. (2001)
SARS–CoV	Fukushi et al. (2005)
HBV	Saha et al. (2005)
Arenavirus	Vela et al. (2007)
JEV	Tani et al. (2010)
Baculovirus	Kaname et al. (2010)
LCMV	Muik et al. (2011)
VACCINE VECTORS	
Influenza Virus	Roberts et al. (1998)
Papillomavirus	Roberts et al. (2004)
Marburg, Ebola, Lassa	Garbutt et al. (2004)
HIV-1	Publicover et al. (2005)
West Nile virus	Iyer et al. (2009)
HBV	Cobleigh et al. (2010)
Norovirus	Ma and Li (2011)
DIAGNOSIS	
Borna disease virus	Perez et al. (2001)
Hantaan Virus	Lee et al. (2006)
SARS–CoV	Fukushi et al. (2006)
Nipah virus	Kaku et al. (2009)

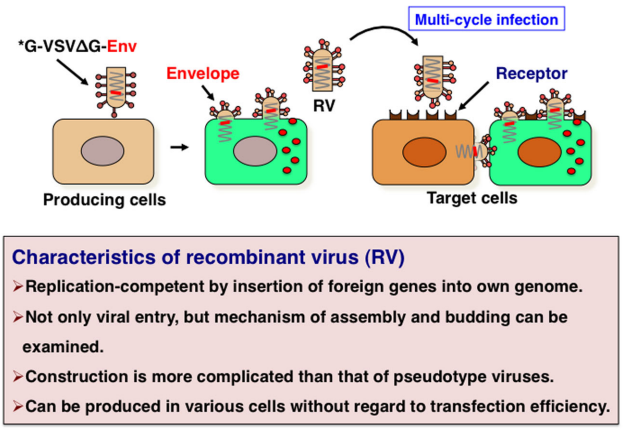

FIGURE 2 | Schematic representation of the production of recombinant VSV. Various mammalian producer cells were inoculated with a VSV G-complemented recombinant virus encoding foreign envelope genes instead of VSV G. The recombinant VSV was able to undergo a fully productive infection generating infectious progeny viruses that could be passaged into naïve cells.

replaced with 10% FBS DMEM, and cells were incubated at 37°C for 48 h. The supernatants were then filtered through a 0.22-μm-pore-size filter to remove vaccinia virus and were applied to 293T or BHK cells that had been transfected with pCAGVSVG 24 h previously. If BHK cells constitutively expressing the bacteriophage T7 RNA polymerase (BHKT7) were utilized, the cells were only transfected with helper plasmids, pIRES-N, pIRES-P, pIRES-L, pIRES-G, and template plasmids using a cationic liposome reagent without the vaccinia virus infection. Recovery of the virus was assessed by examining the cells for the cytopathic effects that are typical of a VSV infection after 24 h. Stock of *G-complemented viruses, i.e., VSVΔG virus or recombinant viruses transiently bearing VSV G protein on the virion surface, were grown from the single plaque on BHK cells transfected with pCAGVSVG and then stored at −80°C. The infectious titers of the recovered viruses were determined by a plaque assay. To generate pseudotype virus, 293T, BHK, or some other type of cells that exhibit a high competency of transfection were transfected with a plasmid expressing the envelope protein using a cationic liposome reagent. After 24 h of incubation at 37°C, cells were infected at an MOI of 0.5 with the *G-VSVΔG–Luci and *G-VSVΔG–SEAP, or 5 with *G-VSVΔG–GFP (RFP). The virus was adsorbed for 2 h at 37°C and then extensively washed four times or more with serum-free DMEM. After 24 h of incubation at 37°C, the culture supernatants were collected, centrifuged to remove cell debris, and stored at −80°C. To generate recombinant virus in various mammalian cells, cells were infected with the *G-complemented VSVΔG–Env at an MOI of 5 for 2 h at 37°C and then extensively washed four times or more with serum-free DMEM. After 24 h of incubation at 37°C, the culture supernatants were collected and stored at −80°C. The infectious titers of the viruses were determined by evaluation of each reporter assay or a focus-forming assay. Further details of the protocol can be found in a recent paper (Whitt, 2010).

APPLICATION IN THE STUDY OF ENTRY MECHANISMS OF HCV

Hepatitis C virus has already infected more than 3% of the worldwide population and 80% of those infected develop persistent HCV infection (Cerny and Chisari, 1999; Theodore and Fried, 2000). Persistent HCV infection often leads to chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. Currently, there are still 1.5 million or more HCV carriers in Japan. In past years, anti-hepatitis C therapy has modestly improved; however, a currently available combination therapy, consisting of interferon and the nucleoside analog, ribavirin, shows a sustained response in only less than half of the treated patients. The development of innovative treatment alternatives for patients infected with HCV is urgently required, and a better understanding of the life cycle of HCV should allow us to improve HCV therapies. However, due to the lack of an *in vitro* cell culture system for the isolation of virus directly from patient sera at present, various surrogate systems such as replicon cells (Lohmann et al., 1999), pseudotype viruses (Lagging et al., 1998; Matsuura et al., 2001; Bartosch et al., 2003; Hsu et al., 2003; Tani et al., 2007), or trans-complement particles (Ishii et al., 2008; Steinmann et al., 2008) have been developed to study each step of HCV infection. Although *in vitro* binding assays using soluble purified envelope proteins or HCV–LPs have identified several candidate receptors for HCV, the final determination of a true entry receptor or co-receptor capable of internalizing HCV may be made using an infection assay. Toward this end, pseudotype virus systems based on VSV and retrovirus or lentivirus have been established and applied to identify entry receptors for HCV. Although it is still unknown how HCV envelope proteins retained in the endoplasmic reticulum (ER) are incorporated into both VSV and retroviruses, which naturally bud from the plasma membrane, significant infectivity of these pseudotype viruses has been exhibited in several human hepatoma cell lines. These infections could be inhibited by treatment with antibodies or soluble proteins against putative receptors or HCV envelope proteins, or by a knockdown of receptor molecules by small interfering RNAs (siRNAs), suggesting that innate HCV infection had occurred. We also successfully generated infectious pseudotype and recombinant VSVs incorporating unmodified HCV envelope proteins in hepatic and non-hepatic human cell lines. These viruses exhibited high infectivity in a human hepatoma cell line, Huh7, which is highly susceptible to infection by cell-cultured HCV (HCVcc). The recombinant virus, but not the pseudotype virus, was able to propagate and form foci only in Huh7 cells. The infection of Huh7 cells with pseudotype and recombinant viruses was inhibited by anti-hCD81 and anti-E2 antibodies and by sera from chronic HCV patients. These viruses, as well as pseudotype retroviruses (HCVpp) or HCVcc, were sensitive to the inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, or bafilomycin A_1, or formation of clathrin-coated pits, chlorpromazine, suggesting that these viruses enter via pH-dependent and clathrin-mediated endocytosis into target cells (Blanchard et al., 2006; Tani et al., 2007). The infectivity of the recombinant virus was inhibited by an ER α-glucosidase inhibitor, *N*-(*n*-nonyl) deoxynojirimycin (*Nn*-DNJ), but not by a Golgi mannosidase inhibitor, deoxymannojirimycin

(Tani et al., 2007). Focus formation of the recombinant virus was also impaired by *Nn*-DNJ treatment. It was obvious that the appearance of infectious or non-infectious viruses was dependent on the cell type as a result of the infectivity of the recombinant viruses generated from various cell lines. Although the precise mechanisms of HCV assembly or budding that cause the differences in infectivity of viruses generated from different cell lines is still unclear, host cellular factors might be involved in the assembly or budding steps in the generation of infectious particles.

APPLICATION IN THE STUDY OF ENTRY MECHANISMS OF JEV

Japanese encephalitis virus, a mosquito-borne zoonotic pathogen, is the leading cause of viral encephalitis in humans, with ∼50,000 cases reported annually worldwide. JEV is an enveloped virus belonging to the family *Flaviviridae* and the genus *Flavivirus*, which also includes Dengue virus, West Nile virus, Yellow fever virus, and Tick-borne encephalitis virus (Gubler et al., 2007). The genome consists of a single-stranded positive-sense RNA of approximately 11 kb, encoding a single large polyprotein, which is cleaved by host- and virus-encoded proteases into three structural (C, PrM, and E) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The envelope protein (E) is a 53-kDa glycoprotein, which is a major component of the virion surface and has been found to be associated with all the biological properties of the virus, such as attachment to cellular receptors, penetration, fusion with the endosomal membrane, host cell range and cell tropism, and neutralization to antibodies. Although a number of cellular components that interacted with E protein, such as heat shock cognate protein 70 (Ren et al., 2007), heat shock protein 70 (Das et al., 2009), vimentin (Das et al., 2011), glycosaminoglycans (Su et al., 2001; Lee and Lobigs, 2002), and laminin (Boonsanay and Smith, 2007), have been shown to participate in JEV binding or penetration, the precise mechanisms remain largely unknown. The pseudotype and recombinant VSV systems have offered us useful tools to focus on the study of entry mechanisms of JEV E proteins by using control viruses harboring an appropriate protein on identical particles. Both pseudotype (JEVpv) and recombinant (JEVrv) VSV bearing the JEV E protein exhibited high infectivity for the target cells, and JEVrv, but not JEVpv, was able to propagate and form foci, as did authentic JEV (Tani et al., 2010). Both JEVpv and JEVrv were neutralized by anti-JEV E antibodies. Treatment of cells with inhibitors for vacuolar ATPase and clathrin-mediated endocytosis reduced the infectivity of JEVpv, suggesting that JEVpv enters cells via pH- and clathrin-dependent endocytic pathways. Treatment of the JEVpv and JEVrv with cholesterol drastically reduced the infectivity, as previously reported on authentic JEV (Lee et al., 2008). In contrast, depletion of cholesterol from the viruses by treatment with methyl β-cyclodextrin enhanced the infectivity. Furthermore, treatment of cells with sphingomyelinase (SMase), which hydrolyzes membrane-bound sphingomyelin to ceramide, drastically enhanced infection with JEVpv and JEVrv (Tani et al., 2010). These enhancements were inhibited by treatment with an SMase inhibitor or C_6-ceramide. Involvement of ceramide in the entry of JEV was confirmed by co-precipitation of the JEV E protein with labeled-ceramide (Tani et al., 2010). In our study, it was demonstrated that cellular lipid components such as cholesterol and ceramide play crucial roles in the entry of JEV. Modification of sphingolipids on the plasma membrane of the cells might be a novel target for the development of antivirals against JEV infection.

APPLICATION IN THE STUDY OF COMPLEMENT RESISTANCE OF BACULOVIRUS GP64

Baculovirus vectors have been shown to exhibit not only a high-level of gene expression in insect cells but also efficient gene transduction into a wide variety of mammalian cells with lower cytotoxicity (Hofmann et al., 1995; Boyce and Bucher, 1996; Shoji et al., 1997). In contrast, the complement systems of animals have been defined to represent a potent primary barrier to *in vivo* application of baculovirus vectors produced in insect cells (Hofmann and Strauss, 1998; Tani et al., 2001, 2003). However, pseudotype viruses based on retroviruses or lentiviruses bearing baculovirus envelope protein GP64 have recently been shown to exhibit efficient gene transduction into mouse organs for long periods compared with those bearing the VSV G protein, which is commonly used for pseudotyping (Kumar et al., 2003; Schauber et al., 2004; Kang et al., 2005; Sinn et al., 2005, 2008). It was considered that serum resistance of the pseudotype viruses bearing GP64 protein is caused by differences between insect and mammalian cells, because pseudotype retroviruses or lentiviruses were generated from mammalian cells, and in contrast, baculovirus was generated from insect cells. Therefore, we generated recombinant VSV bearing GP64 protein in both insect and mammalian cells and examined the role of the GP64 on resistance to inactivation by human or guinea-pig sera (Kaname et al., 2010). Recombinant VSVs generated in human cell lines exhibited the incorporation of human decay accelerating factor (DAF) in virions and were resistant to serum inactivation, whereas those generated in insect cell lines exhibited no incorporation of human DAF and were sensitive to complement inactivation. Recombinant baculoviruses generated in insect cells expressing human DAF or carrying the human DAF gene exhibited resistance to complement inactivation, suggesting that acquisition of resistance to human complement by the incorporation of DAF with baculovirus GP64 represents a step in the development of novel viral vectors for improved gene therapy.

APPLICATION IN THE STUDY OF VIRAL ENTRY AND NEUTRALIZATION TEST OF ARENAVIRUSES

Viral hemorrhagic fever viruses, such as members of the Arenaviridae family, including Lassa virus, Junin virus, Machupo virus, and Chapare virus, cause fulminating disease characterized by acute fever followed by generalized hemorrhagic syndrome that is associated with 90% mortality in the severe forms.

Because these viruses cannot be handled under BSL-2 or -3 containment facilities, pseudotype viruses bearing each GPC envelope protein of various arenaviruses, such as Lassa virus, Junin virus, Machupo virus, Sabia virus, Chapare virus, Guanarito virus, and Lujo virus, were generated as surrogate models for the study of viral infection or neutralization. All of the pseudotype viruses exhibited high susceptibility to various cell lines and were neutralized by sera from patients infected with each virus. Reduction of the infectivity of the pseudotype virus

in the cells treated with various entry inhibitors depended on the species of pseudotype virus, suggesting that several entry mechanisms were involved in the infection of arenaviruses (Tani et al., unpublished data). In studies on serological diagnosis of arenaviruses, cross reaction occurred among species of arenaviruses in enzyme-linked immunosorbent assay or immunofluorescence assay, whereas neutralization tests using pseudotype viruses exhibited a specific reaction with each species of virus (Nakauchi et al., 2009; Iha et al., unpublished data). Although Old or New World arenaviruses have been shown to utilize α-dystroglycan or human transferrin receptor 1, respectively, as one of the cellular receptors, infectivities of the pseudotype viruses have not been consistent with the expression levels of the receptor molecules in our preliminary studies. The infection of pseudotype viruses was not completely inhibited by soluble protein or antibodies of receptor molecules, suggesting that another receptor molecule(s) might be involved in the entry of these viruses. Although further characterization of the pseudotype viruses bearing GPC envelope proteins of arenaviruses will be needed, these viruses are thought to mimic the functional properties of wild type arenaviruses and are suitable for the study of entry mechanisms, including investigation of novel cellular receptor(s), neutralization tests, or vaccine development.

CONCLUSION AND PERSPECTIVES

Up to the present, various viral vectors aimed at gene transfer or therapy have been developed and applied in biological and medical research fields. Pseudotype or recombinant VSV are useful tools as alternative viruses to study entry mechanisms, identification of novel cellular receptors, screening antiviral libraries, or development of serological diagnosis for various kinds of viruses, especially unmanageable BSL-3 or -4 viruses. These viruses have also been applied in targeting vectors to specific cells. VSV vectors with monoclonal antibodies against specific oncogenic proteins or viral receptor molecule(s) incorporated on virion surface have been targeted specifically to cells expressing oncogenic proteins or infected cells expressing the viral envelope proteins, respectively, without any influences on normal or uninfected cells. This raises the possibility of the elimination of cancer cells or chronic viral infections by using acute VSV infection. Genetically engineered VSVs encoding suicide cassettes or immune response genes have also been generated as more specific, safer, and effective agents for cancer therapies. Further studies and applications of VSV vectors will provide us not only with useful tools for virological studies but also various benefits for biological sciences and medical research.

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Tropism and pathogenicity of rickettsiae

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Rickettsiae are obligate intracellular parasitic bacteria that cause febrile exanthematous illnesses such as Rocky Mountain spotted fever, Mediterranean spotted fever, epidemic, and murine typhus, etc. Although the vector ranges of each *Rickettsia* species are rather restricted; i.e., ticks belonging to *Arachnida* and lice and fleas belonging to *Insecta* usually act as vectors for spotted fever group (SFG) and typhus group (TG) rickettsiae, respectively, it would be interesting to elucidate the mechanisms controlling the vector tropism of rickettsiae. This review discusses the factors determining the vector tropism of rickettsiae. In brief, the vector tropism of rickettsiae species is basically consistent with their tropism toward cultured tick and insect cells. The mechanisms responsible for rickettsiae pathogenicity are also described. Recently, genomic analyses of rickettsiae have revealed that they possess several genes that are homologous to those affecting the pathogenicity of other bacteria. Analyses comparing the genomes of pathogenic and non-pathogenic strains of rickettsiae have detected many factors that are related to rickettsial pathogenicity. It is also known that a reduction in the rickettsial genome has occurred during the course of its evolution. Interestingly, *Rickettsia* species with small genomes, such as *Rickettsia prowazekii*, are more pathogenic to humans than those with larger genomes. This review also examines the growth kinetics of pathogenic and non-pathogenic species of SFG rickettsiae (SFGR) in mammalian cells. The growth of non-pathogenic species is restricted in these cells, which is mediated, at least in part, by autophagy. The superinfection of non-pathogenic rickettsiae-infected cells with pathogenic rickettsiae results in an elevated yield of the non-pathogenic rickettsiae and the growth of the pathogenic rickettsiae. Autophagy is restricted in these cells. These results are discussed in this review.

Keywords: *Rickettsia*, tropism, pathogenicity, spotted fever group, typhus group, vector, tick, insect

INTRODUCTION

Rickettsioses in the broad sense are caused by a variety of gram-negative bacteria from the *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, or *Neorickettsia* genera. *Rickettsia* are now further classified into the typhus group (TG) and spotted fever group (SFG; **Table 1**), although *Orientia* used to belong to the scrub TG of *Rickettsia* (Tamura et al., 1995; Fournier et al., 2005). *Coxiella*, the causative agent of Q fever, used to be classified into the *Rickettsiales* order but now belongs to the *Legionellales* order (Weisburg et al., 1989). As *Rickettsia* are obligate intracellular parasites, many of the mechanisms involved in their attachment and internalization into host cells are shared by viruses.

Rickettsiae cause febrile exanthematous illnesses, such as spotted fever, epidemic, and murine typhus, etc. (Dyer et al., 1931; Zinsser and Castaneda, 1933). Until nearly two decades ago, only five species of SFG rickettsiae (SFGR), *R. rickettsii*, *R. conorii*, *R. sibilica*, *R. australis*, and *R. akari*, which are responsible for Rocky Mountain spotted fever (Wolbach, 1919), Mediterranean spotted fever or Boutonneuse fever (Brumpt, 1932), North Asian tick typhus or Siberian tick typhus (Shmatikov and Velik, 1939), Queensland tick typhus (Plotz et al., 1946), and Rickettsialpox (Huebner et al., 1946), respectively, were known to be pathogenic. However, since the isolation and identification of a new SFGR species, *R. japonica*, from Japanese patients with a form of SFG rickettsiosis known as Japanese spotted fever or Oriental spotted fever (Uchida et al., 1988, 1989, 1991, 1992; Uchiyama and Uchida, 1988; Uchiyama et al., 1991), many pathogenic SFGR species have been newly isolated and identified, and some of the previously identified SFGR species have now been recognized as pathogenic (**Table 1**; Beati and Raoult, 1993; Beati et al., 1993, 1997; Kelly et al., 1996; Raoult et al., 1997; Stenos et al., 1998; Fournier et al., 2003, 2005; Mediannikov et al., 2004; Paddock et al., 2004). More recently, *R. bellii* was isolated from amebas, and it was suggested that it should be classified into the ancestral group (AG), which contains *Rickettsia* species that have been demonstrated by phylogenetic analyses to have the oldest genomic features among the previously isolated and identified *Rickettsia* species (Philip et al., 1983; Stohard et al., 1994; Gillespie et al., 2012). *R. bellii* displays weaker pathogenicity in experimental animals than *R. conorii*, although its pathogenicity in humans remains unknown (Ogata et al., 2006). Although the mechanism by which rickettsiae cause disease has not been well established, it is thought that rickettsial pathogenicity involves the infiltration of cells into the vascular environment, hemorrhaging, and thrombosis due to the degeneration of endothelial vein cells caused by the growth of the rickettsiae. Lipopolysaccharides (LPS) on the outer membranes of rickettsiae are also thought to participate in

Table 1 | Classifications, vectors, and reservoirs of *Rickettsia* that are known to be pathogenic to humans.

Antigenic group	Species	Disease	Vector	Reservoir(s)
Spotted fever group	*R. aeschlimannii*	Rickettsiosis	Tick	Unknown
	R. africae	African tick-bite fever	Tick	Ruminants
	R. akari	Rickettsialpox	Mite	Mice, rodents
	R. australis	Queensland tick typhus	Tick	Rodents
	R. conorii	Mediterranean spotted fever or Boutonneuse fever	Tick	Dogs, rodents
	R. felis	Cat flea rickettsiosis	Flea	Cats, rodents, opossums
	R. heilongjiangensis	Far Eastern spotted fever	Tick	Rodents
	R. helvetica	Aneruptive fever	Tick	Rodents
	R. honei	Flinders Island spotted fever, Variant Flinders Island spotted fever, Thai tick typhus	Tick	Rodents, reptiles
	R. japonica	Japanese spotted fever or Oriental spotted fever	Tick	Rodents
	R. massiliae	Mediterranean spotted fever-like disease	Tick	Unknown
	R. parkeri	Maculatum infection	Tick	Rodents
	R. rickettsii	Rocky Mountain spotted fever, Febre maculosa, São Paulo exanthematic typhus, Minas Gerais exanthematic typhus, Brazilian spotted fever	Tick	Rodents
	R. sibirica	North Asian tick typhus, Siberian tick typhus	Tick	Rodents
	R. sibirica mongolotimonae	Lymphangitis-associated rickettsiosis	Tick	Rodents
	R. slovaca	Tick-borne lymphadenopathy (TIBOLA), *Dermacentor*-borne necrosis and lymphadenopathy (DEBONEL)	Tick	Lagomorphs, rodents
Typhus group	*R. prowazekii*	Epidemic typhus, Brill-Zinsser disease	Louse	Humans, flying squirrels
	R. typhi	*Murine* typhus	Flea	Rodents

the eruption, pyrexia, and endotoxin shock observed during the course of rickettsial infection.

It is worth noting that the vector ranges of each rickettsial species are rather restricted; i.e., the vectors for SFGR species are usually ticks (except those for *R. akari* and *R. felis*, which are mites and fleas, respectively) belonging to *Arachnida*. On the other hand, those for TGR species are lice and fleas, which belong to *Insecta* (**Table 1**; Higgins et al., 1996). *R. felis* carries its pRF genes on a plasmid so it does not fully meet the criteria for the SFG or TG. Rather, this indicates that *R. felis* has participated in horizontal gene transfer involving the AG and might be better classified into a transitional group along with *R. akari*, which displays both SFG and TG characteristics (Gillespie et al., 2007, 2012). This plasmid might have been incorporated into the chromosomes of the other *Rickettsia* during the course of their evolution (Gillespie et al., 2007). The mechanisms responsible for the vector tropism of rickettsiae have not been studied in detail.

TROPISM OF RICKETTSIAE TOWARD ARTHROPOD VECTORS AND CULTURED CELLS

Although the relationships between rickettsiae and their vectors are relatively fixed, the mechanisms responsible for the tropism of rickettsiae toward arthropod vectors have not been elucidated. Studies using cell lines derived from arthropods are indispensable for clarifying these mechanisms. In studies using insect cells, Uchiyama reported that the growth of some SFGR species, *R. japonica*, and *R. montanensis*, was restricted in the NIAS-AeAl-2 (AeAl2) insect cell line, which is derived from *Aedes albopictus*, even though SFGR species have been demonstrated to be capable of adhering to and invading these cells (**Figures 1–3**; Mitsuhashi, 1981; Mizuki et al., 1999; Noda et al., 2002; Uchiyama, 2005). Scanning and transmission electron microscopy confirmed these results (**Figure 3**; Uchiyama, 2006). Rickettsiae seem to begin their invasion of AeAl2 cells immediately after adhering to them. The superinfection of SFGR-infected AeAl2 cells with a TGR species on day three of infection resulted in the growth of the TGR species but not the SFGR species. Furthermore, the SFGR-infected AeAl2 cells suffered rapid cell death; however, as no DNA fragmentation, lobed nuclei, or peripheral chromosome condensation were observed, the growth inhibition of these cells was possibly due to their non-apoptotic necrotic cell death. Concerning this issue, induced cell death (subsequently renamed programmed necrosis), which is one of the candidates for the mechanism responsible for growth inhibition, has been found to act in opposition to anti-apoptotic factors (Laster et al., 1988; Holler et al., 2000; Chan et al., 2003; Cho et al., 2009; He et al., 2009). For example, cells infected with Cowpox virus cause tumor necrosis factor (TNF)-induced programmed necrosis, which is opposed by the anti-apoptosis factor CrmA (Chan et al., 2003). When T cells or fibroblastic cells are infected with the Vaccinia virus, apoptosis is inhibited by the anti-apoptotic factor B13R/Spi2; however, TNF-induced programmed necrosis can also occur (Cho et al., 2009). Thus, programmed necrosis might occur when AeAl2 cells are infected with SFGR. Contrary to our results, a previous report found that some non-pathogenic SFGR species, *R. montanensis*, and *R. peacockii*, were able to grow in two mosquito cell lines (the *A. albopictus* cell line Aa23 and the *Anopheles gambiae* cell line Sua5B; Sakamoto and Azad, 2007). The reason for this discrepancy is poorly understood;

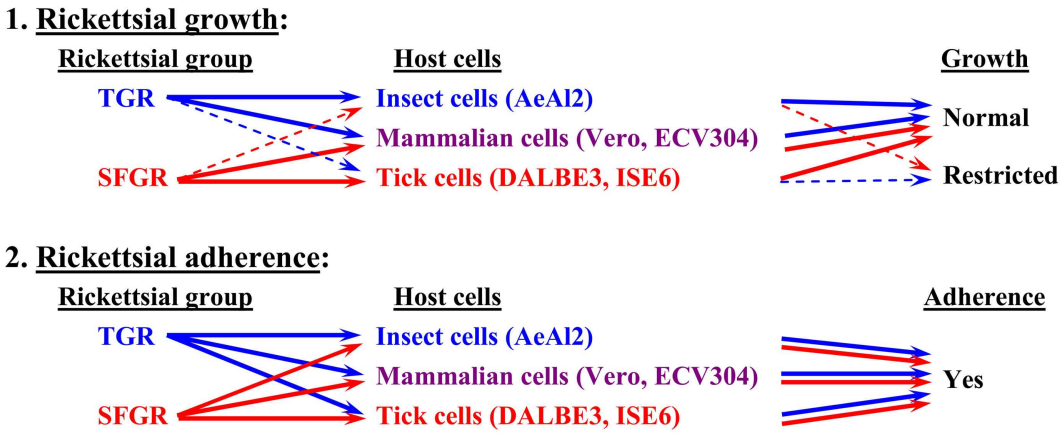

FIGURE 1 | Basic tropism of rickettsiae toward cultured cells. The growth of SFGR and TGR was monitored in cells derived from ticks, insects, and mammals. SFGR grew well in tick cells, while TGR grew well in insect cells. However, the growth of SFGR was restricted in insect cells and that of TGR was restricted in tick cells. Both groups of pathogenic rickettsiae grew well in mammalian cells. Both groups of rickettsiae were confirmed to be capable of adhering to all of the tested cells.

however, the abovementioned growth was only detected by FISH and PCR, rather than in an infective assay. Another report found that the transcription of spoT gene paralogs was suppressed during the maintenance of *R. conorii* in *A. albopictus* (C6/36) cells at 10°C for 38 days. Shifting the temperature to 37°C resulted in a rapid upregulation of *spoT1* gene expression (Rovery et al., 2005). Although *R. conorii* were confirmed to be able to survive in the cells at low temperature, their growth was not directly assayed after the temperature was increased.

As for studies using tick cell lines, several reports have demonstrated the growth of SFGR species in cells derived from ticks (Policastro et al., 1997; Munderloh et al., 1998). In our study (Uchiyama et al., 2009), the DALBE3 cell line from *Dermacentor albipictus* and the ISE6 cell line from *Ixodes scapularis* were inoculated with *R. japonica* and *R. conorii* as SFGR species and *R. prowazekii* and *R. typhi* as TGR species. The SFGR grew well in these tick cells as well as in Vero, HeLa, and ECV304 mammalian cells (**Figures 1** and **2**). On the contrary, the growth of TGR was restricted in these tick cells, even though they successfully adhered to the cells, which was also true for other combinations of rickettsiae and host cells. These findings were confirmed by transmission electron microscopy. Rickettsiae were also found to be able to escape into the cytoplasm from phagosomes after being engulfed by the tick cells. Thus, the observed growth restriction occurred after these steps, although the precise mechanism responsible for it is yet to be elucidated. These results from studies using various combinations of SFGR or TGR and tick or insect cells suggest that the host vector tropism of rickettsiae is at least partially based on host cellular tropism (**Figure 1**).

PATHOGENICITY OF RICKETTSIAE

As shown in **Table 1**, many rickettsial species have displayed evidence of being pathogenic to humans. On the other hand, many other species have not displayed any evidence of being pathogenic to humans, some of which might be weakly pathogenic. To date, various putative factors that might be associated with the pathogenicity of rickettsiae have been proposed; however, the molecular basis for the pathogenicity of rickettsiae is yet to be precisely established.

It is reasonable to think that the degree of growth of *Rickettsia* in human blood vessels; i.e., endothelial cells (EC), primarily determines the severity of their effects on the host, with the exception of *R. akari*, the causative agent of Rickettsialpox, which principally targets macrophages (Walker et al., 2007). Thus, every step of the growth of rickettsiae in host cells could affect their pathogenicity. The events involved in host cell infection by rickettsiae are summarized in **Figure 4**. The first steps involve the adherence of the rickettsiae to host cells and their subsequent invasion of these cells, since *Rickettsia* are obligate intracellular parasitic bacteria. Internalization occurs within minutes, and rickettsiae escape from phagosomes into the cytoplasm via the phospholipase activities of hemolysin C (TlyC) and phospholipase D (Pld; Teysseire et al., 1995; Whitworth et al., 2005). It has been clarified that among the 17 subfamilies of Sca autotransporter proteins, rOmpA (=Sca0), and rOmpB (=Sca5) are involved in host cell adherence and invasion by rickettsiae. rOmpA is one of the major surface antigen proteins of SFGR, and treatment with the antibody against rOmpA or immunization with recombinant rOmpA protected animal models against infection by rickettsiae (Anacker et al., 1985, 1987; McDonald et al., 1987; Li et al., 1988; McDonald et al., 1988; Vishwanath et al., 1990; Sumner et al., 1995; Crocquet-Valdes et al., 2001). The role of rOmpA in the adherence of rickettsiae to host cells has also been examined using cultured cells (Li and Walker, 1998). However, TG rickettsiae do not possess rOmpA, although a remnant (369 bp) of its ORF (6,063 bp) still exists in the equivalent region in *R. prowazekii*. rOmpB, which is the only major surface antigen protein common to the genus *Rickettsia*, was also confirmed to play roles in host cell adherence and invasion by rickettsiae in studies using *Escherichia coli* expressing recombinant *R. japonica* rOmpB on their surface (Uchiyama, 1999; Uchiyama et al., 2006; Chan et al., 2009). rOmpB is well conserved among the *Rickettsia* genus

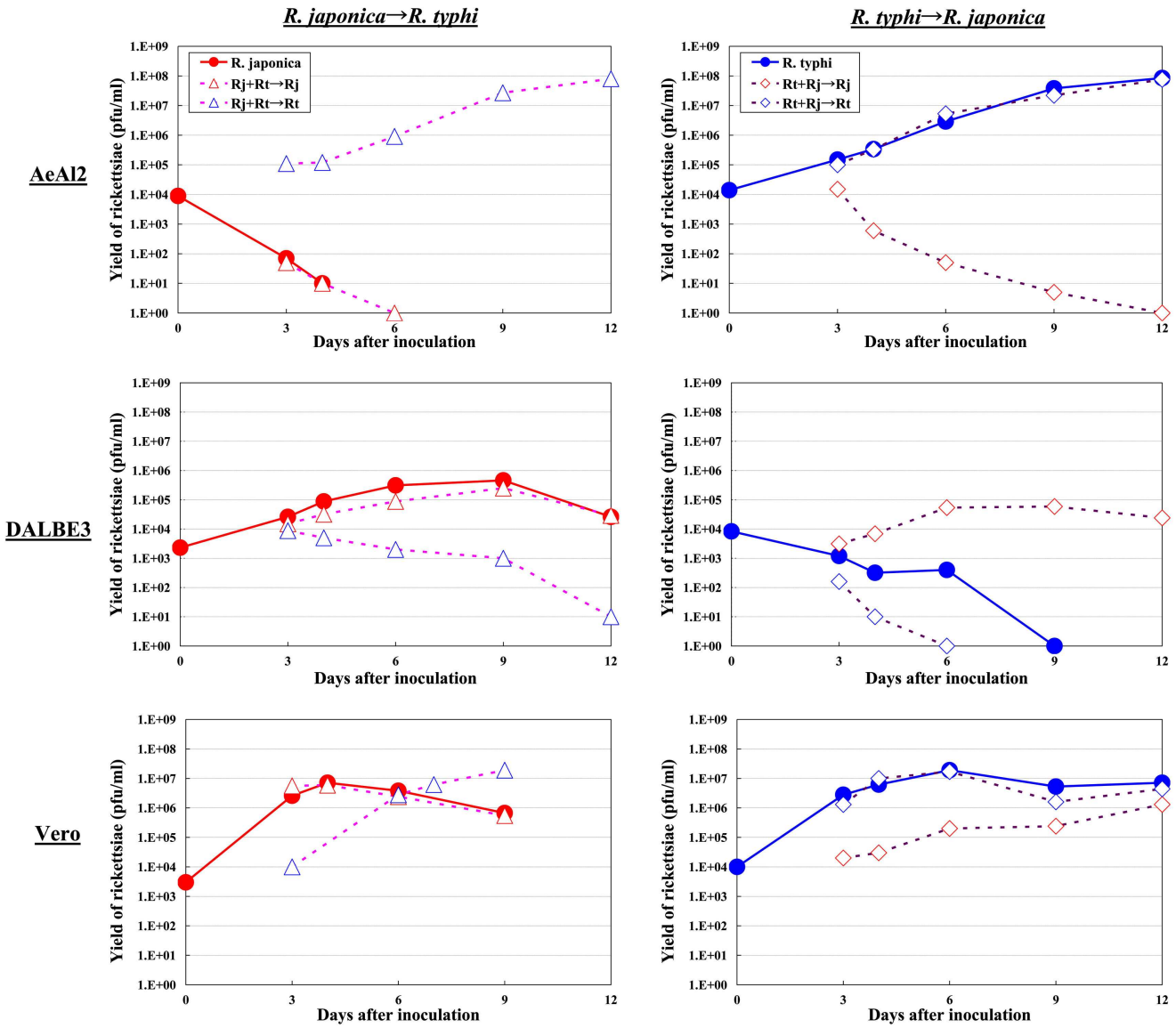

FIGURE 2 | Growth kinetics of SFGR and TGR in insect, tick, and mammalian cells. Various cultured cells were infected with SFGR alone or TGR alone, and the yield of rickettsiae was monitored. Some of the infected cultures were superinfected with TG or SFGR, respectively, on day three of infection, and the growth of each *Rickettsia* species was monitored.

including SFGR and TGR, e.g., the rOmpB amino acid sequences of *R. prowazekii* and *R. conorii* share 70% homology, which might reflect the importance of the molecule for rickettsial growth (Carl et al., 1990; Gilmore et al., 1991; Hahn et al., 1993). rOmpB might also play other roles, e.g., in the maintenance of the structure of the bacteria or as a molecular sieve, etc. rOmpB associates with Ku70 on the plasma membrane (Martinez et al., 2005), and this interaction is sufficient to mediate the rickettsial invasion of non-phagocytic host cells (Chan et al., 2009). Clathrin and caveolin-2-dependent endocytosis are responsible for the internalization of rickettsiae. The recruitment of c-Cbl, a ubiquitin ligase, to the entry site is also required for the ubiquitination of Ku70 (Martinez et al., 2005). *R. conorii* enters non-phagocytic cells via an Arp2/3 complex-dependent pathway (Martinez and Cossart, 2004). Pathways involving Cdc42, phosphoinositide 3-kinase, c-Src, cortactin, and tyrosine-phosphorylated proteins activate Arp2/3, resulting in localized actin rearrangement during rickettsial entry. Furthermore, activation of the *p*38 mitogen-activated protein kinase module facilitates host cell invasion by *R. rickettsii in vitro* (Rydkina et al., 2005, 2008). Recently, it has been clarified that some of the other outer membrane proteins belonging to the Sca family, Sca1, and Sca2, also play roles in host cell adherence and invasion by rickettsiae (Cardwell and Martinez, 2009; Riley et al., 2010). Overlay assays involving biotinylated EC, 2D-PAGE, and mass spectrometry have demonstrated that the β-peptide, Adr1, and Adr2 are also putative rickettsial adhesins (Renesto et al., 2006).

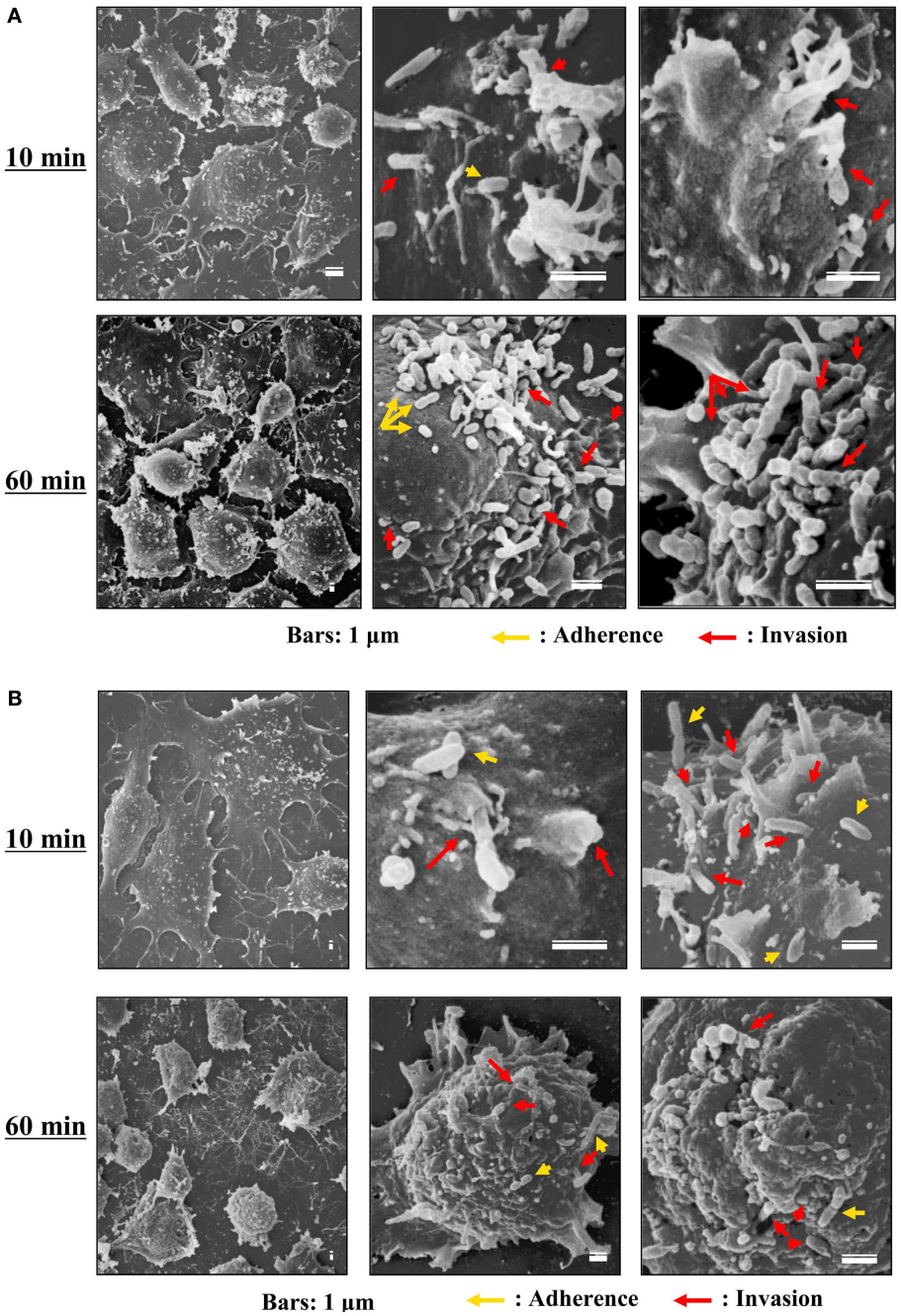

FIGURE 3 | Scanning electron microscopy of cells infected with TGR and SFGR. (A) AeAl2 cells infected with *R. typhi* at 10 and 60 min after infection. **(B)** AeAl2 cells infected with *R. japonica* at 10 and 60 min after infection. Successful adherence to and invasion of AeAl2 insect cells was achieved by both TGR and SFGR soon after their inoculation. The yellow and red arrows indicate adherent and invading rickettsiae, respectively.

In addition to studies of these early events during the course of rickettsial infection, there have been many genomic analyses of the pathogenicity of rickettsiae (Andersson et al., 1998; Li and Walker, 1998; Ogata et al., 2001; Uchiyama, 2003; Joshi et al., 2004; Sahni et al., 2005; Whitworth et al., 2005; Uchiyama et al., 2006; Chan et al., 2009; Fournier et al., 2009; Clark et al., 2011). Rickettsial genomes possess homologs of the *virB* operon, which is known to be related to the type IV secretion system (T4SS) and might be associated with rickettsial pathogenicity. It was reported that Vero cells that had been infected with *R. conorii* displayed upregulated *virB* operon expression when they were exposed to nutrient stress (La et al., 2007). The factors secreted by the T4SS, such as Sec7, LepA, LepB, and patatins, might upregulate the synthesis of nutrients that allow rickettsiae to survive in stressful

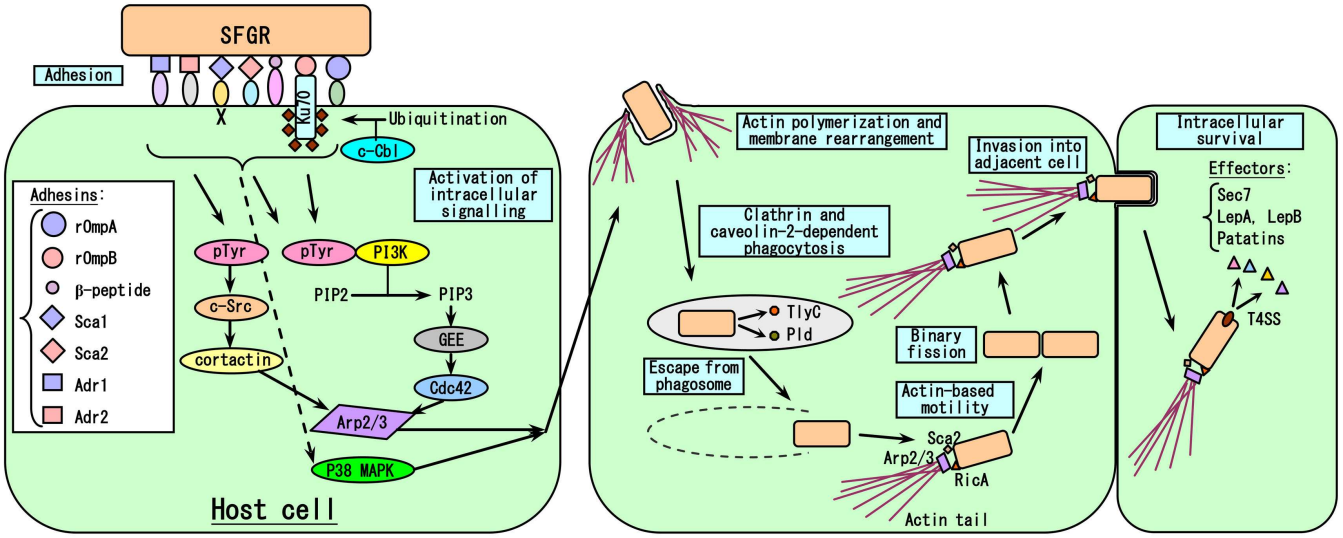

FIGURE 4 | Model of the rickettsia-host cell interactions that occur during the course of infection. The first step in SFGR entry into host cells is the adhesion of rickettsiae to cells due to the binding of many rickettsial adhesins to host cell receptors, followed by the activation of intracellular signaling pathways that induce actin polymerization and membrane rearrangement, causing the attached rickettsiae to be engulfed. Just after clathrin and caveolin-2-dependent phagocytosis, rickettsiae escape from the phagosomes that engulfed them by secreting the phospholipases TlyC and Pld. In the case of SFGR, the surface molecules RicA and Sca2 recruit Arp2/3 to polymerize actin, resulting in the formation of an actin tail, which aids the movement of the bacteria. However, in the case of TGR, *R. prowazekii* does not have an actin tail, while *R. typhi* has a very short actin tail. SFGR invade the adjacent cells very early in the course of the infection. Rickettsiae grow within cells by binary fission. The VirB-related T4SS is essential for the intracellular survival of rickettsiae as it allows them to secrete effector molecules.

environments. Moreover, of the ten *Staphylococcus aureus* genes (*capA–M*) involved in the biosynthesis of capsular polysaccharides, which are related to pathogenicity, three have homologs in the *Rickettsia* genome (Lin et al., 1994).

The infection of cultured human EC with *R. rickettsii* induced the early cell-to-cell spread of the bacteria, resulting in widespread membrane damage and finally cell death (Silverman, 1984). However, EC are not only injured by infection, but also initiate cellular responses such as endothelial activation. Specifically, the infection of EC with *R. rickettsii* or *R. conorii* induces surface platelet adhesion (Silverman, 1986); the release of von Willebrand factor from Weibel–Palade bodies (Sporn et al., 1991; Teysseire et al., 1992); and increased expression of tissue factor (Teysseire et al., 1992; Sporn et al., 1994), E-selectin (Sporn et al., 1993), IL-1α (Kaplanski et al., 1995; Sporn and Marder, 1996), cell-adhesion molecules (Dignat-George et al., 1997), and plasminogen activator inhibitor-1 (Drancourt et al., 1990; Shi et al., 2000). The infection of EC with the TGR *R. prowazekii* resulted in enhanced prostaglandin secretion (Walker et al., 1990). Thus, EC infected with rickettsiae demonstrate procoagulant and proinflammatory features, which might contribute to the severity of rickettsioses. Rickettsial infection of EC also activates nuclear factor (NF)-κB, which inhibits apoptosis and mediates the production of the proinflammatory cytokines IL-6, IL-8, and monocyte chemoattractant protein 1 (Joshi et al., 2004; Bechelli et al., 2009). Conversely, EC activated by IFN-γ, TNF-α, IL-1β, or RANTES degenerate intracellular rickettsiae through nitric oxide production and hydrogen peroxide production (Valbuena et al., 2002; Rydkina et al., 2004).

A comparative study of rickettsial genomes suggested that the inactivation of some genes by genome reduction during the course of their evolution abrogated host-induced rickettsial growth restriction (Blanc et al., 2007). In fact, a conflicting relationship was detected between a smaller genome size and increased pathogenicity in rickettsiae, e.g., *R. prowazekii,* which possesses a smaller genome, causes more severe symptoms than *Rickettsia* species with larger genomes such as *R. conorii* (Fournier et al., 2009; Botelho-Nevers and Raoult, 2011). A comparison of the growth of the virulent and avirulent strains of *R. rickettsii* revealed that the relA/spoT gene is essential for growth restriction (Clark et al., 2011).

The growth kinetics of pathogenic rickettsiae in mammalian cells were compared with those of non-pathogenic rickettsiae. Vero and HeLa cells derived from mammals were inoculated with a non-pathogenic species of SFGR, *R. montanensis* (Bell et al., 1963; Uchiyama et al., 2012). The growth of *R. montanensis* in the mammalian cells was restricted; however, the infection was persistent, and low levels of rickettsiae were produced throughout its course (**Figure 5**). On the other hand, superinfection of the *R. montanensis*-infected cells with the pathogenic *R. japonica* resulted in increased yields of the non-pathogenic *R. montanensis* and *R. japonica* growth. Western blotting confirmed that autophagy had been induced in the cells infected with *R. montanensis* alone. On the contrary, autophagy was restricted in the *R. montanensis*-infected cells that had been superinfected with pathogenic *R. japonica*. These results were consistent with the findings of ultrastructural observations (**Figure 6**). Thus, it is suggested

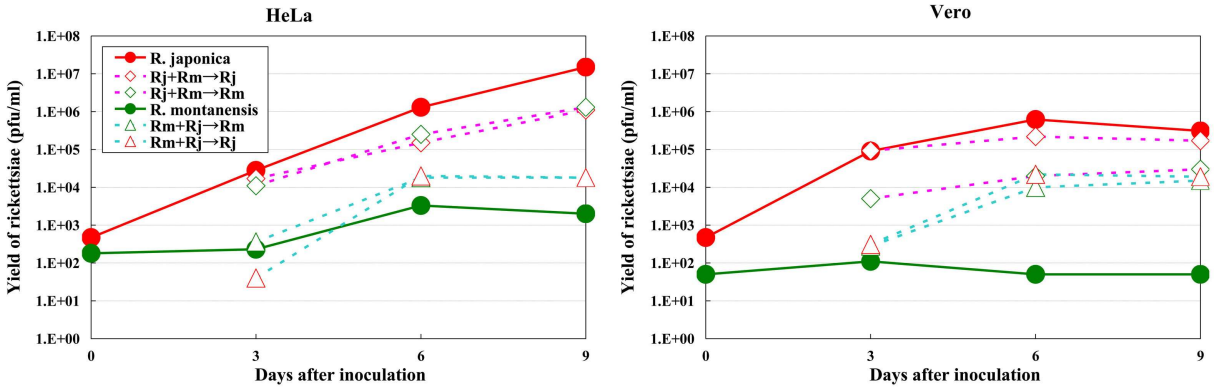

FIGURE 5 | Growth kinetics of non-pathogenic and pathogenic SFGR in mammalian cells. The growth of non-pathogenic and pathogenic rickettsiae was monitored. Some of the cells that had been infected with non-pathogenic *R. montanensis* were superinfected with pathogenic *R. japonica* on day three of infection, and the growth of each *Rickettsia* was monitored. The growth of non-pathogenic SFGR was restricted in mammalian cells. The superinfection of the infected cells with pathogenic SFGR induced an elevated yield of the non-pathogenic SFGR and the growth of the pathogenic species.

that the growth restriction of the non-pathogenic species, *R. montanensis*, was at least partly due to the occurrence of autophagy in the infected cells and that the pathogenic species, *R. japonica*, might secrete an unidentified autophagy restriction factor(s). Although autophagy is one of the innate defense systems against invading microbes, other pathogenic bacteria that display intracellular growth, such as *Shigella*, *Listeria*, and *Burkholderia*, also possess mechanisms for escaping from autophagic degeneration (Sasakawa, 2010). *Shigella* escapes from autophagic recognition by secreting IcsB via the type III secretion system (TTSS; Ogawa et al., 2005). *Listeria* recruits the Arp2/3 complex and Ena/VASP to its surface via the bacterial ActA protein and disguises them from autophagic recognition (Yoshikawa et al., 2009), and *Burkholderia* secretes the BopA protein via the TTSS to evade autophagy (Cullinane et al., 2008). It is also known that the BopA protein shares 23% homology with IcsB of *Shigella*. Another of the putatively non-pathogenic SFGR, *Rickettsia* sp. LON, which was isolated from *Haemaphysalis longicornis* (a tick), but has never been isolated from human spotted fever patients in Japan (Fujita, 2008; Hanaoka et al., 2009), is genetically closely related to *R. japonica* and in fact is classified within the *R. japonica* group. Its growth in mammalian cells was examined in a recent study (Uchiyama and Fujita, 2012). The growth of *Rickettsia* sp. LON is restricted in mammalian cells, as was found for *R. montanensis*. However, its growth can be recovered by superinfection of the pathogenic *R. japonica*. These results further strengthen the hypothesis that the degree of rickettsial growth in mammalian cells basically determines the pathogenicity of *Rickettsia*. Another non-pathogenic *Rickettsia*, *R. peacockii*, which is also known as the East Side agent, was isolated from Rocky Mountain Wood ticks (*Dermacentor andersoni*) from Montana, USA (Bell et al., 1963; Burgdorfer et al., 1981). *R. rickettsii*-carrying *D. andersoni* display a markedly reduced prevalence on the east side of the Bitterroot Valley, while Rocky Mountain spotted fever predominantly occurs on the west side of the valley (Philip and Casper, 1981). Thus, the presence of *R. peacockii* in *D. andersoni* ticks might prevent the transovarial transmission of *R. rickettsii* and limit its spread in the tick population, although it is uncertain whether *R. peacockii* actively interferes with *R. rickettsii* in ticks or whether ticks carrying *R. peacockii* have a reproductive advantage over those carrying *R. rickettsii*. *R. rickettsii* has been demonstrated to have a lethal effect on its tick vector *D. andersoni* (Niebylski et al., 1999). A comparative study has also been performed of the genome sequences of the pathogenic *R. rickettsii* and the non-pathogenic *R. peacockii* (Niebylski et al., 1997; Felsheim et al., 2009). In *R. peacockii*, the genes encoding an ankyrin repeat containing protein, DsbA, RickA, protease II, rOmpA, Sca1, and a putative phosphoethanolamine transferase, which are related to its pathogenicity, were deleted or mutated. The gene coding for the ankyrin repeat containing protein is especially noteworthy as it is also mutated in the attenuated Iowa strain of *R. rickettsii*. The precise mechanisms by which these factors contribute to the pathogenicity of SFGR are yet to be clarified.

PERSPECTIVES

The vector tropism of rickettsiae seems to correspond with their growth in cultured mammalian cells. It has been clarified that the growth restriction of SFGR in AeAl2 cells depends on the non-apoptotic cell death induced after host cell adherence and invasion by rickettsiae. It is important to analyze the mechanisms responsible for this cell death and the cell death inhibition observed in AeAl2 cells infected with TGR. Moreover, the mechanisms responsible for the restriction of TGR growth in tick cells and the abrogation of the growth restriction in tick cells infected with SFGR also need to be elucidated.

A relationship was detected between the ability of *Rickettsia* species to grow in cultured mammalian cells and their pathogenicity; however, the growth abilities of *Rickettsia* species are affected by many host and rickettsial factors during the various stages of rickettsial infection. In order to elucidate the mechanisms governing rickettsiae pathogenicity, it is necessary to compare these factors between pathogenic and non-pathogenic strains.

Although I have attempted to elucidate the relationships between various rickettsiae species and cell types in this review, it is also necessary to clarify the roles of innate and acquired immunity against rickettsiae infection.

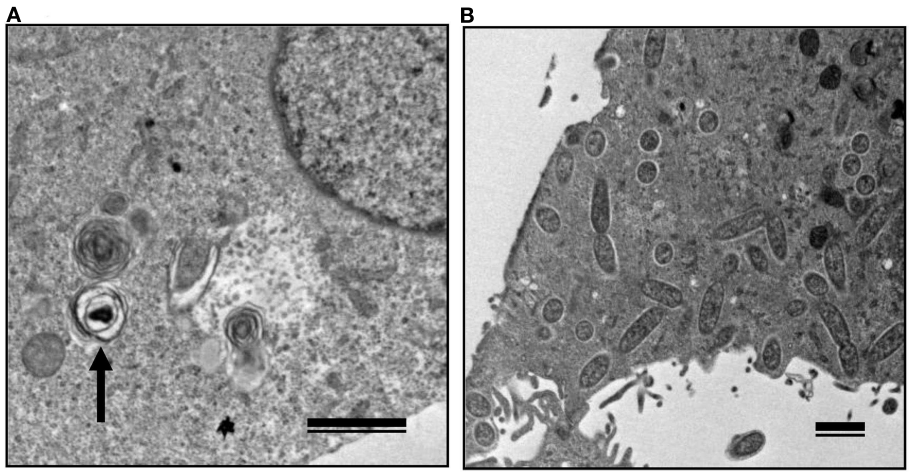

FIGURE 6 | Transmission electron microscopy of Vero cells infected with non-pathogenic and pathogenic SFGR. (A) Vero cells infected with *R. montanensis* alone was observed at 7 days after infection. An arrow marks a degenerating rickettsia in an autophagosome-like vacuole. **(B)** *R. montanensis*-infected cells were superinfected with *R. japonica* on day three of infection and observed at 7 days after the first infection. Many free rickettsiae around 1 μm in length surrounded by halos and those in the course of binary fission were seen in the cytoplasm. Bars, 1 μm.

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