ROLE OF LIPIDS IN VIRUS ASSEMBLY

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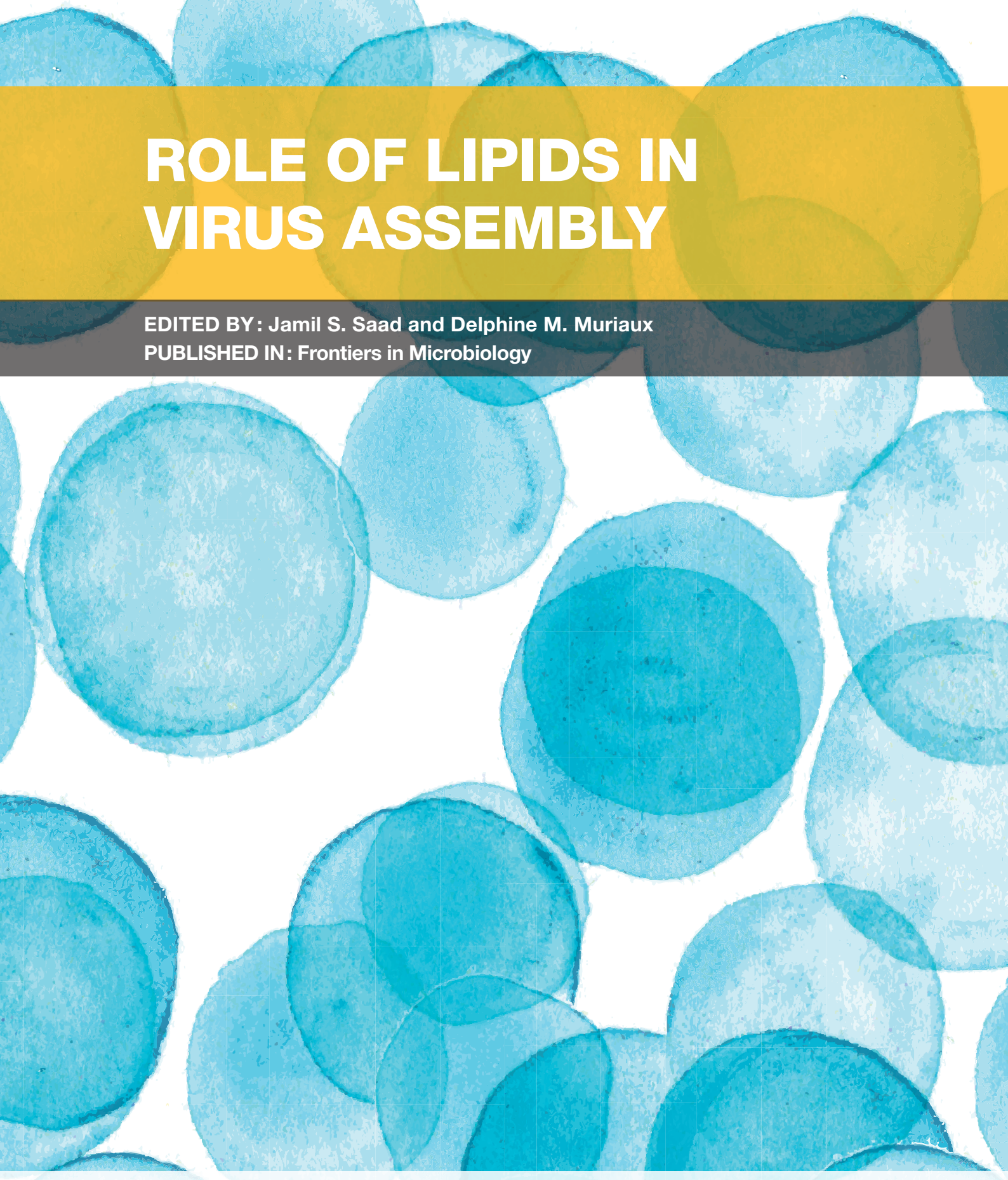

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ROLE OF LIPIDS IN VIRUS ASSEMBLY

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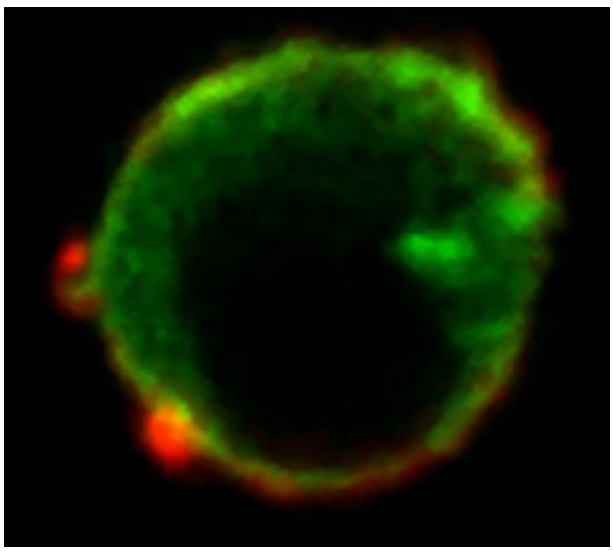

Jurkat T cells expressing HIV-1 Gag-GFP, fixed and labelled with an anti-PI(4,5)P2 antiboby coupled to a Alex555 secondary antibody, and analyzed by confocal fluorescence microscopy imaging. Gag is localized in the cytosol and at the cell plasma membrane (green) whereas PI(4,5)P2 is present only at the cell plasma membrane. Several articles in this special topic address the molecular determinants of Gag-membrane interactions not only for human immunodeficiency virus type-1 (HIV-1) but also for other retroviruses such as Rous sarcoma virus (RSV), Mason-Pfizer monkey virus (M-PMV), and human T-lymphotropic virus type (HTLV-1).
Image by Delphine Muriaux

RNA enveloped viruses comprise several families belonging to plus and minus strand RNA viruses, such as retroviruses, flavoviruses and orthomyxoviruses. Viruses utilize cellular lipids during critical steps of replication like entry, assembly and egress. Growing evidence indicate important roles for lipids and lipid nanodomains in virus assembly. This special topic covers key aspects of virus-membrane interactions during assembly and egress, especially those of retroviruses and Ebola virus (EBOV). Virus assembly and release involve specific and nonspecific interactions between viral proteins and membrane compartments. Retroviral

Gag proteins assemble predominantly on the PM. Despite the great progress in identifying the factors that modulate retroviral Gag assembly on the PM, there are still gaps in our understanding of precise mechanisms of Gag-membrane interactions. Studies over the last two decades have focused on the mechanisms by which other retroviral Gag proteins interact with membranes during assembly. These include human immunodeficiency virus (HIV), Rous sarcoma virus (RSV), equine infectious anemia virus (EIAV), Mason-Pfizer monkey virus (M-PMV), murine leukemia virus (MLV), and human T-lymphotropic virus type (HTLV-1). Additionally, assembly of filoviruses such as EBOV also occurs on the inner leaflet of the PM. The articles published under this special topic highlight the latest understanding of the role of membrane lipids during virus assembly, egress and release.

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EDITORIAL
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Editorial: Role of lipids in virus assembly

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Keywords: retroviruses, HIV-1, Gag, Matrix, membrane, NMR, Ebola, PI(4, 5)P2

Viruses utilize cellular lipids during critical steps of replication like entry, assembly, and egress. Growing evidence indicate important roles for lipids and lipid nanodomains in virus assembly. This special topic covers key aspects of virus-membrane interactions during assembly and egress of two classes, retroviruses and filoviruses. It discusses molecular mechanisms of assembly and budding of retroviruses and Ebola virus (EBOV) and how various membrane components facilitate these events. It is well established that assembly of most of retroviral Gag proteins occurs on the plasma membrane (PM) (Ono et al., 2004; Grigorov et al., 2006; Jouvenet et al., 2006, 2008; Finzi et al., 2007; Welsch et al., 2007; Chukkapalli et al., 2008, 2010; Chu et al., 2010; Hamard-Peron et al., 2010; Chukkapalli and Ono, 2011). Biochemical, *in vivo*, *in vitro*, and genetic data have identified factors that modulate retroviral Gag-membrane interactions. Studies over the last decade have provided insights on the molecular and structural determinants of Gag-membrane binding. The human immunodeficiency virus type-1 (HIV-1) Gag polyprotein adopts a compact "folded over" conformation and exists in the monomeric or low-order oligomeric states prior to targeting to the PM (Datta et al., 2007, 2011; Kutluay and Bieniasz, 2010; Kutluay et al., 2014). Although, it is established that the nucleocapsid (NC) domain of Gag recognizes motifs in the viral RNA genome to mediate packaging, there is compelling evidence that the matrix (MA) domain also binds to cellular RNA to prevent premature Gag targeting to intracellular membranes (Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011; Hogue et al., 2012; Inlora et al., 2014; Kutluay et al., 2014; Olety and Ono, 2014). Upon transport of Gag to the PM, the interaction of MA with RNA is exchanged for an interaction of MA with PM lipids, inducing an extended conformation of Gag and formation of high-order Gag oligomers on the PM. The key to understanding this essential switch is elucidating at the molecular level the interaction of MA with specific PM components. Several retroviruses like Rous sarcoma virus (RSV), equine infectious anemia virus (EIAV), Mason-Pfizer monkey virus (M-PMV), and human T-lymphotropic virus type (HTLV-1) have evolved distinct mechanisms for Gag membrane targeting and assembly. Our understanding of retroviral Gag-PM interaction is incomplete because of the lack of molecular details on how membrane components contribute to the overall membrane binding. Eight out of the nine articles discuss the latest understanding of retroviral Gag-membrane binding. Prchal et al. review the latest developments on the characterization of Mason-Pfizer monkey virus (M-PMV) Gag interactions with the PM (Prchal et al., 2014). M-PMV, which belongs to *Betaretroviruses*, first assembles into virus-like particles (VLPs) in the pericentriolar region of the infected cell and therefore. Structural details of M-PMV MA binding to single phospholipids are discussed. Dick et al. describe the principles that govern Gag interactions with membranes, focusing on RSV and HIV-1 Gag (Dick and Vogt, 2014). The review defines lipid and membrane behavior, and discusses the complexities in determining how lipid and membrane behavior impact Gag membrane binding. Yandrapalli et al. review the role of plasma membrane lipids in HIV-1 Gag targeting and assembly, mainly focusing on membrane biophysics (Yandrapalli et al., 2014). Studies identified the 1,4,5-inositol trisphosphate receptor (IP_3R), a channel mediating release of Ca^{2+} from ER stores, as a cellular factor differentially associated with HIV-1 Gag that might facilitate ESCRT function in virus budding. In a research

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article, Ehrlich et al. show that Gag modulates ER store gating and refilling (Ehrlich et al., 2014). It is shown that Gag accumulation at the plasma membrane required continuous IP_3R activation. Elevation of Ca^{2+} level in the immediate vicinity of the plasma membrane is suggested to drive events that lead to stable membrane localization of assembling Gag. In their review, Alfadhli et al. focus on the functions of retroviral MA proteins, with an emphasis on the nucleic acid-binding capability of the HIV-1 MA protein and its effects on membrane binding (Alfadhli and Barklis, 2014). A review by Maldonado et al. discusses not only retroviral Gag-membrane interactions but also how Gag-Gag interactions contribute to the overall assembly process (Maldonado et al., 2014). Differences among retroviruses in Gag–Gag and Gag–membrane interactions implying various molecular aspects of the viral assembly pathway are described. Mariani et al. discuss the role of Gag and lipids during HIV-1 assembly in CD^{4+} T cells and macrophages (Mariani et al., 2014). Whereas, HIV-1 assembly and budding in macrophages is thought to follow the same general Gag-driven mechanism as in T-lymphocytes, the HIV-1 cycle in macrophage exhibits specific features. How Gag interacts with membrane lipids and what are the mechanisms involved in the interaction between the different membrane nanodomains within the assembly platform are not fully understood. Vlach et al. discuss the structural and molecular determinants of HIV-1 Gag binding to the plasma membrane (Vlach and Saad, 2015). This review emphasizes the structural findings on HIV-1 and HIV-2 MA binding to PM lipids and how these studies may advance our understanding of the overall Gag-membrane binding mechanism. The ninth article of this issue discusses the assembly and budding mechanisms of filoviruses including Marbug (MARV) and EBOV viruses (Stahelin, 2014). EBOV budding occurs from the inner leaflet of the plasma membrane (PM) and is driven by the matrix protein VP40, which binds to anionic lipid membranes. The review by Stahelin describes what is known regarding VP40 membrane interactions and what answers will fill the gaps. Collectively, the articles published under this special topic remarkably enriched our understanding of the role of membrane lipids during assembly, egress and release.

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Interaction of Mason-Pfizer monkey virus matrix protein with plasma membrane

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Budding is the final step of the late phase of retroviral life cycle. It begins with the interaction of Gag precursor with plasma membrane (PM) through its N-terminal domain, the matrix protein (MA). However, single genera of *Retroviridae* family differ in the way how they interact with PM. While in case of *Lentiviruses* (e.g., human immunodeficiency virus) the structural polyprotein precursor Gag interacts with cellular membrane prior to the assembly, *Betaretroviruses* [Mason-Pfizer monkey virus (M-PMV)] first assemble their virus-like particles (VLPs) in the pericentriolar region of the infected cell and therefore, already assembled particles interact with the membrane. Although both these types of retroviruses use similar mechanism of the interaction of Gag with the membrane, the difference in the site of assembly leads to some differences in the mechanism of the interaction. Here we describe the interaction of M-PMV MA with PM with emphasis on the structural aspects of the interaction with single phospholipids.

Keywords: Retrovirus, Mason-Pfizer monkey virus, matrix protein, phospholipids, interaction, plasma membrane

In the late phase of retroviral life cycle, all structural proteins are produced in a host cell as a polyprotein precursor Gag. Such pre-arrangement ensures their equimolar incorporation and proper functioning in a viral particle (Hunter, 1994). Gag proteins of most retroviruses (formerly described as C-type retroviruses) are immediately after the synthesis transported to the plasma surface where they interact with the plasma membrane (PM) and assemble to an immature viral particle simultaneously with its budding.

Mason-Pfizer monkey virus (M-PMV) belongs to *Betaretrovirus* genus of *Orthoretroviridae* subfamily and is also reffered to as SRV type 3(Montiel, 2010). It is a simple simian exogenous, non-transforming, horizontally transferred retrovirus, which causes failure of the immune system of the infected animal. Initially it was isolated from breast tumor of rhesus monkey (*Macaca mulatta*), but as it was learned shortly after its discovery, it is not the direct cause of a carcinoma development (Chopra and Mason, 1970). Although it causes similar disease as simian immunodeficiency virus (SIV), it is not related to it and belongs to different genus. M-PMV was formerly described as B/D-type retrovirus, which means that its Gag proteins are first transported to a periplasmic region of the cell, where they assemble (Vlach et al., 2008). Resulting immature virus particle is then transported to the PM where budding occurs. The membrane interaction of Gag proteins, as well as intracellular transport is facilitated by their N-terminal domain – the matrix protein (MA). MA is localized on the surface of the virus particle and remains associated with the virus membrane after maturation of Gag, which is cleaved by viral protease to individual structural proteins. MAs of most retroviruses are N-terminally myristoylated.

The interaction of MAs with the PM is enabled by a bipartite signal which consists of the myristoyl and a surface displayed patch of basic residues, mostly arginines and lysines. This is a canonical arrangement of the binding epitope shared by most retroviral proteins interacting with phospholipids (Bryant and Ratner, 1990; Zhou et al., 1994; Freed, 1998). While positively charged amino acids of MA interact with phosphate groups of the membrane, the myristic acid is in a close contact with phospholipid long aliphatic chains. Both interactions are additive, i.e., they contribute to the overall affinity of MA toward the PM. The interaction of the myristoyl with the membrane is not strong enough to mediate the membrane binding of MA (Gag) without contribution of other forces (Peitzsch and Mclaughlin, 1993). The basic residues on the surfaces of lipid binding proteins warrant this function as they are responsible for non-specific electrostatic interactions with negatively charged polar heads of phospholipids. However, differently phosphorylated phosphoinositides are present in membranes of various cellular organelles serving as specific markers which are recognized by numerous cargo transferring proteins (Roth, 2004).

Myristoylated human immunodeficiency virus (HIV-1) MA interacts with the membrane by using a mechanism called myristoyl switch (Zhou and Resh, 1996). In cytosol or *in vitro*, the myristoyl of MA is sequestered inside the protein core. However, it is released and serves as one of the interaction epitopes of Gag (virions) upon approaching the membrane of infected cell. The process must be carefully controlled to ensure both the binding of MA to the membrane to enable budding, however, loose enough to allow release of the mature virus from its membrane during the early phase of infection.

In retroviruses, the mechanism was well described for the interaction of HIV-1 MA with PM (Zhou and Resh, 1996; Tang et al., 2004; Saad et al., 2006). Saad reported that the switch was triggered by the interaction with phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], a phospholipid present exclusively in the PM. $PI(4,5)P_2$ binds to a binding site on the surface of MA

and causes myristoyl to be released from the protein and ready for binding. The interaction of HIV-1 MA with $PI(4,5)P_2$ composed of shorter fatty-acid chains (4 and 8 carbons in length), was experimentally proved as suitable for solution nuclear magnetic resonance (NMR) measurements because these soluble $PI(4,5)P_2$ bind in a cleft between the second and fifth helix. The binding has also been confirmed for phosphatidylinositolphosphates (PIP) containing natural fatty-acid residues (C_{18} and C_{20}) either by interaction of MA with artificial liposomes mimicking PM or by blocking $PI(4,5)P_2$ synthesis leading to the HIV-1 virus particles to be unable to assembly on PM (Chukkapalli et al., 2008). The interaction of PIP was also proved for other retroviruses: HIV-2, moloney murine leukemia virus (MoMuLV) and equine infectious anemia virus (EIAV). HIV-2 MA interacts with PIP in a similar way as HIV-1 MA, but it was reported that the interaction with neither C_4 nor C_8 $PI(4,5)P_2$ leads to the release of the myristate (Saad et al., 2008). The authors concluded that the reason was a weaker affinity of $PI(4,5)P_2$ to the HIV-2 MA and further speculated that the rationale behind this phenomenon might be that HIV-2 is less infectious than HIV-1. Both HIV-1 and HIV-2 show stronger preference for $PI(4,5)P_2$ compared to the other, differently phosphorylated PIPs. EIAV MA is naturally non-myristoylated, so its interaction is fully dependent on the interaction of basic amino-acid residues with membrane phospholipids (Chen et al., 2008). Chen has reported that $PI(4,5)P_2$ specifically interacts with EIAV MA and also induces its oligomerization, which promotes the assembly of virus particle. MoMuLV MA also interacts with PIPs, but without any discrimination of $PI(4,5)P_2$. However, in the presence of phosphatidylserine, it exhibits stronger and more specific interaction over other differently phosphorylated PIPs (Hamard-Peron et al., 2010). Similar behavior, i.e., preferential and stronger binding of a chosen phosphoinositide in the presence of other phospholipids, mostly in the form of micelles was also described for proteins bearing pleckstrin homology domain (Sugiki et al., 2012). An important role of different phospholipids for the interaction of HIV-1 MA with the PM has been proposed recently by Vlach and Saad (2013). They found that phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine bound to HIV-1 MA, however, to a different binding site than $PI(4,5)P_2$ and that the interaction was weaker. The authors concluded that this interaction further stabilizes the binding of MA to the membrane.

The first evidence of the interaction of M-PMV MA with $PI(4,5)P_2$ was reported by Stansell et al. (2007). She observed that depletion of $PI(4,5)P_2$ from PM by overexpression of active form of PI-5-phosphatase IV led to 90% decrease of particles release from M-PMV infected cells. Direct evidence of the interaction of M-PMV MA with $PI(4,5)P_2$ was then confirmed by Prchal et al. (2012).

Similarly to HIV-1 and HIV-2 MAs, the interaction of M-PMV MA with $PI(4,5)P_2$ was studied using NMR spectroscopy and soluble forms of $PI(4,5)P_2$ with 4 and 8 carbon fatty-acids. While dibutanoyl $PI(4,5)P_2$ did not interact, dioctanoyl $PI(4,5)P_2$ interacted specifically with K_D of about 100 μM, which is a comparable affinity as that of the interaction of HIV-1 MA with C_4-$PI(4,5)P_2$ (Saad et al., 2006). Similarly as for HIV-2, the interaction did not trigger the myristoyl switch.

The M-PMV MA molecule contains one PIP binding site located between the first, second and fourth helices (**Figure 1**). Comparison of the structures of the myristoylated and non-myristoylated M-PMV MAs showed that this binding site is present only on the surface of the myristoylated protein. Due to a slightly different orientation of the helices in the structure of the non-myristoylated MA, the proper binding pocket is inaccessible for PIP.

The PIP binding site follows the canonical shape of epitopes for binding phosphoinositides, i.e., it is composed of a hydrophobic pocket formed by all four helices and a patch of basic residues on the surface (Roth, 2004). This pocket is connected with the cavity where the myristoyl is sequestered. One of PIP's fatty-acid chains is buried inside this hydrophobic pocket while the phosphates interact with positively charged amino acids forming the basic patch. The structure has been solved only for the complex of MA with C_8-PIP, so it might be expected that one of the naturally long PIP's fatty-acid chains will somehow interfere with the myristoyl which might lead to its exposure from the cavity. The $PI(4,5)P_2$ molecule is sequestered deeper in the protein core, compared to HIV-1 MA, where $PI(4,5)P_2$ remains on the surface of MA (**Figure 2**).The surface part of the interaction site is formed mainly by lysines and arginines from the loop between the first and second helices and terminal parts of the first, second and fourth helices. The electrostatic interaction between positively charged lysine residues (K16, K25, K27, K33, and K74) and negatively charged inositol phosphate groups is important for the interaction of M-PMV MA with the membrane, as it was proven by mutation studies (Stansell et al., 2007). Stansell found that mutations of basic residues in the proximity of PIP binding site influenced both the transport of immature viral particles and their binding to PM. Virus-like particles (VLPs) bearing mutations K16A or K20A budded into intracellular vesicles. This may indicate that the mutations disrupted the recognition

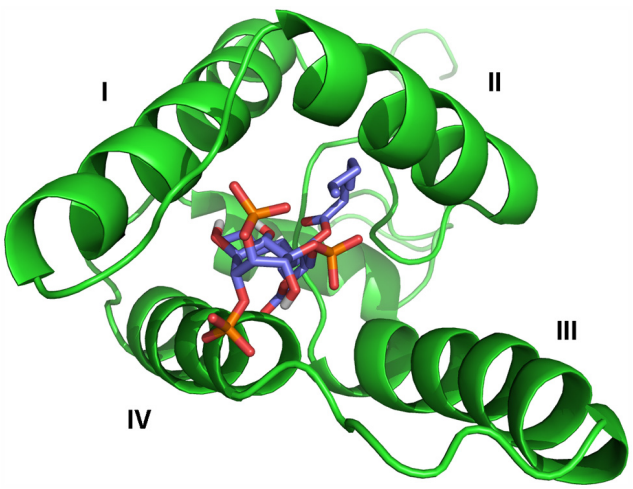

FIGURE 1 | Structural model of the myristoylated MA – C_8-$PI(4,5)P_2$ complex. MA is shown in green, with helices numbered by roman numbers. C_8-$PI(4,5)P_2$ molecule colors correspond to its composition – carbons are shown in blue, oxygens in red and phosphors in orange.

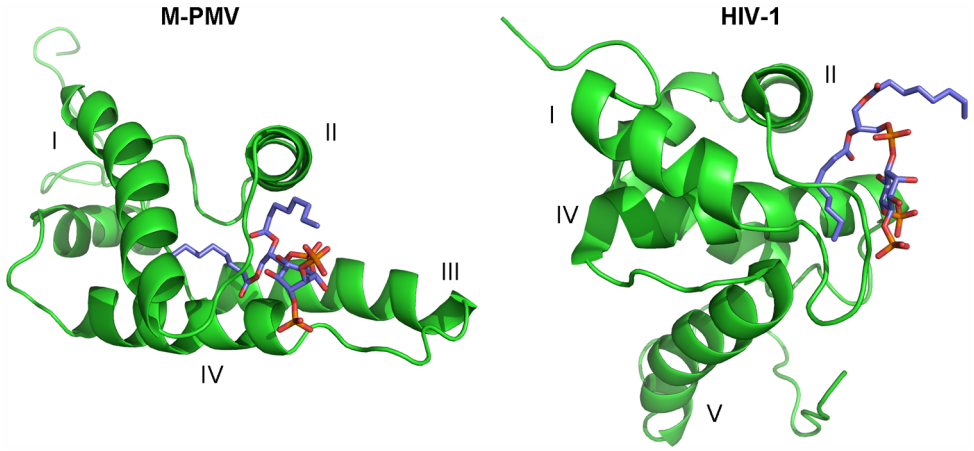

FIGURE 2 | The comparison of the myristoylated M-PMV MA – C_8-PI(4,5)P_2 and HIV-1 MA C_4-PI(4,5)P_2 complex structures. MAs are shown in green, with helices numbered by roman numbers. C_8-PI(4,5)P_2 molecule colors corresponds to their composition – carbons are shown in blue, oxygens in red and phosphors in orange.

of the target membrane, likely by changing the affinity of MA for differently phosphorylated PIPs than PI(4,5)P_2. VLPs bearing R10A, R22A, K27A, K33A, or K39A mutations were accumulated near the PM, indicating that the mutations prevented the interaction of MA with PI(4,5)P_2, or other phospholipids in the membrane. The mutation K25A disrupted some early stages of VLP transport, since they were randomly distributed in the cytoplasm.

Accumulation of VLPs near PM can also be caused by mutations of non-basic amino-acid residues in M-PMV MA. Double mutations T41I/T78I, Y11F/Y28F, and Y28F/Y67F blocked the release of VLPs from the host cell, while single mutations only slowed down the release of VLPs, but failed to fully arrest it (except of T41I mutation, that showed wt-like virus release; Rhee and Hunter, 1991; Stansell et al., 2004). Since all these mutations introduce more hydrophobic amino acids, Stansell speculated that they created a pocket capable of stronger hydrophobic interactions of mutated residues with the myristoyl and thus block its release from the protein core and therefore, prevents the interaction with PM. However, our results based on the known structure of the complex between myristoylated MA and PI(4,5)P_2 show that all the mutated residues are too distant from the myristoyl to interact with it (except for T41) but they are part of the PIP binding site (Prchal et al., 2012). Therefore, it is more likely that the mutations rather prevent the interaction of MA with (membrane) phospholipids, than block the myristoyl switch due to a stronger hydrophobic interaction of the myristoyl with exchanged amino acids.

In summary, the MA interaction with the PM is an essential step of retroviral life cycle that allows virus release. A firm contact of Gag with the PM is mediated by the bipartite signal, where the key player is the interaction of MA with PI(4,5)P_2. This ensures the selectivity for the PM over the membranes of cellular organelles.

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Membrane interaction of retroviral Gag proteins

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Assembly of an infectious retroviral particle relies on multimerization of the Gag polyprotein at the inner leaflet of the plasma membrane. The three domains of Gag common to all retroviruses – MA, CA, and NC – provide the signals for membrane binding, assembly, and viral RNA packaging, respectively. These signals do not function independently of one another. For example, Gag multimerization enhances membrane binding and is more efficient when NC is interacting with RNA. MA binding to the plasma membrane is governed by several principles, including electrostatics, recognition of specific lipid head groups, hydrophobic interactions, and membrane order. HIV-1 uses many of these principles while Rous sarcoma virus (RSV) appears to use fewer. This review describes the principles that govern Gag interactions with membranes, focusing on RSV and HIV-1 Gag. The review also defines lipid and membrane behavior, and discusses the complexities in determining how lipid and membrane behavior impact Gag membrane binding.

Keywords: HIV-1, RSV, lipid, assembly, liquid ordered, plasma membrane

INTRODUCTION

In enveloped viruses the common function of the viral membrane is to help protect the genome. Having a membrane poses two challenges for the virus. First, the genome, in the form of a nucleocapsid, must acquire its membrane from the appropriate cell compartment that will allow virus release into the environment. Second, upon infection of a target cell, the nucleocapsid must escape the membrane surface. The latter process is mediated by viral surface glycoproteins that act as fusion machines. The former process for some viruses takes place after assembly of the nucleocapsid, while in other viruses occurs concomitantly with assembly. For retroviruses, the topic of this review, assembly typically is coupled with acquisition of the membrane, although with some exceptions.

For most retroviruses, expression of the single internal structural protein, Gag, is sufficient for assembly and budding of immature virus-like particles (VLPs) from the plasma membrane (PM). That Gag can seek out the PM and bud in diverse cell types – for example for HIV-1 in mammalian cells as well as in avian cells and insect cells – implies that Gag recognizes general features of the PM. Several exceptions to this generalization are well known. Spuma (foamy) viruses require expression of the viral envelope glycoprotein for budding to occur, and the specific interaction between Gag and Env that underlies this finding has recently been characterized (Goldstone et al., 2013). In this case the immature but not yet enveloped virus particle is assembled near the microtubule organizing center (MTOC), enveloped by budding through the endoplasmic reticulum, and then transported to the PM. Betaretroviruses (formerly called type B and type D retroviruses), for example Mason-Pfizer monkey virus (MPMV), pre-assemble an immature viral core near the MTOC, and the core itself is transported to the PM for envelopment there. These viruses are not considered further in this review. Rather, we focus on the most widely studied retroviruses in the genera called alpharetrovirus (e.g., Rous sarcoma virus, RSV in chickens), gammaretrovirus (e.g., murine leukemia virus in mice, MuLV), deltaretrovirus (e.g., human T-cell leukemia virus, HTLV), and lentiretrovirus (e.g., human immunodeficiency virus type 1, HIV-1). In fact, given the AIDS epidemic over the past two and a half decades and the extensive research it has spawned, more is known about HIV-1 than about any other retrovirus.

Immature retroviruses, i.e., before cleavage of Gag into its constituent domains by the viral protease, have a characteristic morphology as seen by thin section electron microscopy (EM). As early as the 1960s retroviruses were observed to bud from the PM of infected cells, and so the assumption reigned for many years that the PM was the site of assembly and budding. However, in the period ∼2000–2005 an alternative model for budding was suggested for at least some viruses and some cell types [e.g., HIV-1 in macrophages, (Pelchen-Matthews et al., 2003) or MuLV in 293T and HELA cells (Sherer et al., 2003)]. According to this model, some or all virus budding occurs into a late endosome compartment, followed by fusion of that organelle with the PM, releasing the virus particles. This model was based in part on EM observations in HIV-infected macrophages. However, as it turned out, macrophages have enormously convoluted infoldings of the PM, making it impossible to define by EM alone if a membrane is the PM or contiguous with the PM, or is topologically distinct. Although there may be some exceptions, the present view of wild type (wt) HIV budding is that it occurs exclusively at the PM (Jouvenet et al., 2006; Deneka et al., 2007; Welsch et al., 2007). Thus, the budding sites originally interpreted to be internal in a macrophage cell actually appear to be in a specialized membrane compartment that is a deeply invaginated part of the PM (Deneka et al., 2007; Welsch et al., 2007). Similarly, in polarized T cells HIV budding takes place in a highly restricted part of the PM (Hogue et al., 2009; Llewellyn et al., 2010). These observations highlight the conclusion that Gag not only selects the PM, but in fact selects specific parts of the PM. The distinguishing features of these specialized regions remain to be elucidated. In principle such features

could be proteins or lipids, or some aggregate property of these such as membrane phase behavior (see below).

LIPIDS AND MEMBRANES

To better understand the role lipids play in Gag-membrane interactions and the formation of the viral envelope, it is necessary to have an understanding of lipid behavior. Lipids represent a diverse group of molecules involved in energy storage, signaling, and the structure of cellular membranes. Glycerophospholipids, characterized by a glycerol-based head group and two fatty acid chains, represent the predominant group of polar membrane lipids in eukaryotic cells (van Meer et al., 2008). The most common cellular glycerophospholipids are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA). The sterol cholesterol is the most common non-polar membrane lipid.

The PM of cells is an asymmetric bilayer approximately 30 Angstroms thick and represents a minor percent of total cellular membrane lipids. Of the cellular proteins associated with the PM, only a fractions are transmembrane proteins. However, *in vitro* characterization of lipid mixtures has yielded many breakthroughs in the understanding of membranes, and so these studies are useful.

Lipids can be characterized by a number of measurements including their rate of lateral diffusion (translational diffusion coefficient, D_T) and the order, or range of movement, of their acyl chains (S). Defining the characteristics of lipids in a membrane bilayer can also be thought of as defining the phase behavior of the membrane bilayer. Phase behavior refers to the state of motion and order of individual lipids and how this state changes as temperature or composition change (van Meer et al., 2008). Membrane phases include liquid-disordered (L_d), liquid-ordered (L_o), and solid-gel (L_β; **Figure 1**). L_d lipid bilayers generally have lower concentrations of cholesterol and higher concentration of lipids with unsaturated acyl chains (van Meer et al., 2008). The lipids are loosely packed with their chains sampling a large cone of space below the lipid's head group. This cone of space that the acyl chain samples can be represented by a $\Delta\Theta$ (**Figure 1D**). Due to the thin but wide volume of the lipid chains, the L_d bilayer is thin. L_β bilayers are composed mainly of saturated lipids that pack close to each other and have a low $\Delta\Theta$ (van Meer et al., 2008). Additionally, the rate that lipids exchange with each other in the L_β bilayer is at least a 1000-times slower than in L_d bilayers (van Meer et al., 2008). L_o bilayers typically contain a mix of saturated and unsaturated acyl chains and cholesterol, and while the bilayer has a $\Delta\Theta$ similar to that of L_β, its D_T is similar to that of liquid disordered phase (van Meer et al., 2008).

Similar to lipid acyl chains, cholesterol must be shielded from the aqueous environment, but unlike phospholipids cholesterol has only a tiny hydrophilic head group. Shielding of cholesterol in the bilayer is accomplished by straightening of lipid acyl chains (a decrease in $\Delta\Theta$) so that the lipid head group can shield both its own acyl chains and cholesterol (Huang, 2009).

Membrane phase behavior can be determined for mixtures of up to three- and four-components. These data can be displayed in a phase diagram. A typical three-component diagram displays the membrane phase behavior for mixtures of cholesterol, a high Tm (saturated) lipid, and a low Tm (unsaturated) lipid. These diagrams can be used to determine if a given mixture forms one, two, or three phase, and if those phases are L_o, L_d, or L_β. Phase diagrams also define the composition of mixtures that are near critical points between two-phase and one-phase mixtures.

Membrane phases can be nanoscopic or microscopic. Nanoscopic domains are typically characterized using Förster resonance energy transfer (FRET) while macroscopic domains can be observed with an optical microscope by employing giant unilamellar vesicles (GUVs) prepared with fluorescent dyes that partition into different phases (Heberle and Feigenson, 2011). FRET also measures the partitioning of lipid dyes in bilayers (Buboltz, 2007). An advantage of FRET is that preparation of the membranes by rapid solvent exchange (RSE) does not require lipids to transition through a dry phase, a step that is required in GUV preparation that may introduce artifacts (Buboltz and Feigenson, 1999). The use of GUVs to study membrane phase behavior yields striking and convincing evidence for the presence of lipid phases (**Figure 1**).

Formation of distinct, large and visible membrane phases has never been observed in living or fixed cells without extensive crosslinking, but this does not mean that phase coexistence do not occur. The phases can be nanoscopic and so not observable by microscopic techniques. There is indirect evidence for the existence of phase coexistence behavior in cellular membranes. For example, treating cells with cold detergent results in the isolation of a highly ordered, raft-like lipid and protein fraction termed detergent resistant membranes (DRMs). DRMs are enriched in cholesterol and sphingolipids (Brown and Rose, 1992; Simons and Ikonen, 1997) and one study found enrichment of arachidonic acid (20:4) and PE (Pike et al., 2002). In another study, electron spin resonance (ESR), a method that measures the order of lipid bilayers, showed that DRMs isolated from rat peripheral blood cells (RBL-2H3) were liquid ordered (Ge et al., 1999). While the behavior of DRMs has convincingly been defined as raft-like, it remains unclear to what degree DRMs represent a phase present in living cells or alternatively represent an artifact generated during cold detergent treatment of cells (Brown, 2006).

Further evidence for cell membrane phase behavior comes from microscope-based studies that characterize GUV-like membranes isolated from the PM of cells. The first study collected giant PM-derived vesicles (GPMV) from cells by treating cells with paraformaldehyde, which induces membrane blebbing (Baumgart et al., 2007). At a range of temperatures below 25°C GPMVs demonstrated lipid dye partitioning similar to that observed for model membranes with optically resolvable L_o and L_d phases (Baumgart et al., 2007). In a second study GPMVs were collected by the paraformaldehyde method and also by an alternative method that involves osmotic swelling of plasma membrane spheres (PMS) with PBS buffer (Kaiser et al., 2009). The PMSs demonstrated phase separation similar to GPMVs, and the inclusion of a PM protein, the transmembrane protein linker for activation of T cells (LAT) in the L_o phase, also was detected (Kaiser et al., 2009). The L_o phase of PMSs is significantly less ordered than that of the model membrane used for comparison. One explanation for the difference in order is the difference in cholesterol content; the GUVs were prepared with 20% cholesterol

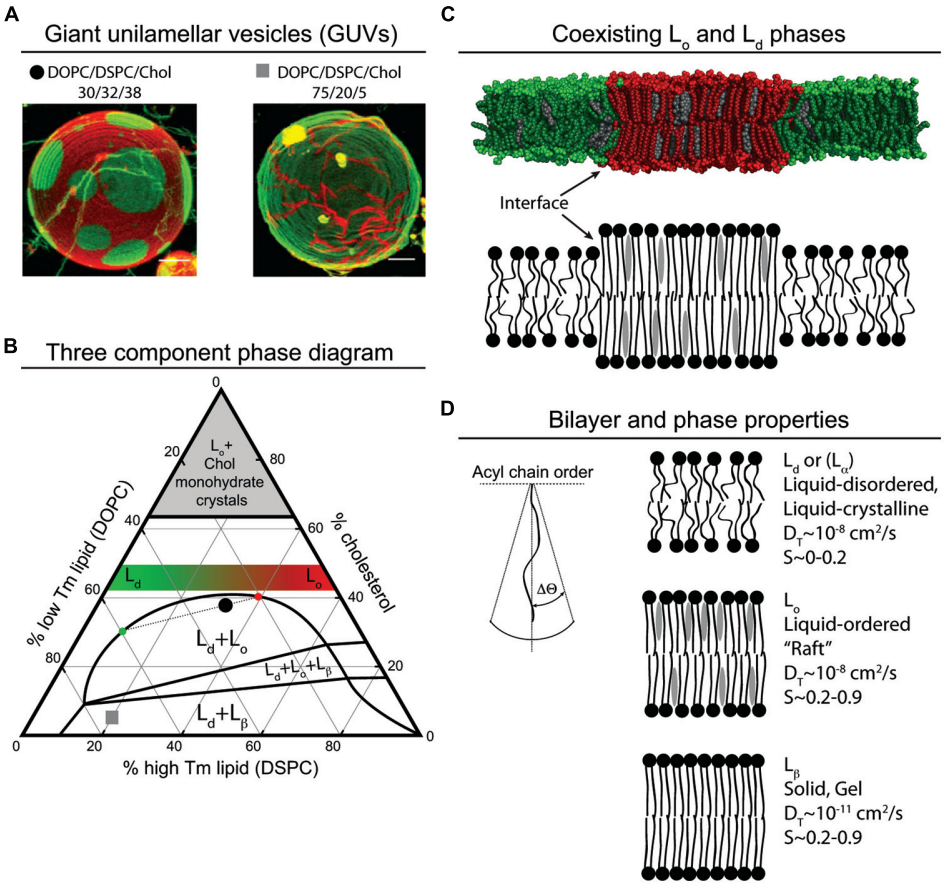

FIGURE 1 | Model membrane phase behavior and lipid parameters. **(A)**Color merged GUV's of simultaneously collected fluorescence emission from C20:0-DiI and (16:0, Bodipy)-PC reconstructed from confocal microscopy z-scans (Zhao et al., 2007). The red emitting C20:0-DiI segregates to both the L_o and L_β membrane phase, and the green emitting (16:0, Bodipy)-PC segregates to the L_d membrane phase. The lipid composition of each GUV is denoted by a black circle and a gray square on the phase diagram **(B)**. **(B)** The phase diagram is adapted from Zhao et al. (2007), Heberle et al. (2010) and Konyakhina et al. (2013). The vertices of the triangle represent pure components: 100% cholesterol, 100% low Tm lipid dioleoyl-PC (DOPC), and 100% high Tm lipid distearoyl-PS (DSPC). Each side of the triangle represents a binary mixture. The left side of the triangle represents lipid mixtures resulting in L_d membranes, and the right side of the triangle represents lipid mixtures resulting in L_o membranes. As the low Tm lipid DOPC is replaced with the high Tm DSPC at a fixed cholesterol concentration, for example, above 42% the membrane phase becomes increasingly ordered as depicted by the green to red gradient trapezoid. The one phase region falls outside of the labeled two and three phase regions. Lipid mixtures that fall in the region of the phase diagram labeled with more than one phase, for example the black circle in the L_o + L_d region, result in membranes with co-existing phases. The dotted line through the black circle represents a tie line. The distance of the black circle from the phase boundaries along the tie line represents the percent of membrane that is L_d and L_o, approximately 25 and 75%, respectively. The intersection of the tie line with the left or right boundary denotes the composition of the respective membrane phase. In this example, the intersection of the tie line on the left side of the boundary corresponds to an L_d membrane with an approximate composition DOPC/DSPC/Chol (60/10/30). The intersection of the tie line on the right hand side of the boundary corresponds to an L_o membrane with an approximate composition DOPC/DSPC/Chol (20/40/40). When preparing GUVs with a charged lipid such as PS, it seems reasonable to exchange a percentage of the PC lipid for PS lipid. Using the black circle as an example, if one replaces all the DOPC (30%) with DOPS (30%), the resulting GUV would have an L_d region with 60% PS and an L_o region with 20% PS. This simplified example of exchanging PC for PS does not take into account the possible effect that lipid head group has on membrane phase behavior. **(C)** Molecular dynamics snapshot of DOPC/DSPC/Chol lipid segregation in a membrane bilayer. Line tension drives the formation of macro domains, due in part to differences in membrane thickness; L_o is thicker than L_d. Because the interface is costs energy, in some lipid mixtures, micro domains coalesce into macro domains to minimize the interface. **(D)** Properties of different membrane phases and acyl chain order (adapted from van Meer et al., 2008).

while the PMSs may have had as much as 45% (Kaiser et al., 2009). In addition to differences in cholesterol content compared to the GUVs, the GPMVs and PMSs were treated with cholera toxin to induce phase separation (Hammond et al., 2005).

NATURE OF RETROVIRAL MEMBRANES

Like all natural membranes, retroviral membranes are made of lipids and proteins. The protein components prominently include the products of the *env* gene, i.e., trimers of the SU-TM complex that is formed in the Golgi. Minor amounts of cellular membrane and other proteins also are found in or associated with virions (Chertova et al., 2006), but to date most of these are without clearly known function. The lipid components of retroviral membranes were first analyzed systematically in the late 1970s and early 1980s for RSV (Quigley et al., 1971, 1972; Pessin and Glaser, 1980; it should be noted here that all alpharetroviruses appear to be strains

of a single species, since they all show ∼95% sequence identity, not counting viral oncogenes picked up from the cell, such as *src*. For convenience, in this review all of these strains are referred to as RSV). These early lipid analyses showed that the viral membrane was enriched in cholesterol and sphingolipids, compared with the PM. Similar results were found for HIV-1 and HIV-2 in the early 1990s (Aloia et al., 1993). A major limitation in interpreting these results is that the PM itself is difficult or impossible to obtain in pure form. Nevertheless, the retroviral composition seems to be enriched in the lipids that later were found to be enriched in membrane microdomains often referred to as "rafts" (Simons and Ikonen, 1997), and that also were found to be enriched in model membranes in the L_o phase (see below). This similarity gave rise to the over-simplified notion that retroviruses "bud from rafts."

Consistent with the importance of rafts for retroviral budding are the results of cholesterol depletion from cells expressing HIV-1 or other Gag proteins. Such depletion can be accomplished by addition of methyl β-cyclodextrin (CD), which tightly binds cholesterol. How is CD, a highly soluble compound that does not enter a lipid bilayer, able to extract cholesterol from the hydrophobic environment inside the membrane bilayer? While at equilibrium cholesterol highly prefers to be in the membrane, according to one model at physiological temperatures cholesterol molecules may escape into the aqueous phase, where the cholesterol is captured by CD. Depletion of cholesterol from the PM is surprisingly rapid, in tens of minutes (Klein et al., 1995; Yancey et al., 1996), depending on the CD concentration. Addition of CD to HIV-1 Gag-expressing cells greatly reduces budding (Ono and Freed, 2001), apparently even in a time window and CD concentration range in which cell viability is not severely compromised. Nevertheless, given the importance of cholesterol for many cellular processes, interpretation of the results of CD depletion should be cautious. By *in vitro* liposome flotation assays, Gag binding is greatly stimulated by the presence of cholesterol, even under conditions where no L_o phase is present (Dick et al., 2012; see below).

Recently more accurate lipid compositions have been determined for HIV-1 by use of mass spectrometry (MS; Brugger et al., 2006; Chan et al., 2008; Lorizate et al., 2013). MS allows not only the lipid type as defined by headgroup to be identified (e.g., PS, PC, or PE) but also the composition of the many kinds of natural fatty acyl chains of each type, which vary in length as well as in the degree of unsaturation. As for earlier reports, a limiting factor in interpreting these results is the purity of the virus and the purity of the PM with which the viral membrane is compared. Several important generalizations emerge from these recent papers. First, HIV and MuLV membranes contain the phosphatidylinositol phosphate PI(4,5)P2, which has been inferred, at least for HIV-1, to be involved in Gag-PM recognition (Chan et al., 2008; see below). Second, retroviral membranes are enriched in PM outer leaflet lipids that are found in L_o-like membrane microdomains (rafts), e.g., sphingomyelin. Third, HIV-1 is highly enriched in a lipid derived from PE, plasmalogen PE, which comprises a major species of PE in the PM (Chan et al., 2008; Lorizate et al., 2013). The significance of the latter is uncertain. Retroviral membranes also are reported to be enriched in cholesterol, compared with the PM (Brugger et al., 2006; Chan et al., 2008). However, a more recent study did not find significant enrichment in cholesterol in the HIV envelope (Lorizate et al., 2013). Interpreting these results is limited by two factors. First, purification of virions away from cellular membranes is difficult. Second, as mentioned above, comparison of the lipid composition of viral membranes with that of the PM is limited by the lack of purity of the isolated PM fraction.

PRINCIPLES GOVERNING THE ASSOCIATION OF Gag WITH THE PM

Several principles have been elucidated that underlie Gag targeting to the PM. First, the matrix (MA) domain of most retroviral Gag proteins is myristoylated. Myristate, a 14-carbon fatty acyl modification at an N-terminal Gly residue of proteins, is quite common in cellular proteins that interact with membranes, and in Gag proteins it is presumed to become inserted into the hydrophobic core of the bilayer. Gag myristoylation is essential for membrane binding (O'Carroll et al., 2012b), a conclusion that stems from the observation that mutation of the N-terminal Gly completely blocks budding and membrane localization of Gag; the Gly is known to be required for the N-myristoyl transferase that attaches this fatty acid to the nascent protein while it is still on the ribosome (Gordon et al., 1991). In the HIV MA domain as well as in some cellular proteins, the myristate can exist in a solvent-exposed state or in sequestered state in which the hydrophobic acyl chain lies in a pocket or groove in the protein (Tang et al., 2004). According to the "myristoyl switch" hypothesis (Ames et al., 1997), the solvent exposed state for monomeric MA is much less populated than the sequestered state, for example as shown by NMR (Tang et al., 2004). Thus the myristate-sequestered protein has low membrane binding potential. Myristate exposure can be triggered by multimerization (Tang et al., 2004) or by PI(4,5)P2 binding (Saad et al., 2006; see below), which in turns leads to membrane binding. One of the several *in vivo* observations that bolster the biochemical analyses *in vitro* is based on an MA deletion mutant form of HIV Gag. If Gag has no MA domain at all, but does have an ectopic exposed myristate just upstream of the CA domain, membrane binding and budding can occur (Borsetti et al., 1998; Reil et al., 1998).

While the myristoyl switch hypothesis is generally accepted, according to a recent molecular dynamic simulation, myristate exposure and subsequent membrane insertion can occur even without multimerization or PI(4,5)P2 binding (Charlier et al., 2014). The mechanistic implications of these results remain to be explored. Furthermore, a single fatty modification on a monomeric protein is known to be insufficient to stably lock a protein into a membrane (Silvius et al., 2006). In addition, some Gag proteins, like those of alpharetroviruses (e.g., RSV) and the lentivirus equine infectious anemia virus (EIAV), are not myristoylated at all. Both of these observations imply that retroviral Gag proteins also rely on other membrane binding signals.

A second principle for Gag-PM binding and budding is based on electrostatics. The MA domains of retroviruses share a high degree of structural homology. The N-terminal domain (NTD) of MA is composed of 5–6 major alpha helices that fold into a globular shape (Hill et al., 1996; Murray et al., 2005). The globular head of MA positions a number of basic amino acids on one surface resulting in a basic patch that is oriented towards the

PM. This basic patch interacts electrostatically with the negatively charged inner leaflet of the PM. Most genera of retrovirus have such a basic patch, with the net surface charge of MA domains differing from neutral to +3 to +6, as in the case of EIAV, RSV, and HIV-1, respectively (Murray et al., 2005). The basic patch of HIV-1 MA also mediates binding of RNA, which may modulate electrostatic interaction of Gag with the PM (Alfadhli et al., 2009, 2011; Chukkapalli et al., 2010, 2013; Dick et al., 2013). To what extent this finding can be generalized to other retroviruses remains to be carefully explored.

The MA domain of RSV Gag is not myristoylated, and so RSV MA serves as a good model to probe electrostatic interactions between retroviral MA domains and membranes. Single and double mutations of basic to acidic amino acids in RSV MA result in a decrease or a loss, respectively, of Gag localization to the PM and of virion release (Callahan and Wills, 2000). In the background of a double basic to acidic mutant, mutations elsewhere in MA that return the net surface charge to +3 restore virion release (Callahan and Wills, 2000), indicating that the exact placement of the basic side chains in the structure is not important. Mutating two acidic amino acids to basic amino acids results in increased viral release (Callahan and Wills, 2000). This mutant Gag protein, dubbed super-M (super membrane binder, SM), fails to traffic through the nucleus of the cell, packages 1/10th of the viral genomic RNA (vgRNA) of wt particles, releases virus particles more rapidly than wt, and is non-infectious (Callahan and Wills, 2003). It remains unclear if the decrease in vgRNA packaging is due to the rapid viral release or to the defect in nuclear trafficking (Callahan and Wills, 2003).

Liposome flotation analyses of RSV MA and Gag strengthen the hypothesis thatits membrane binding is driven by electrostatics. RSV MA binding to liposomes composed of physiological amounts of the negatively charged phospholipid PS is decreased to undetectable levels as salt is increased from 75 to 500 mM NaCl (Dalton et al., 2005). A MA mutant with two basic lysine residues changed to the neutral asparagine is significantly defective in membrane binding (Dalton et al., 2005). Increasing the lipid concentration of PS increases the amount of protein that associates with liposomes (Chan et al., 2011). While some retroviral MAs bind specifically to PIPs, RSV MA has no known PIP binding pocket. However, RSV MA responds strongly to the presence of PIPs as measured by liposome flotation. This apparent discrepancy may be due to the high charge density of PIPs at physiological pH, and thus the observed increase in binding to membranes with PIPs may be electrostatic.

Compared with the net +3 charge of RSV MA, HIV MA has a net +6 charge. Interestingly, the first 31 residues of MA can function independently as a membrane-binding region, dependent on the basic residues and the myristate, as demonstrated by the membrane binding of a MA-Src chimera (Zhou et al., 1994). The MA-Src chimera likely does not maintain structural components required for forming the PIP binding pocket.

Similar to RSV MA, altering the number of basic residues in HIV MA alters HIV MA membrane binding. For example, mutation I18K/L20K results in nearly double the amount of membrane-associated Gag protein compared with wt as measured by flotation of postnuclear supernatants of virus expressing cells (Ono et al., 2000). The mutation K29T/K31T results in a three-fold reduction of virus release and K29E/K31E results in Gag accumulation at Golgi membranes in cells (Freed et al., 1995; Ono et al., 2000). Individual mutations of the basic residues K18, R20, and R22 each results in a dramatic decrease in viral infectivity. However, these mutants still produce virions but with lower levels of Env incorporation, which may account for the decrease in infectivity (Bhatia et al., 2007). Interestingly, mutations K29T/K31T and K29E/K31E do not significantly reduce binding to PC/PS membranes in a standard liposome flotation assay, but they do decrease binding to membranes that contain PI(4,5)P2 (Chukkapalli et al., 2008). Taken together these results show that electrostatics cannot solely explain the interaction of HIV-1 Gag with membranes.

A third principle, at least for some Gag proteins, such as HIV-1 and MuLV (Ono et al., 2004; Hamard-Peron et al., 2010; though perhaps not for all Gag proteins; Chan et al., 2011; Inlora et al., 2011) is the involvement of PI(4,5)P2 in MA interaction with bilayers. The Summers lab was the first to show that the HIV-1 MA domain binds specifically to water soluble (C4) and (C8) chain analogs of PI(4,5)P2 (Saad et al., 2006). Specific interactions between MA and PI(4,5)P2 was confirmed by protein footprinting (Shkriabai et al., 2006). Since this phosphoinositide is a marker for the PM, the specificity of binding *in vitro* was taken to imply that PI(4,5)P2 plays a critical role in HIV-1 Gag PM localization (Freed, 2006; Saad et al., 2006). This conclusion was supported by experiments in which PI(4,5)P2 was depleted in Gag-expressing cells, by means of a transfected 5-phosphatase, which resulted in abrogation of budding and relocalization of much of Gag to internal membranes (Ono et al., 2004). Also consistent with an important role for PI(4,5)P2 in HIV-1 budding was the finding that both HIV-1 and MuLV viral membranes incorporate relatively high levels of this phosphoinositide (Brugger et al., 2006; Chan et al., 2008). Reports of the influence of PI(4,5)P2 for RSV are inconsistent. We showed that depletion of PI(4,5)P2 *in vivo* did not significantly alter Gag PM localization or virus release, under the same conditions that HIV PM localization and budding were knocked down in parallel (Chan et al., 2011). However, using a more sensitive assay for changes in PM localization, the Parent lab demonstrated that PI(4,5)P2 depletion *in vivo* did reduce virus release (Nadaraia-Hoke et al., 2013). However, in that study HIV was not tested in parallel, and hence plausibly RSV is less sensitive to PI(4,5)P2 depletion than HIV. *In vitro*, we have reported that PI(4,5)P2 at low molar membrane concentrations does enhance binding of RSV Gag to membranes. Thus it remains unclear what role PI(4,5)P2 has for RSV Gag PM binding *in vivo*.

Much of the binding energy for PI(4,5)P2 and MA comes from hydrophobic interactions with acyl chains (Anraku et al., 2010), consistent with the observation that PI(4,5)P2 species with eight carbon-acyl (C8) chains bind much more tightly to HIV-1 MA than species with four carbon-acyl (C4) chains (Saad et al., 2006). From these several observations, a model emerged in which HIV-1 MA extracts ("flips out") the sn2 acyl chain from hydrophobic environment in the PM and places it in a hydrophobic groove in the protein. Clearly from NMR results, this is what happens in the *in vitro* system based on shortened acyl chains. At the same time, the myristate chain normally sequestered in an MA pocket then "flips" into the membrane (Freed, 2006). This "flipping out"

and "flipping in" model has gained wide currency. The model recently has been further elaborated with a report that not only for PI(4,5)P2, but also for PS, PC, and PE the sn2 acyl chains have specific binding sites on the surface of HIV-1 MA (Vlach and Saad, 2012).

Nevertheless, the "flipping "in/out" hypothesis remains an uncertain model for several reasons. Direct evidence for the specificity of binding *in vitro*, i.e., preference for PI(4,5)P2 over other phosphoinositides, rests on phospholipid molecules with short C4 and C8 chains, which are not found in biological systems. In quantitative assays for interaction of MA with liposomes containing biologically relevant phosphoinositides, little specificity for PI(4,5)P2 is observed (Chukkapalli et al., 2008; Chan et al., 2011). Furthermore, in liposomes approximating the composition of the inner PM leaflet, i.e., containing high levels of cholesterol and PS, HIV Gag binding is robust even without any phosphoinositides. The inner leaflet of the PM is highly negatively charged, and thus electrostatic interactions could account for much of the observed specificity toward the PM, as it does for proteins engineered to bind solely on the basis of charge (Yeung et al., 2008). For MuLV, the enhanced binding of MA to membranes by PIP2 was dependent on the presence of PS, suggesting that PIP binding is dependent on the net negative charge of the membrane from PS (Hamard-Peron et al., 2010). Finally, the published data on the effects of PI(4,5)P2 depletion by 5-phosphatase on retrovirus budding all are steady state measurements made many hours after the phosphatase has been expressed by transfection. Given the extremely dynamic nature of PIP pools, and the importance of PIPs for a multitude of cellular processes, it will always be difficult to interpret PI(4,5)P2 depletion experiments, unless systems are used which allow rapid and controlled recruitment of the phosphatase to the PM (Balla, 2007). Final acceptance of the flipping model probably will await crosslinking experiments or similar studies that directly detect that the sn2 acyl chain is no longer in the membrane but in the MA pocket.

A fourth and poorly understood factor in targeting of Gag to the PM is cellular trafficking. HIV-1 Gag, which is the best understood in this context, has binding sites for the clathrin adaptor proteins AP1 (Camus et al., 2007), AP2 (Batonick et al., 2005), and AP3 (Dong et al., 2005). Perhaps the best understood is the AP3 binding site in the MA domain. While ablation of this sequence does not completely abrogate PM binding and budding, it is reported to greatly reduce both of these readouts (Dong et al., 2005). According to a more recent report, MA does not directly interact with AP3, making interpretation of the earlier results difficult (Kyere et al., 2012). Thus, the mechanism by which adapter proteins actually work to promote PM interaction remains unknown.

Finally, a fifth principle that helps explain Gag-PM targeting is Gag multimerization. An intrinsic property of Gag is the propensity to multimerize, a process that is dependent of Gag concentration and on the interaction of NC with nucleic acid. At low concentrations, HIV Gag with a dimerization constant of 1–10 mM (Gamble et al., 1997; Datta et al., 2007b) is found to be largely cytoplasmic (Fogarty et al., 2013). As the concentration of Gag increases in the cytoplasm the fraction of Gag found at the PM increases, likely because Gag multimerization increases (Fogarty et al., 2013). In the cytoplasm the predominant fraction of Gag is monomeric with a subfraction being dimeric (Kutluay and Bieniasz, 2010). Consistent with the model that the PM induces Gag assembly, there is an enrichment of higher multimeric complexes of Gag at the PM (Kutluay and Bieniasz, 2010).

Gag interaction with the PM presumably also promotes Gag multimerization. Knocking out HIV Gag membrane association by preventing myristoylation, by mutating the terminal glycine residue, results in a loss of viral particle assembly (O'Carroll et al., 2012b). Nevertheless, at sufficiently high concentrations of Gag, assembly is detected in the cytoplasm, independent of Gag membrane association (O'Carroll et al., 2012a,b).

Fluorescent correlation spectroscopy (FCS) measurements of cytoplasmic RSV Gag-GFP movement show that in the RSV system, Gag is in large complexes prior to localization to the PM (Larson et al., 2003). However, these complexes contain only a few Gag proteins (Larson et al., 2003), implying the involvement of cellular proteins, perhaps like those implicated in HIV assembly (Lingappa et al., 1997). FRET measurements of RSV Gag-YFP and Gag-CFP show that Gag-Gag association occurs prior to membrane binding of Gag.

Fusion of the Gag membrane binding domain, MA, to proteins with defined multimerization states allows for a measurement of the effect of multimeric state of MA on membrane binding, both *in vivo* and *in vitro*. For example, for HIV MA, dimerization enhances membrane binding *in vitro* and *in vivo* (Dalton et al., 2005; Dick et al., 2013). While for RSV MA dimerization is not sufficient to promote substantial PM binding *in vivo*, hexamerization leads to strong PM association (Dick et al., 2013). Overall, these findings suggest that Gag multimerization is an early, critical step in the association of Gag with the PM in cells.

Taken together these results are consistent with a model in which early steps of Gag multimerization occur prior to membrane localization. Once these small Gag multimers associate with the membrane, additional Gag molecules rapidly associate with the Gag multimer resulting in the assembly of a virus particle. Because at typical Gag levels, assembly into a complete virion does not occur in the cytoplasm, the PM must provide a feature required for assembly. This feature may be a restrictive (2 dimensional) environment with locally high concentrations of Gag compared with cytoplasm. Additionally, or alternatively, the PM may provide a critical component that promotes multimerization, such as a cellular protein, a lipid, or a membrane domain.

The discussion above, like most published discussions of Gag-membrane interaction, focus on the N-terminal MA domain, since it is the known module that is able to bind membranes *in vivo* and *in vitro*. However, some evidence suggests that the NC domain also may play a role in membrane interaction, at least in the case of HIV-1.

Size exclusion chromatography (SEC) and small angle X-ray scattering (SAXS) of HIV-1 Gag show the Gag polyprotein to be in a compact, horseshoe conformation (Datta et al., 2007a). This conformation is also observed for dimers of Gag (Datta et al., 2011). These conformation studies raise the possibility that both the MA and NC domain interact with the cellular PM prior to or during virion assembly. Consistent with the SEC and SAXS data, low angle neutron reflectometry (LANR) studies of HIV-1 Gag on a supported bilayer show Gag is compact, and both the MA

and NC domains interact with the supported bilayer (Datta et al., 2011). The addition of a short oligonucleotide, too short to induce assembly of Gag into VLPs, results in the extension of the Gag protein (Datta et al., 2011). This extended structure is interpreted as MA bound to the membrane and NC bound to the oligonucleotide (Datta et al., 2011). Taken together these observations have led to a model in which both ends of Gag bind to the PM. Upon the binding of vgRNA to NC, which outcompetes NC binding to the PM, Gag takes on an assembly competent extended conformation. A limitation of these studies is that the experiments were done with non-myristoylated Gag. Further evidence will be needed to assess this model critically.

ASSAYS FOR Gag-MEMBRANE INTERACTION AND CHALLENGES IN INTERPRETING THE RESULTS

Many methods have been used to study Gag-membrane interactions, each with its own limitations. Quantitative parameters are particularly challenging to elucidate. In overview, the relevant experiments can be grouped into two classes: *in vivo* studies with transfected or infected cells, and *in vitro* studies with purified or specifically labeled proteins and artificial or natural membranes.

Gag-PM LOCALIZATION *IN VIVO*

The most straightforward and very common method to visualize Gag subcellular localization is fluorescence microscopy of GagGFP fusions (or more rarely, immunofluorescence). With some exceptions, such studies typically give information on the steady state distribution of Gag, and do not report on Gag trafficking from the site of synthesis in the cytoplasm to the PM, or to other locations. At steady state the PM is enriched in Gag, but the cytoplasm also has abundant levels of Gag, depending somewhat on the species of retrovirus (Fogarty et al., 2013).

Gag-MEMBRANE INTERACTION *IN VITRO*

Probably the most standard method to visualize Gag or MA interaction with membranes *in vitro* is liposome sedimentation or flotation. Liposomes that have bound protein are either pelleted by high speed centrifugation, or alternatively after addition of sucrose are floated upwards through a sucrose step gradient, also by high speed centrifugation. The former version is less robust because aggregated protein also will pellet to the bottom of the tube. These techniques are low throughput, requiring one gradient to obtain one data point.

Liposome flotations are relatively easy to set up, involving the preparation of uni- or multi-lamellar vesicles (ULVs and MLVs, respectively), both of which will float to the top of a sucrose gradient. Preparation of vesicles typically involves the combination of two or more lipids at defined molar concentrations in an organic solvent, in most cases chloroform. The lipid mixture is dried down to a film and resuspended in an aqueous buffer. Depending on the concentration and composition of the lipid mixture, the resuspended lipids may require multiple freeze thaw cycles to fully rehydrate the film. At this stage the lipids are in multi-layered (multi-lamellar) membrane vesicles ranging in size from 100 to 1000 nm. Because the vesicles are multi-lamellar, it is not possible to predict the area of membrane that is available to bind proteins. In addition, the concentration of each lipid in the outer layer of membrane may not be the same as in the inner layer. For example, in a MLV charged lipids like PS or PIPs may preferentially segregate to the outermost layer that is exposed to the aqueous environment, resulting in an effectively higher concentration of the charged lipid. Repeated extrusion of MLVs through a membrane of defined pore size results in ULVs with a defined range of size (about $\pm$20 nm for 100 nm pores), and thus known concentrations of lipid are available to surface-binding proteins.

One disadvantage of preparing MLVs and ULVs by the dry film method is that at high concentrations (the exact concentration varying depending on the lipid mixture) cholesterol forms monohydrate crystals when the organic solvent is removed during the drying process. The cholesterol crystals may not fully rehydrate, resulting in a final membrane with a lower than the intended cholesterol concentration. RSE was developed to overcome the cholesterol monohydrate crystal artifact (Buboltz and Feigenson, 1999). RSE takes advantage of the low vapor pressure of organic solvents compared with the vapor pressure of aqueous buffers. When lipids dissolved in organic solvent are mixed with an aqueous buffer under a vacuum the organic solvent evaporates and the lipids are effectively transferred to the aqueous buffer. RSE of lipids results in a mixture of ULVs and MLVs in the range of 100 nm in diameter (Buboltz and Feigenson, 1999).

Liposome flotation can be performed with crude or purified protein. Using purified protein provides a greater amount of control over protein concentration and buffer conditions of the protein-membrane binding reaction. Dalton et al. (2007) found that binding of RSV and also HIV MA protein to liposomes is largely dependent on the ionic strength of the buffer. Additionally, they showed that for both viruses MA dimerization results in at least an order of magnitude increase in tightness of liposome interaction (Dalton et al., 2005, 2007). Purified protein-based flotations have also been used to show that increasing the multimeric state of RSV MA to a hexamer (as previously mentioned) results in a large increase in affinity for liposomes (Dick et al., 2013). Also, RNA can inhibit binding of purified RSV and HIV MA to liposomes, but this result is dependent on low (50 mM NaCl) ionic strength (Dick et al., 2013).

Some proteins are not tractable in the bacterial expression systems typically used for generating protein. For example, purification of myristoylated HIV Gag in concentrated form is difficult or impractical. An alternative method largely pioneered by the Ono lab (Chukkapalli et al., 2008) is the generation of 35SMet labeled protein by *in vitro* translation in a commercially available reticulocyte extract. Advantages of this system include the presence of physiological concentrations of protein, nucleic acid, and ions, as well as efficient myristoylation, which is essential for HIV Gag-membrane interaction. However, *in vitro* translation is not without its downsides. Most important, in assays such as membrane binding, it is very difficult to sort out the possible confounding effects of cellular proteins, RNA, or metabolites. In addition, because of the high concentrations of nucleic acid in the reticulocyte reaction, Gag proteins that are induced to assemble in the presence of nucleic acid may not be monomeric, or may be aggregated. Not knowing the multimeric state of the protein makes interpretation of membrane binding results difficult in light of the importance of multimerization on MA's interaction with membranes.

To visualize by fluorescence microscopy the interaction of Gag with membranes of defined lipid composition, standard liposomes obviously are not suitable because of their small size. Instead, for this purpose GUVs can be used. GUVs are a common tool to study the phase behavior of lipids. Because GUVs are so large (in the range of 10–50 microns in diameter) they allow real time analysis of the binding of fluorescently tagged proteins. For example, GUVs were used to study the binding of ESCRT proteins, known to be involved in viral budding, to HIV-1 Gag assembly sites (Carlson and Hurley, 2012). Taking advantage of the observable phase behavior of GUVs, Keller et al. (2012) studied the binding of artificially multimerized HIV MA to GUVs having both L_o or L_d membrane domains. The multimeric MA was found to preferentially to the L_d domain. However, the interpretation that MA favored L_d because of the lipid phase itself in this case is not persuasive because the relative partitioning of acidic lipids into the L_o and L_d is not known.

A major hurdle of using GUVs to study electrostatic protein-membrane interactions is that phase diagrams for PS- and PIP-containing lipid mixtures do not exist. This makes the interpretation of results generated using lipid mixtures containing PS and PIP difficult. Lipid head groups influence lipid phase behavior, and so one cannot make the assumption that replacing some fraction of a PC lipid with a PS lipid will leave the phase behavior unchanged. The uncertainty of lipid phase behavior in PS mixtures can be compounded by the addition of PIPs such as PI(4,5)P2. Under some buffer conditions and in some lipid mixtures, PIPs do not mix with other lipids, instead forming their own phase (Redfern and Gericke, 2005; Kooijman et al., 2009; Wang et al., 2012). Not knowing what the behavior is of PIPs in membranes is a major limitation for correctly interpreting experimental results for PIP-containing mixtures.

Another difficulty in working with GUVs is their sensitivity to osmotic pressure. Because the electrostatics of Gag binding to membranes is so heavily influenced by ionic strength, it is important to choose buffer and salt conditions that are biologically relevant. Unfortunately, preparation of GUVs is significantly influenced by the ionic strength of the solution. Typically GUVs are made in the absence of ions or at very low ionic strength. Matching the osmotic strength of the GUVs with the osmotic strength of the protein and at the same time maintaining a physiological ionic strength takes careful planning.

PERSPECTIVES

Key principles that direct the binding of Gag to membranes include electrostatics, fatty acid modification of MA, multimerization of Gag, interaction with lipids such as PI(4,5)P2, and the ability of MA to sense the hydrophobic core of the membrane. However, much remains to be learned about these key signals. What are some of the critical issues?

While we know that HIV-1 Gag membrane binding is sensitive to the presence of PI(4,5)P2 in membranes (Ono et al., 2004; Chukkapalli et al., 2008; Chan et al., 2011), and that *in vitro* as little as 2% PI(4,5)P2 can increase Gag membrane binding by 10-fold (Dick et al., 2012), we understand surprisingly little about how PIPs behave in membranes. Evidence is emerging from studies of model membranes that PIPs cluster in membranes (Redfern and Gericke, 2005; Kooijman et al., 2009). Strong intermolecular and intramolecular hydrogen bond networks drive the formation of the PIP clusters, and under certain conditions these clusters represent a separate membrane phase (Redfern and Gericke, 2005; Kooijman et al., 2009; Wang et al., 2012). These PIP clusters might serve as specialized binding sites for retroviral Gag proteins. If PIP clustering proves to be a biologically relevant type of lipid organization, characterizing it will be extremely important.

Many Gag-membrane binding studies have been based on lipid mixtures that are not representative of the lipids found in the inner leaflet of the PM. Therefore, developing a model inner leaflet lipid mix and studying the properties of this mix should be a priority. For example, model membranes are typically made with the neutral outer leaflet lipid PC instead of the inner leaflet lipid PE. PC and PE have large differences in head group size, which could dramatically affect protein binding. In addition, cholesterol is frequently not included in model membrane mixes, nor are lipids that represent smaller fractions of the membrane such as sphingomyelin, plasmalogen-PE, and PI. Not only is a model inner leaflet lipid mix rarely used to study protein-membrane interactions, but essentially no information is available on the phase behavior of such a lipid mixture. Efforts should be made to characterize biologically relevant lipid mixtures.

The binding of any protein to a membrane involves a limited number of principles. So the study of one protein-membrane interaction may shed light on how other proteins interact with membranes. Therefore future work in the field of retroviral protein membrane binding may have broad implications to cellular biology.

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Lipid domains in HIV-1 assembly

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In CD_4^+ T cells, HIV-1 buds from the host cell plasma membrane. The viral Gag polyprotein is mainly responsible for this process. However, the intimate interaction of Gag and lipids at the plasma membrane as well as its consequences, in terms of lipids lateral organization and virus assembly, is still under debate. In this review we propose to revisit the role of plasma membrane lipids in HIV-1 Gag targeting and assembly, at the light of lipid membranes biophysics and literature dealing with Gag-lipid interactions.

Keywords: HIV-1, Gag, PIP2, lipid domains, lipid molecular shape

INTRODUCTION

In a very oversimplified view, assembling could be seen as: retrieving all of your partners at the right place on the right time. In the case of enveloped retroviruses such as HIV, this means retrieving in a sophisticated spatio-temporal concerted mechanism:

- genomic RNA and cellular t-RNA nucleotide primers
- structural (Gag and GagPol) and other (protease, transcriptase and integrase) proteins or polyproteins
- lipid membrane embedded envelop glycoproteins (Env).

in order to correctly produce a potential infective new virion.

The main molecular constituents of HIV-1 are Gag polyprotein (50% of the virion mass) and the viral envelop membrane lipids (30%) exclusively issued from the host cell (for review see Carlson et al., 2008). Gag is a polyprotein that has the ability to induce on its own the formation of virus like particle (VLPs) without any requirement of other viral or cellular components (except lipid membrane) (Gheysen et al., 1989; Campbell and Rein, 1999). Although Gag has been reported to interact with a cellular motor protein (Tang et al., 1999; Martinez et al., 2008) and with other components of vesicular trafficking pathway (Dong et al., 2005; Camus et al., 2007), it is not clear whether Gag is targeted to the plasma membrane or simply reaches the plasma membrane by diffusion through the cytosol. It has been shown that Gag molecules do not multimerize extensively before they reach the membrane (Kutluay and Bieniasz, 2010) and that they arrive at the plasma membrane as dimers or monomers that will multimerize onto eventual nucleation sites composed of Gag-RNA complexes (Jouvenet et al., 2009; Ku et al., 2013). Other major components of the viral infectivity are the Env glycoproteins, they reach the plasma membrane independently of Gag. Env is constituted of two different subunits gp120 and gp41, the later being a transmembrane protein. The gp41 protein is twice palmitoylated and is considered to be targeted to the so called lipid "rafts" membrane domains (Patil et al., 2010).

Undoubtedly, Gag is the main pillar of HIV assembly. It recruits the constituents of HIV virions and orchestrates their assembly while multimerizing onto the inner leaflet of the plasma membrane. Although assembly should appear as a very simple mechanism, many questions concerning Env recruitment and incorporation into virions remain unsolved. Different studies have shown that the cytoplasmic tail of the gp41 and the N-terminal part of Gag are both necessary for Env incorporation into virions, suggesting therefore an interaction of these two proteins, whether direct or through a cellular protein intermediate (for review see Murakami, 2008). Nevertheless, since HIV-1 only displays a tenth of glycoprotein trimers (Zhu et al., 2006) for thousands of Gag molecules (Turner and Summers, 1999; Briggs et al., 2004), it can still be questioned whether or not Gag and Env interact directly? Moreover, it is still to be known if there is a sequential order importance for the control of viral assembly.

In every polymerization by addition, the triggering of the reaction, called initiation step, is the formation of an activated monomer. The propagation of the polymerization will depend on local concentration of the "reactants" i.e., the number of monomers surrounding the activated monomer. Although it is not established that Gag multimerization needs an activated form of it, a simple guess shows that the reduction of dimensionality principle (Adam and Delbrück, 1968) should favor enhanced kinetics of the multimerization process. Therefore, it is clear that lipid membranes could play an important role in the assembly process. Moreover, the lateral compartmentalization of lipid membranes could locally induce higher concentrations of Gag monomers and facilitate the retrieval of Env proteins. Due to their physical and chemical heterogeneity, the detailed role of cellular plasma

membrane in the assembly process of HIV-1 is still a source of controversy.

It is the aim of this review to remind basic concepts in lipid membrane organization and domains formation and to introduce the concept of lipid molecular shape and its consequence on the bilayer curvature. Thereafter, the role of lipids in HIV-1 assembly will be considered, by looking at the interaction of Gag with the plasma membrane at the molecular (atomistic) level. Finally the current model of HIV-1 assembly at the cell plasma membrane will be discussed.

PLASMA MEMBRANE: BASIC PHYSICAL PROPERTIES

LATERAL SEGREGATION AND LIPID DOMAINS

Our current view of plasma membrane mainly derives from the fluid mosaic model proposed 40 years ago by Singer and Nicolson (1972) arguing for proteins embedded in a homogeneous sea of lipids. Nevertheless the possibility of lipids segregating laterally to form "domains" in model membrane was reported at the same time (Phillips et al., 1970; Shimshick and McConnell, 1973). Later on, other types of membrane domains induced by protein-lipid interactions were proposed to explain membrane-mediated processes (Marcelja, 1976; Sackmann et al., 1984). Meanwhile, Israelachvili also proposed a model that accounts for the need of membrane proteins (peripheral and transmembrane) and lipids to adjust to each other due to packing effects as well as thermodynamics (i.e., adjust hydrophobic and hydrophilic areas and height) (Israelachvili, 1977), inducing thereby lateral heterogeneities. Based on this, Mouritsen and Bloom (1984) proposed the hydrophobic mismatch model where hydrophobic matching conditions can lead to elastic distortions of the lipid matrix, therefore, resulting in clustering of adapted lipid molecules around a transmembrane protein. Finally, another model accounting for the role of structural peripheral proteins and sugars (cytoskeleton and glycogalix) has been proposed by Sackmann (1995). In this model, cytoskeleton as well as glycocalix could decrease the lateral diffusion of lipids and therefore induce their micro-compartmentalization. This has been widely documented in the case of the cortical actin networks and is known as the membrane-skeleton "fence" (Kusumi et al., 2012).

Although all these membrane models give many possible explanation of the observed lateral heterogeneity and domains existence in lipid membrane, the most popular in bioscience nowadays is based on lipid demixing and named the "rafts" model. Initially reported by van Meer and Simons (1988), "rafts" were considered to be microdomains ($r\sim$ 100–300 nm) enriched in sphingolipids and cholesterol that are functionally associated to specific proteins involved in trafficking and cell signaling (Simons and Ikonen, 1997). Since then, "rafts" spawned thousands of projects and papers up to a point where nowadays a membrane protein is often classified as being a "raft" or a "non-raft" component of the membrane. However, accurate physical explanation of the "raft" hypothesis is still lacking and its definition has been revisited many times to end up with the most recent one as being "fluctuating nano-scale assemblies ($r\sim$ 20–50 nm) of sphingolipid, cholesterol and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking" (Lingwood and Simons, 2010). They are claimed to exist in an ordered phase (or "raft-phase") different from the liquid ordered phase observed in model membrane systems. The thermodynamic term phase relies on a system at equilibrium and it remains to be established if the plasma membrane is near local equilibrium at some time scales in order to permit real phase separation. Another problem with the "raft" hypothesis is that they have mainly been observed using detergent based extraction methods. It is clear that these methods will always isolate from biological membranes the proteins (and their associated lipids) partitioning into the detergent, thereby inducing formation of domains. Even Lingwood and Simons themselves concluded that detergent extraction methods do not isolate pre-existing membrane domains (Lingwood and Simons, 2010). Finally, while conclusive experiments about the existence of rafts in the plasma membrane remain elusive it is clearly established (Ipsen et al., 1987) that, in model systems containing cholesterol, liquid ordered (lo) and liquid disordered (ld) phase coexists. It is also important to state that, as originally proposed by Sackmann (1995), cytoskeleton (Ehrig et al., 2011a; Sens and Turner, 2011) as well as trafficking (Turner et al., 2005) could play a major role in the lateral segregation of lipids.

Biological lipid membranes are not only characterized by their lateral heterogeneity but also by their asymmetric transverse lipid distribution. Each of the plasma membrane monolayer (outer and inner) significantly differ in their chemical composition. It is generally accepted that the outer leaflet is enriched in sphingolipids (SL) and phosphatidylcholine (PC) whereas the inner leaflet is enriched in phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositols (PI, PIP, PIP_2, PIP_3). This transverse asymmetry can also be defined in term of acyl chains saturation/unsaturation distribution. Indeed, mono or poly-unsaturated acyl chain are mainly found in PE, PS and $PI(P)_x$ whereas saturated ones are esterified on PC and SL. The transverse partitioning of cholesterol is unclear but surprisingly seems to be in favor of the cytoplasmic leaflet in different cells (Devaux and Morris, 2004; Wood et al., 2011). These observations point out key questions about the existing lateral heterogeneities in the plasma membrane. How can a "raft" exist in the inner leaflet of the plasma membrane since it is depleted in sphingolipids and enriched in unsaturated acyl chains? Can lipid domains of different nature and composition exist in both leaflets? Finally, if they do exist, how are they coupled? Indeed, there is no theoretical problem with the existence of Lo domains in both leaflets of the plasma membrane, although the physical and chemical properties of these domains must be different. For example, liquid ordered (Lo) domains could exist in PS/PE dominated inner leaflet, provided these lipids are saturated or bear only one unsaturated fatty acid restricted to the *sn-2* position. Indeed several papers have reported that the inner leaflet domains containing PC/Chol or PE/Chol would be less stable than the outer leaflet "rafts." This has also been observed in model membranes (Samsonov et al., 2001). Thus Lo domains in the outer and the inner membrane leaflet of biomembranes should not necessarily spontaneously match to each other. On the opposite, some studies performed on asymmetric model system at the thermodynamic equilibrium tend to show the opposite result and end up in a coupled macroscopic

phase separation (Lo/Ld) on both leaflets (Allender and Schick, 2006; Wan et al., 2008; Kiessling et al., 2009).

MOLECULAR SHAPE AND CURVATURE

Another topic of interest for retroviral assembly is the role of spontaneous local curvature of the membrane. A major regulator of this local curvature is the lipid average molecular shape (Israelachvili, 1977). This molecular shape can be defined by a simple geometric property of the molecule (Israelachvili-Mitchell-Ninham packing parameter: $P = v/al$), where v is the molecular volume, a is the cross section area of the head group and l is the length of the molecule (mainly due to acyl chains) (see **Figure 1**). In a dynamic aggregate, those values should be considered as average molecular properties. Although the role of the average molecular shape in the spontaneous curvature of lipid bilayers is more and more questioned (Cooke and Deserno, 2006), value of P turns out to be very useful to predict the structure of lipid assemblies. For example, it is clearly seen from **Figure 1** that if one of the two leaflets of the membrane start having different average P value, the bilayer will suffer from built-in curvature stress.

Another way to describe the membrane curvature in a continuous model is the bending energy. This latter relies on both the spontaneous curvature (which can be seen as the average value of the molecular shape) and the bending stiffness of the membrane (basically, the thicker the membrane, the higher the bending stiffness). It is clear that average curvature plays a role in lipid sorting although it is hard to define if lipid sorting induce curvature or the opposite (for an extensive review see Callan-Jones et al., 2011). Nevertheless, many reports have shown that bending energy of a homogeneous tri-component model membrane submitted to inhomogeneous curvature can be reduced by enriching the highly curved region in liquid disordered lipids (van Meer and Lisman, 2002; van Meer and Sprong, 2004). It then appears that lipid sorting induced by curvature is a collective effect more than a single molecule effect. Moreover, it is clear that membrane attached proteins (such as Gag) plays a role in this average curvature and the associated lipid sorting. It is therefore highly probable that HIV-1 virus budding, which induces a positive curvature of the membrane, should occur with different average molecular shape on both leaflets of the membrane, i.e., with different lipid composition on both leaflet. In their review (Callan-Jones et al., 2011) stated that "In cell membranes, it is very unlikely that individual lipids, unassisted by interactions with themselves or with proteins, can be enriched in curved regions simply based on their shape alone." This questions the role of individual molecular spontaneous curvature in the curvature based lipid sorting in cells and on the opposite, this reinforce the role of proteins and lipid domains in inducing or responding to curvatures.

To summarize, on both side of the membrane, lipid domain formation and stabilization are due to a combination of lateral segregation or phase separation, induced or spontaneous curvature, transverse distribution asymmetry. This has been recently shown in the outer leaflet in the case of "raft" domains (Meinhardt et al., 2013).

ROLE OF LIPIDS DOMAINS IN HIV-1 ASSEMBLY

As stated in the introduction, the minimal component required for HIV-1 assembly at the plasma membrane, is the viral Gag protein. Its MA domain is mainly responsible for membrane interaction and targeting to the plasma membrane (Ono et al.,

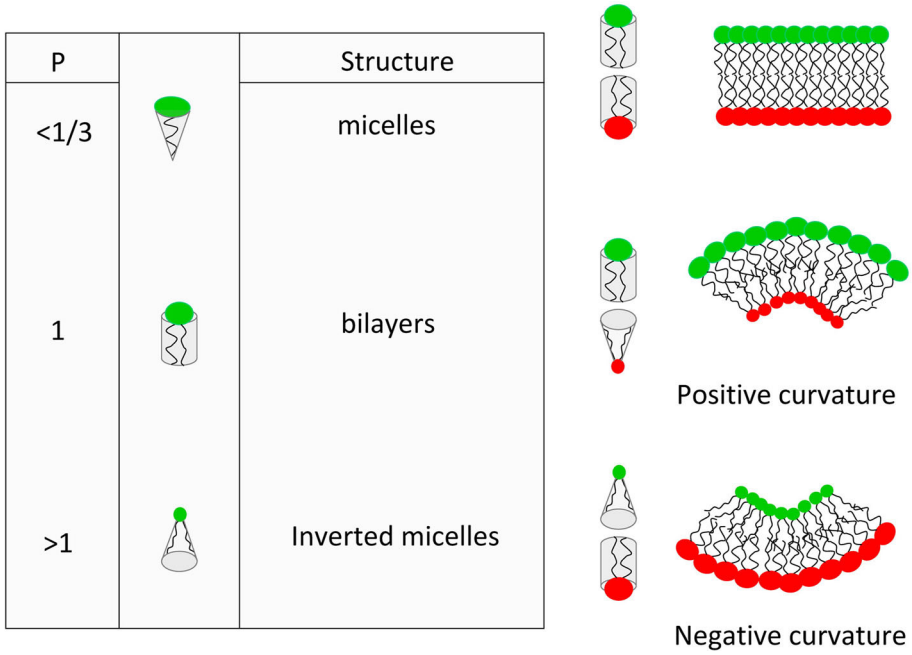

FIGURE 1 | Basic concepts of the lipid average molecular shape and its effect on spontaneous curvature of lipid membranes.

2000), although MA alone seems to exhibit a lower affinity for membrane than Gag (Zhou and Resh, 1996). MA domain has a bipartite motif of interaction with lipid membranes like many other proteins of the same class (for review see Resh, 1999). Indeed, HIV-1 Gag interacts with acidic lipids such as phosphatidyl inositols phosphates(PIP_x) and phosphatidylserine (PS) by means of a polybasic region—called HBR (Highly Basic Region)—(Zhou et al., 1994; Freed et al., 1995; Ono and Freed, 1999). Amongst the (PIP_x), the phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) is considered to be the more specific (Ono and Freed, 2004; Chukkapalli et al., 2008; Hamard-Peron et al., 2010) but phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) or phosphatidylinositol 3,4,5 triphosphate ($PI(3,4,5)P_2$) also bind efficiently (Anraku et al., 2010). As it is the case for other polybasic proteins (Ben-Tal et al., 1997; Murray et al., 1997), one can expect that this electrostatic interaction occurs at long range distances ($d > 1.5$ nm) and can be considered as the attractive force between the lipid membrane and the Gag protein. In addition to its HBR region, Gag is also myristoylated at its N-terminus. This myristoylation is responsible for tightening the attachment of Gag to the plasma membrane by insertion into the membrane.

Elucidation of HIV MA structure bound to $diC_4PI(4,5)P_2$ or $diC_8PI(4,5)P_2$ has shown a switch of the myristoyl from an hydrophobic pocket of MA into the membrane. This switch is thought to be induced by electrostatic binding to $PI(4,5)P_2$ (Saad et al., 2006, 2007; Ono, 2010a). Nevertheless, it appeared that the myristate can spontaneously be released from its hydrophobic pocket in the vicinity of lipid membranes or upon trimerization of the protein (Tang et al., 2004; Valentine et al., 2010; Charlier et al., 2014). While Valentine et al. suggested that Gag released myristate could probe the membrane by successive insertion/exclusion until finding its $PI(4,5)P_2$ target, the coarse grain molecular modeling of Charlier et al. (2014) suggest in contrast, that insertion of the myristate occurs after non-specific electrostatic attraction to the membrane and permits the Gag protein to find a correct orientation to capture the $PI(4,5)P_2$ head in the HBR. Recent NMR data obtained by Vlach and Saad (2013) suggested that other lipid such as PS, PE and PC could reinforce the interaction of Gag with plasma membrane by direct binding to a different site of Gag.

The role of pre-existing lipid domains in the interaction of Gag with the plasma membrane and in HIV-1 assembly has been studied for many years.

Different experiments based on:

- detergent solubilization (Nguyen and Hildreth, 2000; Lindwasser and Resh, 2001; Ono and Freed, 2001; Holm et al., 2003)
- cholesterol depletion (Ono and Freed, 2001; Ono et al., 2007)
- immunofluorescence co-localization (Nguyen and Hildreth, 2000; Holm et al., 2003; Ono et al., 2005)
- lipidomics (Bruegger et al., 2006; Chan et al., 2008; Lorizate et al., 2013).

have suggested a potential role of "rafts" in the assembly of Gag. Although lipidomic studies differ one from each other, they have mainly shown that HIV lipid envelope was highly enriched in PIP_x, but also slightly in sphingomyelin (Chan et al., 2008; Lorizate et al., 2013). Results are contradictory regarding enrichment in cholesterol and PS (Chan et al., 2008; Lorizate et al., 2013).

TEM have also been proposed to be the site for HIV-1 assembly (Booth et al., 2006; Nydegger et al., 2006; Thali, 2009). TEM has been shown to co-localize with Gag in T cells (Jolly and Sattentau, 2007; Grigorov et al., 2009) and tetraspanin components are found to be incorporated into HIV-1 particles, especially CD81 (Grigorov et al., 2009). GM3, for example, is described to be highly present in TEM (Hemler, 2005; Yanez-Mo et al., 2009) and is enriched in the virus lipid envelope compared to the plasma membrane (Chan et al., 2008).

Although the functional goal of assembling into TEM or "rafts" has still not been elucidated, different molecular mechanisms (mainly for assembly into "rafts") have been proposed. Moreover it has been suggested that Gag could induce the coalescence of clustered rafts and TEMs at its own assembly site (Hogue et al., 2011).

"Rafts" are considered to be mainly enriched in saturated lipids, therefore the major problem of Gag partitioning into "rafts" is its interaction with $PI(4,5)P_2$, which naturally bear a long unsaturated acyl chain in its sn-2 position. In our opinion, the most subtle, elegant and detailed model to solve this controversy is coming from the NMR structure of MA with $di\text{-}C_8PI(4,5)P_2$ (Saad et al., 2006). In this mechanism, the unsaturated sn-2 acyl chain of the $PI(4,5)P_2$ is sequestered in a hydrophobic cluster of MA amino acids concomitantly to the myristate switch, whilst the saturated sn-1 acyl chain remains in the plasma membrane. The sequestration of the unsaturated fatty acid of the $PI(4,5)P_2$ out of the hydrophobic part of the membrane thereby allows the complex MA-$PI(4,5)P_2$ to partition into rafts (Ono, 2010b; Simons and Gerl, 2010). This model has been recently reinforced by the suggestion that Gag could sense cholesterol and liquid-ordered acyl-chains environments (Dick et al., 2012) and by new NMR experiments performed by Vlach and Saad (2013). These new NMR data show the existence in MA of a second lipid binding site inducing sn-2 acyl chain flipping into a new associated hydrophobic pocket whatever the bound lipid is (PS, PC, PE). Since sn-2 position is usually the place where unsaturated acyl chains hold in lipids, this new model shows that MA is able to locally deplete the complex MA-bound lipids of unsaturated acyl chains. As a result, the complex, mainly bearing saturated acyl chains, could therefore partition faster into lipid "rafts" despite the lack of direct interaction with sphingomyelin (Vlach and Saad, 2013).

Nevertheless partitioning of Gag into "rafts" is still a matter of controversy since "rafts," as they are defined, can almost exclusively exist in the outer leaflet of the plasma membrane, explaining therefore the lack of direct interaction of MA with sphingomyelin. Recently, Keller et al. (2013) have nicely shown that a myristoylated multimerizable Gag bound to $PI(4,5)P_2$ containing model membrane exclusively partition into liquid disordered domains, not ordered ("rafts") ones. Moreover, starting from the NMR structure established by Saad et al. (2006), Charlier et al. (2014) have performed coarse grain molecular dynamics of Myr-MA in the presence of a lipid bilayer whose composition approach

the inner leaflet of the plasma membrane. Our study shows that, in this configuration, the unsaturated sn-2 acyl chain of the $PI(4,5)P_2$ never flipped out of the membrane into an hydrophobic pocket of Gag. It is important to notice that, although it is not discussed in the paper of Charlier et al. (2014), PS was seen to bind at the site where Saad et al. have seen it by NMR (Vlach and Saad, 2013), but, here again, without any flipping of its sn-2 acyl chain. Moreover, in the light of what is described on page 3 of this review, it is worth wondering how interesting it would be to trap acyl chains into hydrophobic pockets of Gag during HIV-1 assembly. An oversimplified guess shows that removing acyl chains from the plane of the membrane will locally change the molecular curvature of the complex and will induce, during assembly, a negative curvature opposite to the positive curvature expected for budding. Whereas there is no doubt that the viral lipid envelop is enriched in sphingomyelin and cholesterol it is still unclear at which step of the assembly this enrichment occurs. Indeed, these two recent studies clearly questions the role of "raft" as a pre-existing platform where virus assembly occurs.

A question that still remains is the possibility for Gag to be targeted at pre-existing inner leaflet domains. Some studies have suggested that $PI(4,5)P_2$ could spontaneously aggregate into nanodomains (Johnson et al., 2008; Ellenbroek et al., 2011; Salvemini et al., 2014). But it has also been shown that $PI(4,5)P_2$ is sequestered by proteins in the cell (for review see McLaughlin et al., 2002). In our opinion, based on different other studies regarding the effect of membrane bound proteins on lipid phase separation (Ehrig et al., 2011a,b; Witkowski et al., 2012), it is more than likely that, as we already proposed (Kerviel et al., 2013), assembly induces lipid domain, not the opposite. Using coarse grained molecular dynamics of the interaction of Myr-MA with inner lipid leaflet we have shown a potential enrichment of $PI(4,5)P_2$ all around the protein (Charlier et al., 2014), leading to putative enriched acidic lipid nanodomain formation as we already suggested in Kerviel et al. (2013).

Based on micro-emulsion theory, it has been recently demonstrated (Shlomovitz and Schick, 2013) that local fluctuations of curvature could induce asymmetric lipid domains (in term of lipid composition) in both leaflet. The theory predicts that inner PS enriched domains could face outer SL enriched domains ("rafts"), which turns out to be very nice in terms of HIV-1 assembly... Unfortunately, in this configuration (PS domains facing SL domains), the induced curvature is negative, i.e., opposite to viral budding. More generally, the role of pre-existing lipid domains in favoring virus assembly is unclear, whatever their composition and origin are. Indeed, a recent study on the dynamics of the interaction of Gag with TEM domains has shown Gag multimerization to be responsible for trapping CD9 into the domain of assembly instead of Gag targeting through CD9 on preexisting TEMs (Krementsov et al., 2010). This suggests that during retroviral assembly, Gag is trapping membrane components instead of being trapped at specific pre-existing domains. These controversial data shows that the role of lipids during HIV-1 assembly is far from being elucidated.

CONCLUSION

One of the key questions regarding the role of membranes in HIV-1 assembly is the time-ordering of events across the membrane. Is there an induction of inner leaflet lipid domains during multimerization process domains or is Gag targeted to pre-existing coupled outer and inner leaflet domains in order to rapidly assemble. Indeed, in the released virus, the ratio Gag to Env is largely in favor of Gag, it seems therefore reasonable to expect that HIV-1 assembly is an "inside out" process, not an "outside in," i.e., Gag may be driving the assembly from the inside, not pre-existing outer "rafts" domains with Env trapped into (for a scheme of the process see **Figure 1** in Mariani et al. in this special issue). Nevertheless, at the moment, it appears that the respective roles of lipid domains and viral proteins during HIV-1 assembly are still entangled. It thus remains an exciting challenge for virologists as well as for biophysicists to remove this degeneracy.

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Tsg101 regulates PI(4,5)P_2/Ca^{2+} signaling for HIV-1 Gag assembly

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Our previous studies identified the 1,4,5-inositol trisphosphate receptor (IP3R), a channel mediating release of Ca^{2+} from ER stores, as a cellular factor differentially associated with HIV-1 Gag that might facilitate ESCRT function in virus budding. Channel opening requires activation that is initiated by binding of 1,4,5-triphosphate (IP3), a product of phospholipase C (PLC)-mediated PI(4,5)P_2 hydrolysis. The store emptying that follows stimulates store refilling which requires intact PI(4,5)P_2. Raising cytosolic Ca^{2+} promotes viral particle production and our studies indicate that IP3R and the ER Ca^{2+} store are the physiological providers of Ca^{2+} for Gag assembly and release. Here, we show that Gag modulates ER store gating and refilling. Cells expressing Gag exhibited a higher cytosolic Ca^{2+} level originating from the ER store than control cells, suggesting that Gag induced release of store Ca^{2+}. This property required the PTAP motif in Gag that recruits Tsg101, an ESCRT-1 component. Consistent with cytosolic Ca^{2+} elevation, Gag accumulation at the plasma membrane was found to require continuous IP3R activation. Like other IP3R channel modulators, Gag was detected in physical proximity to the ER and to endogenous IP3R, as indicated respectively by total internal reflection fluorescence (TIRF) and immunoelectron microscopy (IEM) or indirect immunofluorescence. Reciprocal co-immunoprecipitation suggested that Gag and IP3R proximity is favored when the PTAP motif in Gag is intact. Gag expression was also accompanied by increased PI(4,5)P_2 accumulation at the plasma membrane, a condition favoring store refilling capacity. Supporting this notion, Gag particle production was impervious to treatment with 2-aminoethoxydiphenyl borate, an inhibitor of a refilling coupling interaction. In contrast, particle production by a Gag mutant lacking the PTAP motif was reduced. We conclude that a functional PTAP L domain, and by inference Tsg101 binding, confers Gag with an ability to modulate both ER store Ca^{2+} release and ER store refilling.

Keywords: HIV-1, Gag assembly, L domain, Tsg101, PI(4,5)P_2, IP3R, Ca^{2+} signaling, ER-PM junction

INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) functions in the cell as plasma membrane anchor and substrate to phospholipase C (PLC) (McLaughlin and Murray, 2005). Together, these two functions give PI(4,5)P_2 influence over multiple processes in the cell despite being a minor plasma membrane lipid component. Our work and that of others indicate that both functions of PI(4,5)P_2 are required for HIV-1 virus production, specifically, Gag assembly. Intact PI(4,5)P_2 at the plasma membrane targets Gag to this site: When PI(4,5)P_2 is depleted by adventitious expression of 5-ptase IV, the characteristic plasma membrane localization of Gag is not detected (Ono et al., 2004; Chukkapalli et al., 2008; Fernandes et al., 2011); when PI(4,5)P_2 accumulation is routed to endosomes by adventitious expression of the Arf6/Q67L mutant, Gag is mostly associated with endosomes (Ono et al., 2004). Both conditions are inhibitory to virus particle formation (Ono et al., 2004; Chukkapalli et al., 2008; Fernandes et al., 2011) in agreement with prevailing models that assembly occurs at the plasma membrane (Ehrlich and Carter, 2012; Sundquist and Krausslich, 2012). Underpinning PI(4,5)P_2-mediated Gag membrane anchoring are direct contacts between PI(4,5)P_2 and residues in the MA and CA domains in Gag (Saad et al., 2006; Shkriabai et al., 2006). Additionally to this role, our studies (Ehrlich et al., 2010, 2011) demonstrate that Gag assembly requires PI(4,5)P_2 in its capacity as substrate to PLC. PLC-catalyzed hydrolysis of PI(4,5)P_2 (Rhee, 2001; Suh et al., 2008) is part of a universal pathway for increasing the concentration of Ca^{2+} ions in the cytosol (Berridge, 2009). We found that inactivation of PLC enzymatic activity abolished localization of Gag to plasma membrane and formation of the assembled Gag particle despite an abundance of intact PI(4,5)P_2 at the plasma membrane (Ehrlich et al., 2010) while activation of PLC led to enhancement of particle formation (Ehrlich et al., 2011). We interpret this requirement for the function of PI(4,5)P_2 as PLC substrate to indicate active participation of Ca^{2+} signaling machinery in Gag assembly.

Through a proteomic search aimed at identification of cellular factors that might participate with HIV-1 Gag and endocytic sorting complexes required for transport (*ESCRT*) in facilitating virus budding, we identified inositol (1,4,5)-trisphosphate receptor (IP3R) as a protein enriched with other Ca^{2+} signaling proteins in plasma membrane-enriched sub-cellular fractions when Gag was expressed. **Figure 1** outlines the central role of IP3R in PI(4,5)P_2-dependent Ca^{2+} signaling (Patterson et al., 2004a; Banerjee and Hasan, 2005; Mikoshiba, 2007). IP3R forms a transmembrane Ca^{2+} ion-specific channel on the membrane of the endoplasmic reticulum (ER), the major organelle for intracellular storage of Ca^{2+}, where it gates release of the ion into the cytosol. IP3R channel opening requires activation that is initiated by the binding of inositol trisphosphate (IP_3) which is produced upon hydrolysis of PI(4,5)P_2. IP3R-mediated release of Ca^{2+} from the ER store (store emptying) reduces the luminal ER Ca^{2+} concentration triggering ER store-refilling through *s*tore-*o*perated *Ca*$^{2+}$ *e*ntry (*SOCE*; Smyth et al., 2010; Vaca, 2010). During SOCE, the apposition of ER tubules to the plasma membrane permits ER-resident Ca^{2+} sensor proteins, called *s*tromal *i*nteracting *m*olecules (*STIMs*), to directly bind and activate plasma membrane-resident Orai Ca^{2+}influx channels (Park et al., 2009). Employing a variety of experimental strategies, we found that productive virus assembly and IP3R function were directly correlated. Agonists known to promote PI(4,5)P_2-dependent Ca^{2+} signaling stimulated Gag accumulation on the plasma membrane and budding of the assembled Gag particles while antagonists that interfered with signaling inhibited both (**Table 1**). Some requirements, e.g., for Gαq and events linked to SOCE, became apparent following disruption of the PTAP Late (L) domain. The PTAP L domain serves as docking site for the cellular protein, Tsg101, which is required for efficient virus particle budding (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). Here, we show that modulation of ER Ca^{2+} release and ER store refilling are components of productive Gag assembly and that these events are facilitated by recruitment of Tsg101 by the PTAP L domain in Gag.

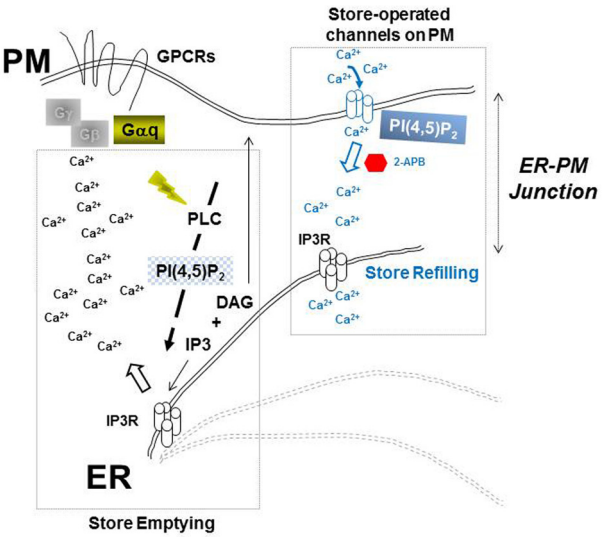

FIGURE 1 | PI(4,5)P_2-dependent Ca^{2+} machinery. Ca^{2+} store emptying (**left**) and store re-filling (**right**) at dynamic ER-PM junctions (dashed lines) are highlighted as key events of IP3R-gating at the prototypical Ca^{2+} store in the ER.

MATERIALS AND METHODS

PLASMIDS AND ANTIBODIES

Plasmids encoding pCMV-Gag-EGFP (WT HIV-1 Gag C-terminally tagged with green fluorescent protein, Hermida-Matsumoto and Resh, 2000), WT Gag-HA and Δp6-Gag-HA (Jin et al., 2007) were kind gifts of M. Resh and J. Jin, respectively. Plasmids encoding P7L-Gag-GFP, Y36S-Gag-GFP, and P7L/Y36S-Gag-GFP were engineered by site-directed mutagenesis using Gag-GFP and P7L-Gag-GFP as templates (Medina et al., 2011). Primary antibodies were: polyclonal rabbit anti-HIV-1 CA (Ehrlich et al., 1992); anti-HIV-1 p6 (a kind gift of S. Campbell); anti-IP3R type 1 (Affinity BioReagents); anti-IP3R type 3 (BD Biosciences); anti-p85 α-subunit of phosphatidylinositol 3-kinase (PI3K, Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-PI(4,5)P2 (Abcam); rabbit anti-IP3R type 2 and anti-actin (Sigma). Secondary antibodies were obtained from: goat anti-rabbit IgG IRDye 800 (Rockland); goat anti-rabbit IgG IRDye 680LT (LI-COR); goat anti-mouse IgG Alexa Fluor 680; TRITC-tagged secondary IgG (Molecular Probes); and gold-conjugated donkey anti-rabbit IgG (Jackson Immuno Res Labs).

CELL CULTURE, TRANSFECTION, AND HARVEST

COS-1 and COS A2.5 (Babé et al., 1995) cell culture, DNA transfection using XtremeGene reagent (Roche) and harvest of cells and tissue culture media were done as previously described (Goff et al., 2003; Ehrlich et al., 2011). For 2-aminoethoxydiphenyl borate (2-APB, Calbiochem) treatment: Stock solutions were prepared using dimethyl sulfoxide (DMSO) as solvent. Treatment media with DMSO only or with 2-APB at 50, 100, and 150 μM final concentration in Ca^{2+}-free DMEM was prepared fresh prior to use. At 24 h post-transfection, the tissue culture media was aspirated and replaced with treatment media and the plates returned to the incubator. After 24 h, tissue culture media was collected, passed through a 0.45 μm filter for use in VLP isolation and the cells were scraped and pelleted for use in preparing cell lysates.

PROTEIN DELIVERY WITH BIOPORTER REAGENT

COS-1 cells were grown on coverslips in 6-well plates. For early antibody delivery, media was aspirated and replaced with 500 ul of DMEM and delivery mixture (antibody mixed with the BioPORTER reagent, Genlantis) prepared according to manufacturer instructions was added dropwise to the cells. After 4 h in the 37°C incubator, the delivery mixture was removed, replaced with 2 ml of fresh complete media and the cells transfected with DNA encoding Gag-GFP following the usual transfection protocol. Following an additional 24 h incubation period, the coverslips were processed for analysis by deconvolution confocal microscopy. For late antibody delivery, the cells were transfected with DNA encoding Gag-GFP following the usual protocol. After 24 h of incubation, tissue culture media was aspirated, replaced by

Table 1 | Observations Linking HIV-1 Budding to PI(4,5)P_2-PLC-IP3-IP3R-Ca^{2+} Signaling Cascade.

Agonists that promote virus budding
Extracellular Ca^{2+}/Ionomycin-induced rise in cytosolic Ca^{2+} [a,b,c]
Thapsigargin-induced ER store depletion [a]
IP3R-FLAG adventitious expression [a]
Antagonists that inhibit virus budding
5-phosphatase IV-induced PI(4,5)P2 depletion from plasma membrane [d,e]
U73122-mediated inhibition of PLC activity [a]
High affinity-binding IP3R fragment-induced sequestration of IP3 [a]
Targeted antibody blockade of IP3 binding [a]
siRNA-mediated IP3R depletion [a]
siRNA-mediated PLCγ depletion [f]

[a] Ehrlich et al., 2010; [b] Perlman and Resh, 2006; [c] Grigorov et al., 2006; [d] Fernandes et al., 2011; [e] Ono et al., 2004; [f] Ehrlich et al., 2011.

500 ul of DMEM and freshly prepared delivery mixture was added drop-wise. At the end of 4 h, the delivery mixture was removed and replaced with 2 ml of fresh complete media. Following an additional 24 h incubation, the coverslips were processed for deconvolution confocal microscopy. Cells on slides were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, incubated with TRITC (*red*)-tagged secondary antibody and the nucleus stained with DAPI (Molecular Probes). Slides were mounted and fluorescent signals viewed and captured with an inverted fluorescent/dic Zeiss Axiovert 200 M microscope operated using Axiovision software.

Ca^{2+} MEASUREMENTS

Intracellular free Ca^{2+} ion was determined in cultures of cells that were mock-transfected cells, transfected with WT Gag or the Δp6Gag mutant as previously described (Guo et al., 2005). Briefly, cells were detached with a buffer (Hank's Buffered Salt Solution, HBSS) stream, counted, incubated with Fura-2 AM (Sigma), pelleted, washed, and resuspended in HBSS, with or without EGTA. Measurements were done at room temperature in an ISS spectrofluorometer with continuous stirring of the sample. Samples were excited at 340 and 380 nm and fluorescent signals emitted at 510 nm were recorded at intervals over a 2 min period. The ratio of fluorescence emitted at 340 and 380 nm was converted to free calcium ion concentration $[Ca^{2+}](nM)$.

SUCROSE GRADIENT FLOATATION

Pelleted cells were washed three times with cold PBS, swollen in 1 ml of cold hypotonic buffer (10 mM Tris, pH 7.4, 1 mM $MgCl_2$) containing protease inhibitors and disrupted with a Dounce homogenizer. The homogenate was spun for 10 min at 1000 × g to obtain a post-nuclear supernatant which was subsequently centrifuged at 27,000 × g to collect a membrane-rich pellet (P2). The P2 pellet was resuspended in 200 µl of a 40% sucrose solution (w/v in PBS), placed at the bottom of the centrifuge tube, and overlaid step-wise with 200 µl of 40, 30, 20, and 10% sucrose solutions. The samples were centrifuged at 72,000 rpm in a TLA-100 rotor for 60 min. All centrifugations were done at 4°C. Aliquots of the floatation gradient were collected from the top of the centrifuge tube.

WESTERN ANALYSIS

Proteins were separated by electrophoresis in 10% (unless otherwise stated in the text) SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane or dot-blotted. Following incubation with appropriate primary and secondary antibodies, protein bands were visualized and quantitated (where needed) using an infrared-based imaging system (Odyssey, LI-COR Biosciences). Release efficiency was defined as the ratio of the signal intensity value for the VLP-associated Gag to the sum of the values for VLP-associated Gag plus cell lysate-associated Gag (VLP/[VLP + Cell Lysate]).

IMMUNO-ELECTRON MICROSCOPY

Cells grown on ACLAR film were fixed in 4% paraformaldehyde/0.1% EM grade glutaraldehyde (Electron Microscopy Sciences) in PBS and processed for resin embedding following standard protocols. The thin sections were incubated with rabbit anti-IP3R type3 polyclonal antibody and subsequently with gold-conjugated donkey anti-rabbit antibody. Sections were counterstained with uranyl acetate and lead citrate and viewed with a FEI Tecanal BioTwinG2 electron microscope.

DECONVOLUTION CONFOCAL MICROSCOPY

Cells on coverslips were fixed in 3.7% formaldehyde (Fisher) for 20 min, and then permeabilized in 0.1% Triton X-100. All images were captured on an inverted fluorescence/differential-interference contrast Zeiss Axiovert 200 M deconvoluting fluorescence microscope operated by AxioVision version 4.5 (Zeiss) software. Ten to 20 optimal sections along the *z* axis were acquired in increments of 0.4 µm. The fluorescence data sets were deconvoluted by using the constrained iterative method (AxioVision). Images shown are of the central focal plane unless otherwise stated. To quantify relative co-localization of signal from two (*red and green*) channels, Pearson correlation coefficients were obtained using Image J quantification software downloaded from NIH website http://rsbweb.nih.gov/ij/index.html. Co-localization analysis was performed using the co-localization finder plug-in. The significance level of correlation coefficients was assessed by reference to http://www.jeremymiles.co.uk/misc/tables/pearson.html.

TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF) MICROSCOPY

COS-1 cells were grown on 25 mm cover slips coated with poly-lysine and co-transfected with WT Gag-GFP and SS KDEL-RFP plasmids. Cell imaging was done after a 20 h incubation period. Immediately prior to imaging, the cover slip was rinsed in Imaging Buffer (130 mM NaCl, 2.8 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM HEPES, pH 7.4), then mounted in an open bath chamber with ∼1 mL of Imaging Buffer. Cells were imaged with an Olympus IX-81 microscope equipped with an Olympus TIRF Launch, Olympus 60× Plan Apo, $N =$ 1.45 TIRF objective, 2× optovar, Photometrics DV2 dual-view image splitter, and Andor iXon CCD camera. Fluorescent proteins were excited with Olympus Cell∗ digital lasers with AOTF shutters at 488 and 561 nm. The objective was equipped with a Semrock LF488/561-A-000 filter cube, with 482/563 excitation filter, 523/610 emission filter, and 488/561 dichroic mirror. The

dual-view was equipped with Chroma 11-EM GFP/RFP (565 dcxr) filter cube, with D520/30 and D630/50 m emission filters. The TIRF angle and laser AOTF shutters were controlled with the native Olympus Cell^TIRF software, and images were recorded with Metamorph Premier (Molecular Devices) software. Image frames were acquired with alternating 488 and 561 nm excitation, with 100 ms exposures at 2 Hz. For image analysis, the red and green channels of cell images were aligned using the calculated alignment of an image of Fluospheres 505/515 (Invitrogen) yellow-green emitting, 100 nm polystyrene beads captured immediately prior cell imaging and aligned using in-house Matlab-based software (Mathworks). Aligned red and green images were overlaid in Metamorph.

RESULTS

CELLS EXPRESSING GAG EXHIBIT HIGHER CYTOSOLIC $[Ca^{2+}]_i$, DERIVED FROM INTRACELLULAR STORES

The concentration of free, unbound Ca^{2+} ion in the cytosol of the cell ($[Ca^{2+}]_i$) is maintained at low levels (~100 nM) due to the combined action of the binding of the Ca^{2+} ions to buffers and protein adaptors, its translocation into intracellular stores and its extrusion out of the cell (Berridge et al., 2003; Hanson et al., 2004; Clapham, 2007). We (Ehrlich et al., 2011) previously provided evidence that $[Ca^{2+}]_i$ in cells expressing WT Gag was higher than in mock-treated cells or in cells expressing a budding-defective Gag mutant. The mutant, P7L-Gag, possesses a single residue change in the primary L domain (P_7TAP to L_7TAP) that impairs Tsg101 binding to the site (Demirov et al., 2002). That earlier study, where we utilized a cell imaging-based assay for measuring free unbound Ca^{2+} ions in the cytosol, indicated that Gag expression was accompanied by a significant increase (~1.5-fold) in $[Ca^{2+}]_i$. Here, we confirm that finding, using an assay that examines the entire population of transfected cells (**Figure 2**). A 1.5-fold increase in $[Ca^{2+}]_i$ was observed in cells that had been transfected with DNA encoding WT Gag over the level measured for cells expressing Δp6 Gag, a mutant missing PTAP and the other L domains, and over the level obtained for mock-transfected cells. Detection of this difference in the assay of the culture indicates that most of the cells in the culture underwent the change. Moreover, as was the case in the single cell imaging-assay, the higher $[Ca^{2+}]_i$ was observed in the presence or absence of 2 mM EGTA, a cell-impermeant chelator of Ca^{2+} ions, indicating that the increase in $[Ca^{2+}]_i$ did not require influx of the ion from the extracellular environment. The results indicate that (i), Gag expression leads to an increase in cytosolic Ca^{2+} through release of the ion from intracellular stores; (ii), the L domains housed in the p6 region of Gag are determinants of the increase and (iii), this change occurred in a large number of the cells in the culture.

IP3R ACTIVATION IS REQUIRED THROUGHOUT THE ASSEMBLY PERIOD TO RETAIN GAG AT THE PLASMA MEMBRANE

ER store Ca^{2+} that is released to the cytosol is rapidly taken up by cellular Ca^{2+} binding buffers and proteins (Schwaller, 2010; Yanez et al., 2012). Our finding that $[Ca^{2+}]_i$ in Gag-expressing cells is above basal level at steady state indicates that Gag assembly induces Ca^{2+} store release events to occur in the cell. We had previously shown that IP3R is required for Gag association with the plasma membrane (Ehrlich et al., 2010). We therefore asked whether IP3R mediation of Ca^{2+} release from the ER store is required for accumulation of Gag at the plasma membrane as well. To test this, we employed the BioPORTER reagent as a protein delivery vehicle to introduce anti-IP3R antibody targeted specifically to the activation domain of IP3R either simultaneously with DNA transfection or 24 h later (**Figure 3**, early or late addition, *respectively*). To account for possible non-specific effects, control antibody (polyclonal antibody directed at the p85 α-subunit of PI3K) was delivered to transfected cells. We have used both of these anti-IP3R and control antibodies in microinjection experiments where each was co-injected with Gag-encoding DNA into cells that were subsequently examined by deconvolution confocal microscopy and have demonstrated that anti-IP3R antibody, but not control antibody, prevented association of Gag at the plasma membrane (Ehrlich et al., 2010). In the current experiment, COS-1 cells grown on coverslips and transfected with Gag-encoding DNA at $T = 0$ were treated with an antibody-BioPORTER mixture either immediately or 24 h later. Thus, for the early addition protocol, the coverslips were processed for deconvolution confocal microscopy at 24 h post-DNA transfection; for the late addition protocol, samples were processed at 48 h post-DNA transfection. The early interference protocol control samples (**A1** panels) indicated that Gag was detected at the plasma membrane in 100% of cells examined

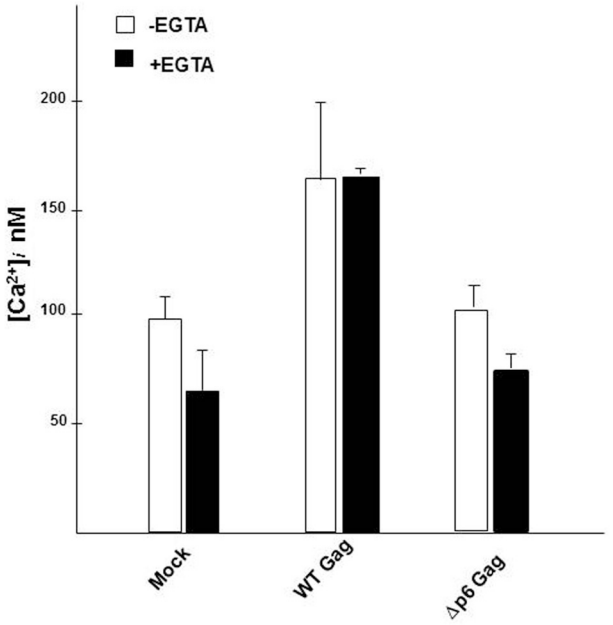

FIGURE 2 | Gag expression induced elevation of cytosolic Ca^{2+} concentration. Mock-transfected cultures of COS cells or cultures transfected with WT Gag or Δp6 Gag were assayed for Ca^{2+} in the absence (*open symbol*) or presence (*filled symbol*) of EGTA. Each bar represents the average value determined for $[Ca^{2+}]_i$ in 3 independent trials using triplicate samples. In each trial, $[Ca^{2+}]_i$ was assayed every 6 s over a 2 min period. The standard error of the mean was ±3% for the mock-treated samples and ±5% for the *gag*-transfected samples.

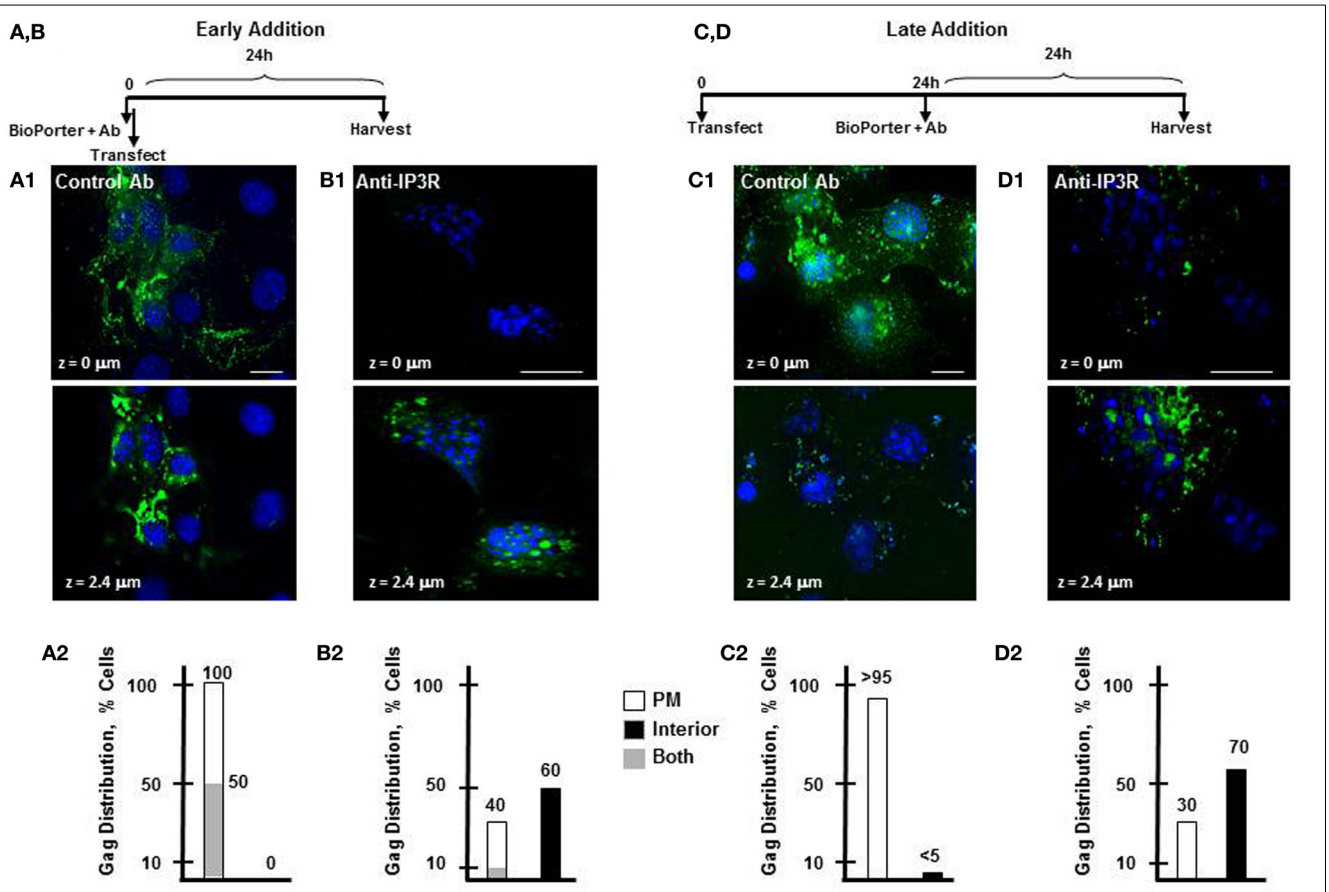

FIGURE 3 | Interference with IP3R function prevents Gag accumulation on the plasma membrane. (A,B and C,D) Schematic diagram of protocol for early **(A,B)** and late **(C,D)** delivery of anti-IP3R antibody. **(A1)** Control antibody, or **(B1)**, anti-IP3R rabbit antibody directed to the highly conserved IP3 binding domain on IP3R were delivered into cells 4 h prior to transfection of DNA encoding Gag-GFP. Twenty-four hours later, the cells were prepared for deconvolution confocal microscopy. **(C1,D1)** Cells were first transfected with DNA encoding Gag-GFP DNA and after 24 h, subjected to protein transduction of control antibody **(C1)** or the anti-IP3R antibody **(D1)**. After an additional 24 h incubation, cells were fixed and processed as above. **(A2–D2)** Quantitative analysis of cells containing Gag at the plasma membrane exclusively (*open bars*), in the cell interior exclusively (*filled bars*), or in both locations (*gray bars*), based on examination of 80–100 cells, $n = 2$. Dapi stain (*blue*) was used to label the nucleus. The figure shows sections through the z plane of cells representing the plasma membrane-proximal ($z = 0$) and the cell interior ($z = 2.4$). Bars indicate 10 µm.

(100/100, panel **A2**). In half of these cells, Gag was additionally in the cell interior (panel **A1** top, cell periphery, $z = 0\,\mu m$; panel **A1** bottom, cell interior, $z = 2.4\,\mu m$). Comparable results were obtained in the transfected cells that received buffer only (i.e., mock-treated; *not shown*). That Gag was mostly at the plasma membrane is consistent with the known rapid trafficking of Gag to the plasma membrane and its primary localization to that membrane compartment at steady state (Hermida-Matsumoto and Resh, 2000; Jouvenet et al., 2006; Perlman and Resh, 2006). In cells receiving anti-IP3R antibody (**B1** panels), Gag was mainly detected in the cell interior z section (panel **B1**, bottom, $z = 2.4$) in contrast to the results obtained in the control sample, where all cells had Gag at the cell periphery (panel **A1**, top, $z = 0$). Analysis indicated that Gag was detectable at the plasma membrane in only 40% of cells examined (32/80, panel **B2**) with the remainder of the cells showing Gag exclusively in the perinuclear region in contrast to the control. In some cases, Gag was detected in round intracellular vacuoles, reminiscent of the results obtained in previous studies where immuno-gold tagged Gag was found to accumulate in 200–500 nm vesicles in the cell interior following siRNA-mediated IP3R depletion (Ehrlich et al., 2010). The late interference protocol control samples (**C1** panels) showed Gag at the plasma membrane in essentially all cells examined (95/100, panel **C2**, $z = 0$). In contrast, in cells receiving anti-IP3R antibody (**D1** panels) Gag was detected mainly in the cell interior z section (panel **D1**, bottom, $z = 2.4$). Analysis revealed Gag at the plasma membrane only in 30% of cells examined (30/100, panel **D2**) and mainly in the interior in the other cells. This result indicates that anti-IP3R delivery at 24 h post DNA transfection caused the Gag molecules that were already at the plasma membrane at this time (see **A1** panels) to be removed from the plasma membrane. We conclude that IP3R activation is continuously required for association and retention of the Gag form that was on the plasma membrane. This provides a possible explanation for the elevation in cytosolic Ca^{2+} detected in cells supporting productive Gag assembly.

ER AND GAG ARE IN PROXIMITY AT THE CELL PERIPHERY

The higher cytosolic Ca^{2+} in WT Gag expressing cells, whether measured in single cells (Ehrlich et al., 2011) or in the cell culture (cf. **Figure 2**) suggests an L domain-mediated modulation of store Ca^{2+} release activity. By analogy to proteins that have been shown to influence IP3R channel activity such as RACK1 (Patterson et al., 2004b) or 80K-H (Kawaai et al., 2009), the ability to influence IP3R channel activity infers physical proximity of Gag to IP3R. Although we have not observed Gag co-localization with the ER body in the cell interior, we previously showed that Gag co-localizes with FLAG-tagged IP3R on the plasma membrane and in ER-derived tubules near the cell periphery (Ehrlich et al., 2011), providing opportunity for proximity to ER store-released Ca^{2+}. As it is generally recognized that ER-plasma membrane (ER-PM) junctions, i.e., dynamically formed regions where the ER is in close apposition to the plasma membrane, are a feature of all eukaryotic cells (Friedman and Voeltz, 2011), we examined cells by *t*otal *i*nternal *r*eflection *f*luorescence (*TIRF*) to determine whether the peripheral ER, revealed by the luminal marker ss-RFP-KDEL, was sufficiently close to plasma membrane-localized Gag-GFP to make feasible a functional link with IP3R channels on ER membrane (**Figure 4**). TIRF (reviewed in Steyer and Almers, 2001) allows visualization of only those fluorescent signals near the plasma membrane where signal is generated only in fluorophores that are positioned in the near plasma membrane region (i.e., from the coverslip surface to ∼100 nm above the coverslip) as illustrated in the cartoon (panel **A**). The images (panel **B**) revealed that fluorescent signals were evident in the TIRF field from both the Gag-GFP on the plasma membrane (*green*) and the ER lumen marker ss-RFP-KDEL (*red*) whether cells were examined at 8 h post-transfection (panel **B1**) or 24 h post-transfection (panels **B2,B3**). Consistent with previous studies (Jouvenet et al., 2008), Gag was detected both as a diffuse signal and as discrete puncta. The latter were previously demonstrated to exhibit the characteristics expected of genuine higher-ordered Gag assemblages or VLPs while the former were suggested to be low oligomeric Gag forms (Jouvenet et al., 2008). As expected based on previous studies (Jouvenet et al., 2008), at the early time Gag exhibited a diffuse distribution; later both diffuse and punctate signals were detected. At 24 h post-transfection, the majority of cells are expected to exhibit a predominantly punctate signal as shown in panel (**B3**); an image from a cell exhibiting fewer puncta (panel **B2**) was included to show that both low oligomer and higher-ordered Gag assemblages were detected in the region. Control studies using mCherry-FP as a marker for cytosol (panel **C**) revealed co-localization of Gag with this marker, indicating that cytosol components also were accessible to Gag in the ER-PM junctional region. This is as expected since Ca^{2+} is released into this space. At both times post-transfection, image overlays revealed yellow puncta, indicating sites of red and green signals in very close proximity suggesting the presence of assembling Gag and VLPs in the plasma membrane regions encompassed by the ER-PM junctions. It should be noted that ER-PM junctions are detectable in most cells (Friedman and Voeltz, 2011), making it difficult to ascertain whether Gag possesses any inducing effect on junction formation. Nevertheless, ER-PM junctions present themselves as sites where IP3R channels on tips of peripheral ER can conceivably get within interaction distance of plasma membrane-localized Gag molecules.

That IP3R can come close to plasma membrane-localized Gag was suggested by our earlier observation that adventitiously expressed IP3R-FLAG co-localized at the cell periphery with WT Gag but, interestingly, not with P7L-Gag (Ehrlich et al., 2011). We wanted to eliminate the possibility that the observed co-localization might be unique to adventitious expression and therefore examined endogenous IP3R for proximity to PM-localized Gag. To assess Gag proximity to endogenous IP3R, we examined mock-treated cells or cells transfected with DNA encoding Gag by immunoelectron microscopy (IEM; **Figure 5**). Cells grown on ACLAR were transfected with DNA encoding WT Gag and at 36 h post-transfection the ACLAR was processed as described in Materials and Methods and examined for IP3R-tagged gold signal. Cells supporting productive Gag assembly were identified by detection of cell-associated virus-like particles (VLPs). Cells were examined for IP3R-3 using a primary mouse monoclonal antibody that recognized the IP3R isoform and secondary donkey anti-mouse polyclonal antibody tagged with 15 nm gold particles. An average number of 11 (210/19) or 14 (224/16) gold particles was detected in the cytoplasm of mock- or *gag*-transfected cells, *respectively* ($n = 2$). No gold was detected in cells depleted of the IP3R-3 target of the antibody by siRNA-mediated interference prior to *gag*-transfection (*data not shown*). Similarly, gold was not detected when the primary antibody was omitted (*not shown*). In the mock-transfected cells, the gold was almost exclusively found in the cell interior (panel **A**). In contrast, in cells in the *gag*-transfected culture, gold particles were detected at the cell periphery (panel **B**, narrow arrows) and in budding (panel **C**) or released (panel **D**) VLPs (broad arrows). Detection of gold signal on VLPs, suggesting virion-association of IP3R, is consistent with previous proteomic analysis that revealed IP3R encapsidation in purified infectious virus (Chertova et al., 2006). Quantification of the gold-tagged IP3R distribution (panel **E**) indicated that the level of plasma membrane-associated IP3R was >20-fold higher in *gag*-transfected cells (12%) compared to mock-treated cells (0.5%). The level was >50-fold higher if compared to gold particles associated with both the plasma membrane and VLPs. The results indicate a significant re-distribution of IP3R in cells expressing Gag, consistent with our previous findings using FLAG-tagged IP3R (Ehrlich et al., 2011).

As described above, interference with IP3R function by introduction of antibody targeted to its activation site prevented Gag accumulation on the plasma membrane in a majority of cells whether the blockade occurred early or later in the Gag assembly period (cf. **Figures 3B2,D2**). TIRF analysis detected Gag at ER-PM junctions early and later (cf. **Figure 4**). As a further test for Gag-IP3R proximity, we examined cells by confocal microscopy. Anti-IP3R antibody was delivered into mock- or DNA-transfected cells, cells were fixed, permeabilized as described in the legend to **Figure 3**, and then incubated with a fluorescently-tagged secondary antibody prior to examination by confocal microscopy (**Figure 6**). While the IP3R signal (*red*) in mock-transfected cells was diffusely distributed in the perinuclear region or throughout the cell interior (panel **A**) as previously reported by our laboratory and others (Vermassen et al., 2004; Ehrlich et al., 2011),

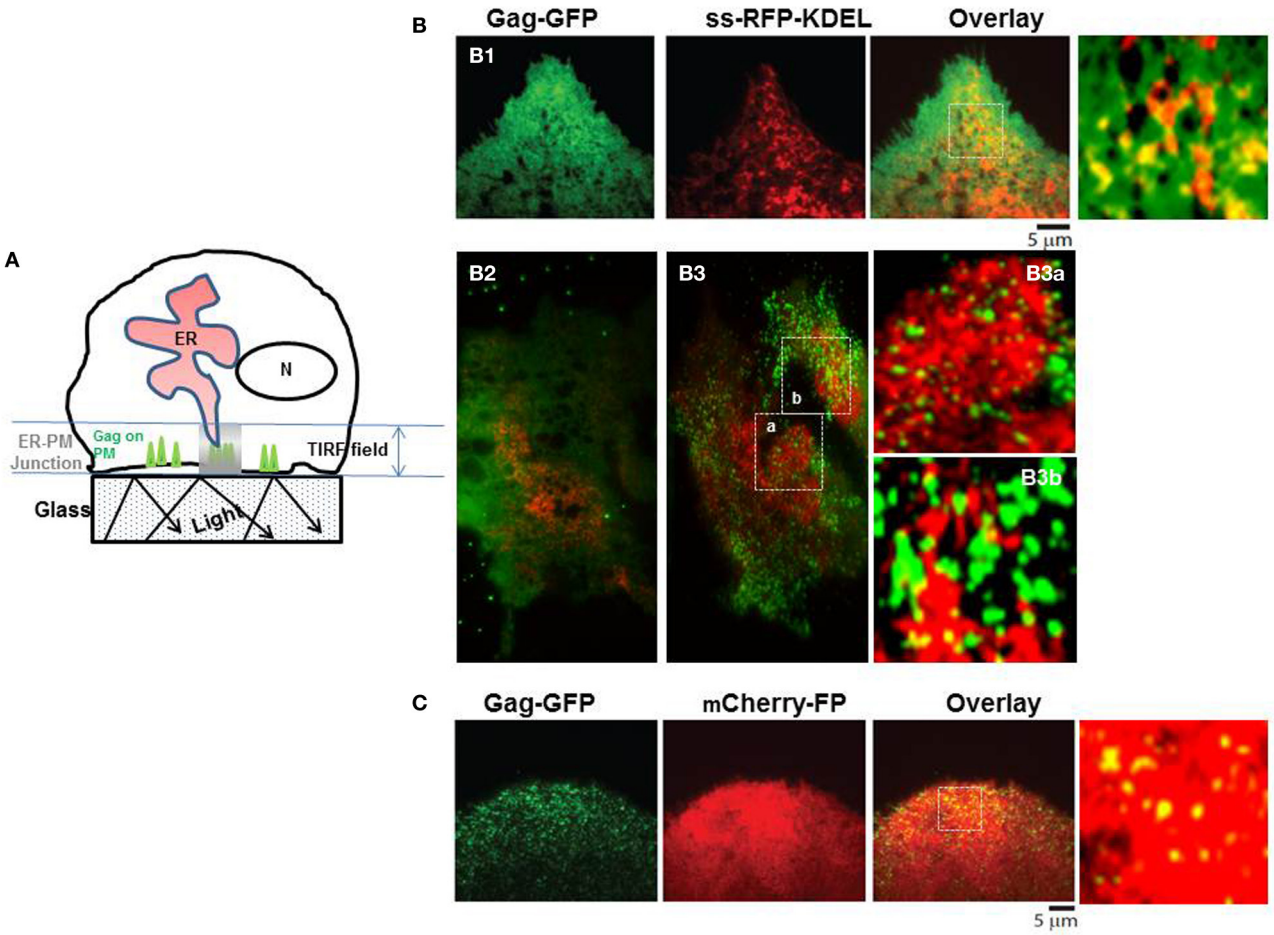

FIGURE 4 | TIRF microscopy of peripheral ER in proximity to Gag at the plasma membrane. (A) Schematic drawing depicting area of signal capture in TIRF field. Panels **(B,C)** show representative images of Gag-GFP fluorescence (green) at an early (8 h, **B1**) and a later (24 h, **B2,B3,C)** time point. SS-KDEL-RFP (*red*) labels the ER **(B)**; mCherry-FP (red) labels the cytosol **(C)**. Indicated regions (white box) in **(B,C)** are enlarged in **(B1,B3,C)**. Overlay shows that some puncta are yellow, indicating co-localization of red and green signals.

the cells in the *gag*-transfected culture exhibited a more punctate signal (panels **B,D**) with some of the signal detected at the cell periphery (bracketed, panels **B,E**). As in TIRF, in some cases, an overlay of the punctate Gag (*green*) and IP3R (*red*) signals produced yellow, suggesting that the proteins co-localized (panels **B–D**, 16/50 cells = 32%, Pearson's coefficient of correlation = 0.7). In other instances, close apposition but not co-localization was observed (panels **E–G**). In either case, these findings are consistent with the results of TIRF and IEM and support the conclusion that Gag and IP3R are in proximity at the cell periphery.

IP3R AND GAG CO-IMMUNOPRECIPITATE

We determined whether the apparent proximity of Gag and IP3R at ER-PM junctions permitted their coimmunoprecipitation from cell lysates prepared by Dounce homogenization of cells in the presence of 1% Triton X-100. Most of the IP3R in such lysates can be expected to derive from the ER body and the network of ER tubules. Cells transfected with DNA encoding WT Gag and mock-transfected cells were Dounce homogenized in lysis buffer containing 1% Triton X-100, clarified by centrifugation at 1000 × g and incubated with pre-immune serum or antibodies that recognized IP3R-1 or Gag (**Figure 7**). Analysis of lysate (panel **A**) indicated that both mock and transfected samples possessed equivalent amounts of full-length and IP3R fragments that were recognized by an antibody targeted to an epitope at aa1829–1848 (panel **A1**). In contrast, the anti-CA antibody recognized Gag in the lysate prepared from transfected cells but not from the mock-treated cells (panel **A2**). Anti-IP3R1 antibody pulldown (panel **B**) showed Gag in IP3R1-specific immune-precipitates from the lysate of transfected cells but not from the lysate derived from the mock-transfected cells (panel **B1**). The antibody brought down a nearly identical panel of IP3R-related fragments with major forms >95 kDa (panel **B2**). In the reciprocal pulldown experiment (panel **C**), antibody that recognizes the p6 region in Gag formed an immune precipitate that mostly contained a ∼100 kDa IP3R-related fragment (panel **C1**, *lane 2*) and Gag (panel **C2**, *lane*

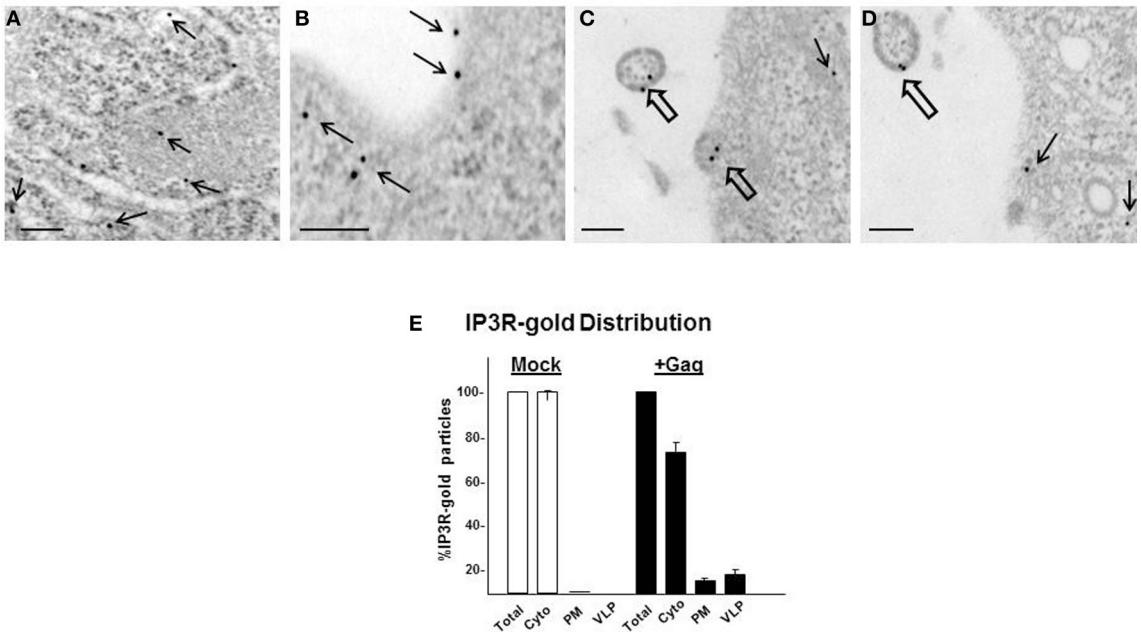

FIGURE 5 | Gag and IP3R are in proximity at the plasma membrane. (A) Immunogold labeling of endogenous IP3R-3 in mock-transfected cells. **(B–D)** Cells transfected with DNA encoding WT Gag. Panels show representative images with IP3R-tagged gold detected at or near the plasma membrane (*thin arrows*) and in budding or fully released VLPs (*thick arrows*). Bar: 100 nm. **(E)** Distribution of gold-tagged IP3R in mock-treated cells and cells expressing Gag.

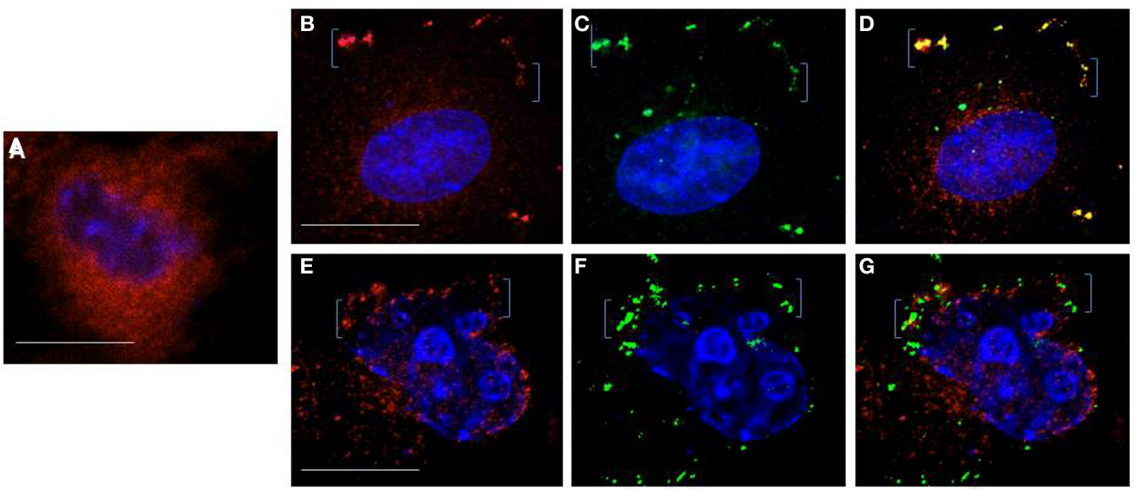

FIGURE 6 | Immunostaining of IP3R. Anti-IP3R antibody delivered into cells using Bioporter reagent was used to detect endogenous IP3R in mock-treated cells **(A)** or cells that were transfected with DNA encoding Gag-GFP (**B–D** and **E–G**). Twenty-four hours post-transfection, cells were fixed, permeabilized, incubated with TRITC-(*red*)-tagged secondary antibody and examined by deconvolution confocal microscopy to visualize IP3R (*red*) and Gag-GFP (*green*). Brackets indicate regions of Gag-IP3R proximity at the plasma membrane. Bar: 10 μm.

2). This fragment, which was consistently observed, most likely was derived by truncation of the intact 300 kDa IP3R protein, as the latter was detected in relatively small amounts (cf. **Figure 7C1**). As the apparently more stable 100 kDa IP3R-related protein was co-precipitated reproducibly, we conclude that, in the cell, Gag was in close proximity to IP3R and the ER Ca^{2+} stores.

IP3R DIFFERENTIALLY ASSOCIATES WITH WT GAG AND P7L-GAG

Because the ∼100 k fragment was not well-represented in the total cell lysate as shown in the anti-IP3R1 antibody reprobe (**Figure 7B2**), we speculated that it might have originated from the ER-PM junction rather than the ER body or ER tubular network. Gag is associated with membranes that should be separable from Golgi and ER by differential centrifugation. To examine

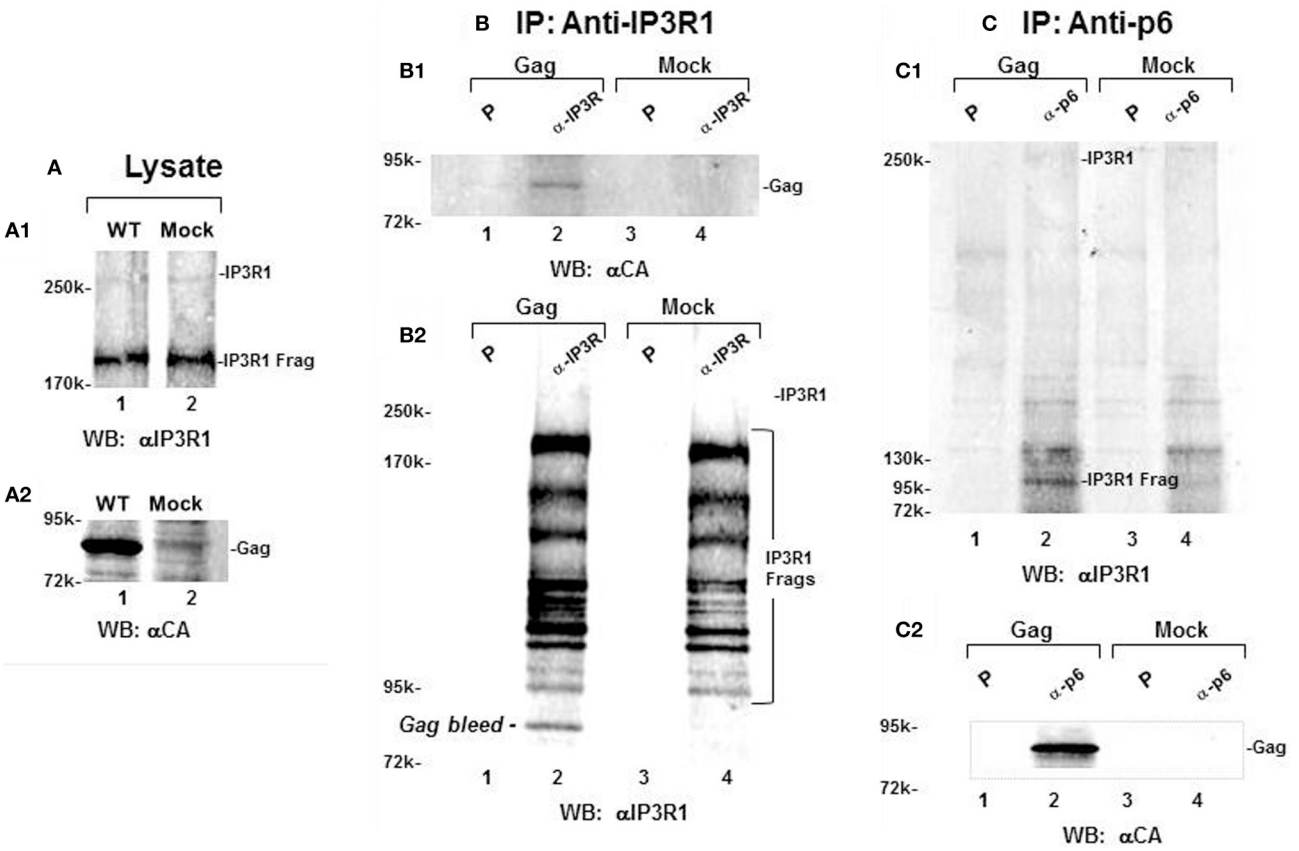

FIGURE 7 | Gag and IP3R can be reciprocally immunoprecipitated. **(A)** Total lysate from cells transfected with Gag (*lane 1*) or from mock-transfected cells (*lane 2*) probed for IP3R (**A1**, *top*) and Gag (**A2**, *bottom*). **(B,C)** Immunoprecipitation of Gag by anti-IP3R antibody **(B)** or by anti-p6 antibody **(C)**. Total lysates were mixed with either preimmune serum (P, *lanes 1,3*) or anti-IP3R antibody (*lanes 2,4*) and immune precipitates were analyzed by Western blotting for IP3R-1 **(B2,C1)** and for Gag **(B1,C2)**. Reciprocal pulldown is shown for Gag by anti-IP3R1(**B1**, *lane 2*) and for a 100 kDa IP3R-related fragment by anti-p6 antibody (**C1**, *lane 2*).

this possibility, cells were Dounce-homogenized in the absence of Triton X-100, followed by centrifugation at 1000 × g to obtain a post-nuclear supernate (S1). Further centrifugation of the S1 fraction at 27,000 × g provides a membrane-enriched fraction (P2; Goff et al., 2003). A P2 fraction prepared from cells that had been transfected with DNA encoding WT Gag or P7L-Gag was used to further assess Gag-IP3R subcellular proximity (**Figure 8**). Panel (**A**) shows a Western analysis of the P2 fraction for IP3R and Gag following solubilization of the fraction in buffer with 1% Triton-X100. In contrast to the total cell lysate in which the anti-IP3R1 antibody detected numerous IP3R fragments (cf. **Figure 7B2**), the P2 fraction almost exclusively contained the ∼100 kDa IP3R-related fragment (panel **A**). Interestingly, the lysates derived from cells expressing WT and P7L-Gag exhibited a clear a difference in the amount of this fragment. To examine the basis for this difference, further separation of membranes in the P2 fraction was conducted through floatation on sucrose. As shown in panel (**B**), WT Gag and P7L-Gag were both detected in gradient fractions 16–20 but only the WT protein floated to the lighter density region of the gradient (fractions 11–15). This bias in WT Gag floatation was confirmed by Western analysis of pooled fractions 12–14 (panel **B2**). Using the WT Gag-containing pool of fractions 12–14 in immune-precipitation experiments (panel **C**) showed that addition of anti-IP3R antibody pulled down Gag (panel **C1**, *lane 1*) and, reciprocally, addition of anti-Gag antibody pulled down the ∼100 kDa IP3R-related fragment (panel **C2**, *lane 1*). Neither pull-down was observed with use of pre-immune serum (*lane 2*). This observation implicates Tsg101 binding in facilitating Gag localization to a membrane context where the Gag and IP3R proteins are in proximity.

L DOMAIN-DEPENDENT MODULATION OF STORE REFILLING

Store Ca^{2+} release is essential for a large number of cellular processes (Clapham, 2007). Both the continuous requirement for IP3R mediation of store Ca^{2+} release (cf. **Figure 3**) and the high level of cytosolic Ca^{2+} originating from internal stores (cf. **Figure 2** and Ehrlich et al., 2011) would suggest that the process of Gag assembly empties the Ca^{2+} store. Thus, cells supporting productive Gag particle formation have either adapted to operating on empty stores or engage in store refilling. Store refilling is characterized by the presence of plasma membrane-proximal puncta that are recognized to consist of ER-resident

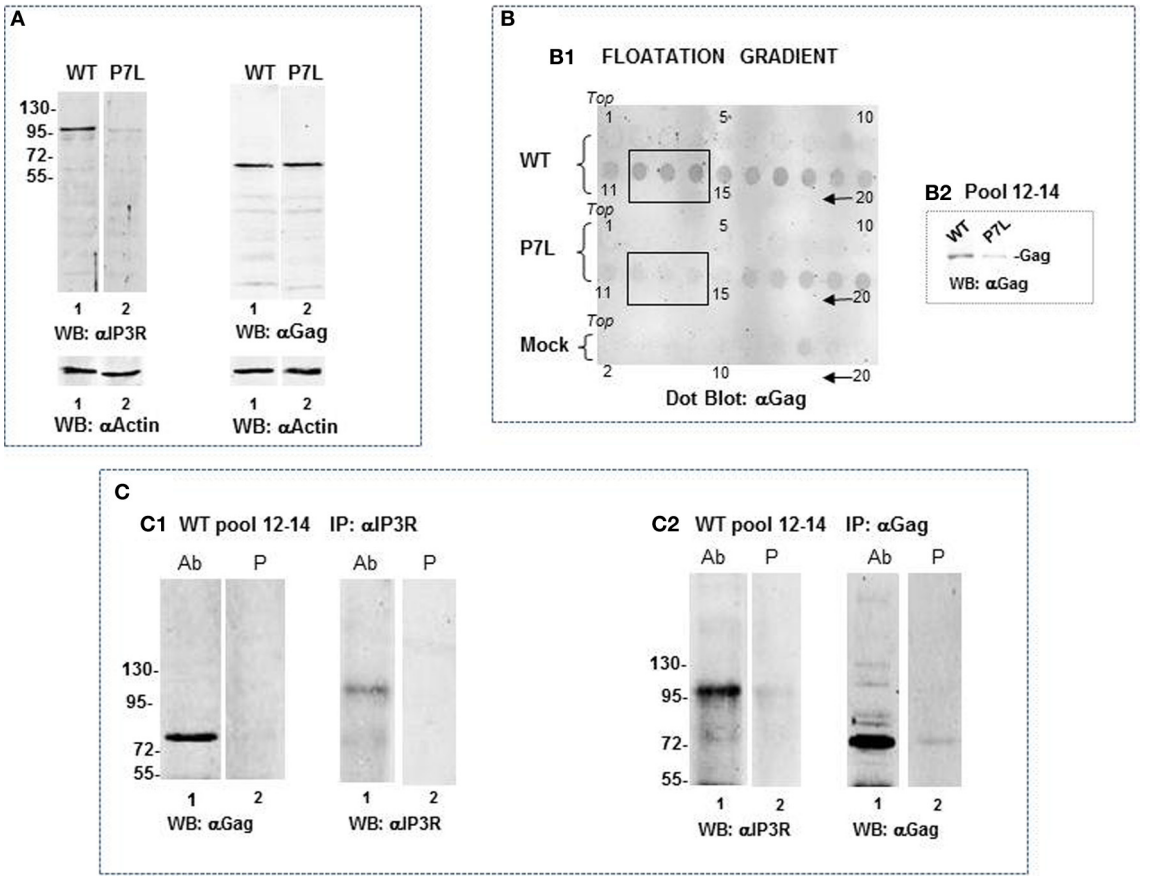

FIGURE 8 | Differential association of IP3R with WT-Gag and P7L-Gag. (A) Western analysis of P2 fraction prepared from cells transfected with DNA encoding WT Gag or P7L-Gag. IP3R probing shows ∼100 kDa IP3R-related fragment as predominant species detected by antibody. Gag probing shows equivalent amounts of wild-type and mutant Gag in P2 fraction. **(B)** Floatation on sucrose density gradient of P2 fraction prepared from cells that had been transfected with DNA encoding WT Gag (*top*), transfected with P7L-Gag (*middle*), or mock-transfected (*bottom*). Fraction 1 is the top of the gradient. **(B1)** Dot blot analysis of 5 ul of gradient fractions (every fraction for WT and P7L samples and every other fraction for the mock-treated sample). Fractions 12–14 were pooled and analyzed for Gag by standard Western analysis to confirm the presence of Gag and absence of P7L in the light density membranes **(B2)**. **(C)** Co-immunoprecipitation experiment using the pool of fractions 12–14 from WT Gag sample. Western analysis shows detection of Gag in the anti-IP3R1 antibody immunoprecipitate (**C1**, *lane 1*) and detection of the ∼100 kDa IP3R-related fragment in the anti-Gag antibody immunoprecipitate (**C2**, *lane 1*).

STIM1 with its C-terminal tail engaged in interaction with PM-resident PI(4,5)P_2 headgroups and the Orai Ca^{2+} influx channel (Walsh et al., 2009; Carrasco and Meyer, 2011). We therefore used plasma membrane PI(4,5)P_2 as an indicator of store refilling to compare its status in naïve COS-1 cells and cells supporting productive and non-productive Gag VLP formation. **Figure 9** shows the plasma membrane proximal (z plane = 0 μm) and interior (z plane = 1.2 μm) regions of cells examined by deconvolution confocal microscopy using an anti-PI(4,5)P_2 antibody. PI(4,5)P_2 was detected in the cell interior in all cases, i.e., whether the cell was mock-treated (panel **A**), expressed WT Gag constitutively (panel **B**) or transiently (panel **C**), or expressed P7L-Gag (panel **D**). However, the phospholipid was apparent in a plasma membrane-proximal location only in the cells expressing WT Gag (panels **B,C**). A quantitative analysis of 30 cells (panel **E**) indicated that PI(4,5)P_2 was detected at or near the plasma membrane (*filled symbol*) in the cells expressing Gag at a ∼3- to 5-fold higher frequency than in the mock-treated cells or the cells expressing the P7L-Gag mutant ($n = 2$). Taken together, the results suggest that, compared to mock-transfected cells or cells expressing P7L, more of the cells supporting productive Gag particle formation have the components for store-refilling in place. As this condition requires a functional PTAP L domain, these observations implicate Tsg101 binding in the store refilling event related to Gag assembly.

To further examine the relationship between viral budding and store refilling, the effect of blocking store refilling on VLP production was tested (**Figure 10**). All cells undergo store refilling with the store operated Ca^{2+} entry (SOCE) as the ubiquitous refilling mechanism that relies on functional and physical coupling between the ER-resident Ca^{2+} sensor protein (STIM1) and the plasma membrane-resident Orai Ca^{2+} influx channel (Smyth et al., 2010; Vaca, 2010). The agent, 2-APB at 50 μM and higher concentrations potently and selectively inhibits Ca^{2+} influx by interrupting this coupling (Cheng et al., 2011; Yamashita et al., 2011). As shown in panel **(A)**, WT Gag VLP release was not perturbed by 2-APB treatment (*lanes 1–4*). Following

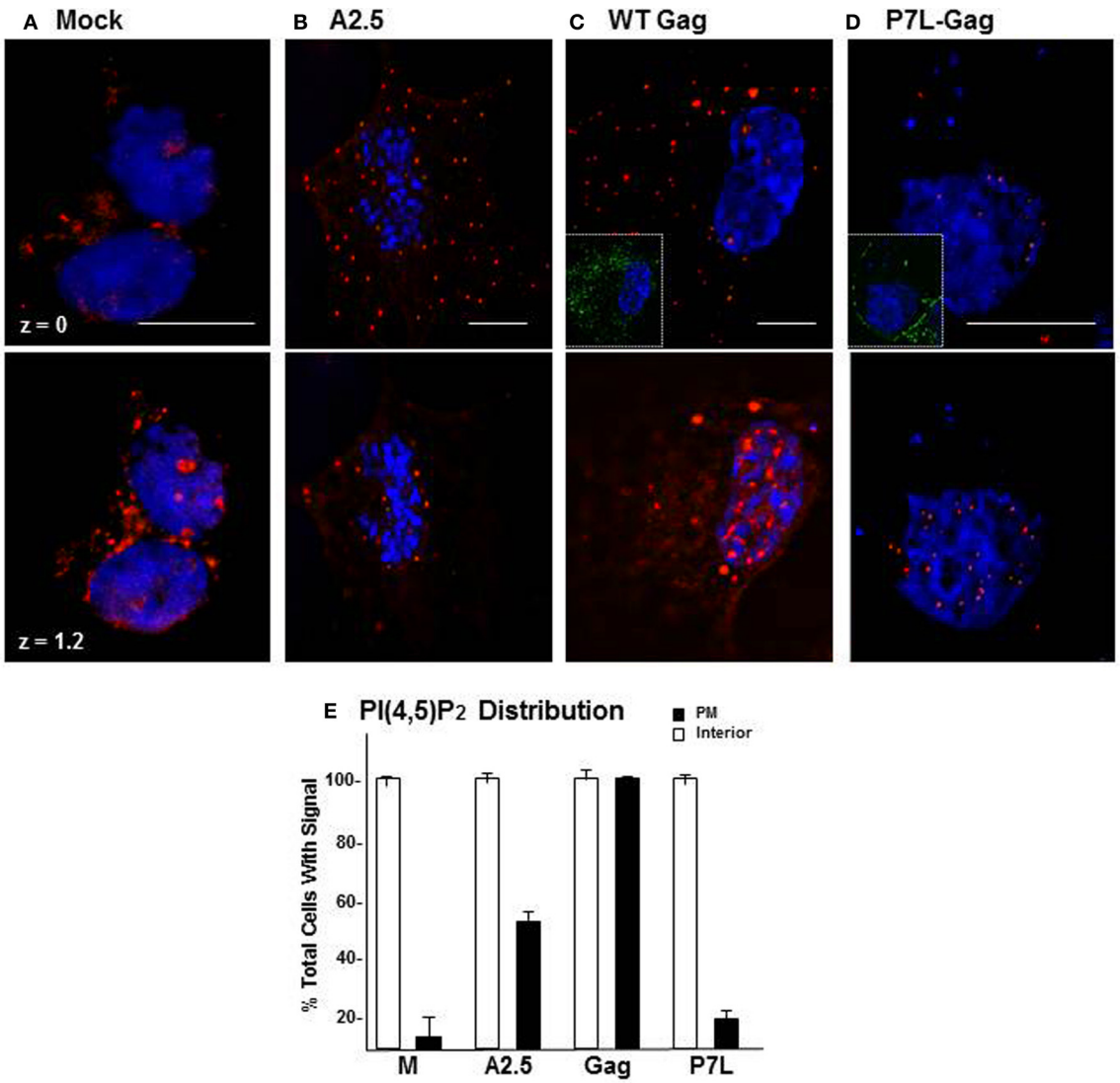

FIGURE 9 | Accumulation of PI(4,5)P_2 in Gag expressing cells is L domain dependent. Mock-treated COS-1 cells **(A)**, COS-12A2 (A2.5) cells that express Gag constitutively **(B)**, or COS-1 cells transfected 24 h with DNA encoding WT Gag **(C)** or P7L-Gag **(D)** were examined for PI(4,5)P_2 by deconvolution confocal microscopy. The phospholipid was detected by staining with an anti-PI(4,5)P_2 primary antibody and visualized by indirect immunofluorescence using a TRITC-labeled secondary antibody (*red*) targeted to the primary antibody. Insets in **(C,D)** show Gag expression in the cells as detected using primary antibody that recognized the capsid domain in Gag and a FITC-labeled secondary antibody targeted to the primary. The bar measures 10 μm. **(E)** Distribution of PI(4,5)P2 in cells.

deletion of the entire L domain-bearing p6 region (Δp6-Gag), Gag exhibited dose-dependent 2-APB sensitivity (*lanes 5–8*). To map the determinant of resistance to 2-APB within the p6 region, a test of the effect of the treatment was done using Gag L domain mutants (panel **B**). The p6 region bears both L domain-1 (PTAP), which recruits Tsg101 (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001) and L-domain-2 ($LY_{36}PX_nL$), which recruits Alix (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). To determine the 2-APB sensitivity of budding directed mainly by L domain-1, L domain-2 was impaired with a Ser substitution for Y36. This single mutation in Gag was sufficient to abrogate Alix binding (Watanabe et al., 2013). The Y36S mutation had little effect on budding efficiency in the presence or absence of 2-APB (*lanes 1–4*). In contrast and as previously reported (Ehrlich et al., 2011), the P7L mutant exhibited dose-dependent sensitivity to 2-APB (*lanes 5–8*). Although the Y36S mutation in itself had little impact on release efficiency, when combined with the P7L mutation the resulting Gag double mutant (P7L/Y36S) is released at very low efficiency (Medina et al., 2011). Under this condition, budding is directed by determinants in the C-terminal region of the CA domain in Gag and facilitated by the ubiquitin E3 ligase Nedd4-2s (Chung et al., 2008; Usami et al., 2008). Interestingly, the double mutant P7L/Y36S exhibited greater sensitivity to 2-APB than either parent alone (*lanes 9–12*). Thus, addition of 2-APB reduced VLP release efficiency for all

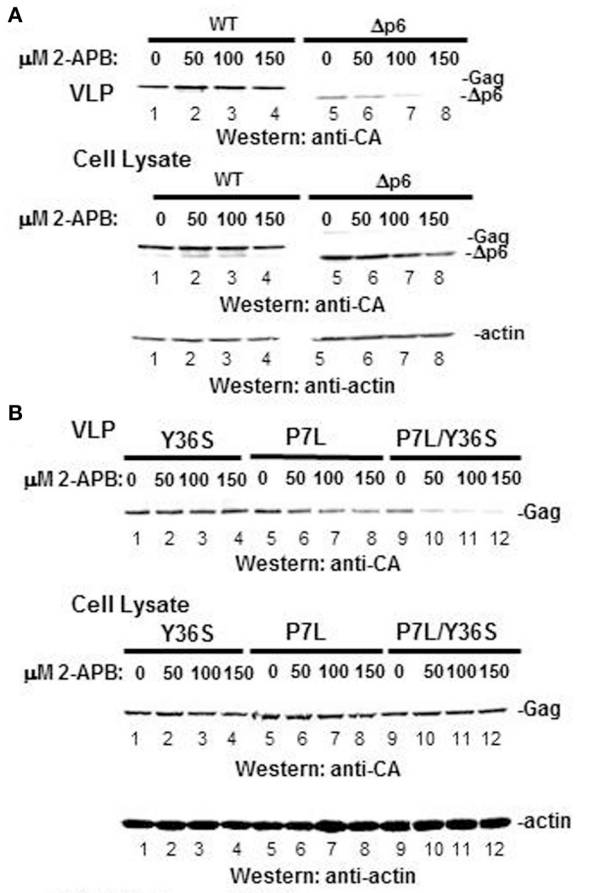

FIGURE 10 | L domain-dependent resistance to 2-APB-mediated inhibition of SOCE. COS-1 cells were transfected with DNA encoding: **(A)** WT Gag (WT, *lanes 1–4*) or a Gag mutant missing the entire C-terminal p6 region (Δp6, *lanes 5–8*); **(B)** Gag with a Tyr36 to Ser substitution in L Domain-2 in p6 (Y36S, *lanes 1–4*); Gag with Pro7 to Leu substitution in L Domain-1 (P7L, *lanes 5–8*); or Gag with both substitutions (P7L/Y36S, *lanes 9–12*). At 24 h post-transfection, the tissue culture media was replaced with treatment media containing DMSO or increasing concentrations of 2-APB. After 24 h, tissue culture media and cells were harvested for Western analysis of Gag VLP (*top*), cell-associated Gag (*middle*), and actin (*bottom*). **(C)** VLP release efficiency. To facilitate sample comparison, more cells were used for samples containing the P7L and P7L/Y36S mutants.

budding pathways except the one mediated by Tsg101 (panel **C**). That WT Gag VLP production was able to circumvent the block to STIM1-Orai coupling imposed by 2-APB reflects functional modulation of the store refilling mechanism by the Gag-Tsg101 complex.

DISCUSSION

The N-terminal MA domain in the Gag polyprotein is the major determinant of plasma membrane binding (reviewed in Chukkapalli and Ono, 2011). The positive charges in MA, the N-terminal myristate moiety and the PI(4,5)P_2 binding pocket in MA all contribute significantly to Gag targeting and plasma membrane binding. Based on previous studies *in vitro* using model membranes, MA can be expected to preferentially direct Gag binding to acidic phospholipids (Ehrlich et al., 1996; reviewed in Scarlata and Carter, 2003; Chukkapalli and Ono, 2011). Given that the main components of rafts are uncharged, we previously suggested that MA would direct Gag outside of rafts (Scarlata and Carter, 2003). A recent report documents the exclusion of multimerized Gag from ordered membrane domains, i.e., lipid rafts, in model membranes (Keller et al., 2013). The authors suggested that stable plasma membrane localization of Gag might be more complex than previously thought and possibly involve additional protein machinery. Supporting this notion, there is increasing evidence that the raft-enriched membrane domain from which Gag assemblages are known to bud is derived through Gag-initiated plasma membrane alterations (Krementsov et al., 2010; Ono, 2010; Kerviel et al., 2013). If so, then the targeting of Gag to PI(4,5)P_2, a phospholipid found in both raft and non-raft domains (Calloway et al., 2011), may only be one of several events required to secure stable localization of Gag at the plasma membrane. Our studies demonstrate that IP3R-mediated Ca^{2+} signaling is required for stable plasma membrane localization of Gag (Ehrlich et al., 2010, 2011). This makes IP3R a component of the additional protein machinery alluded to by Keller et al. (2013).

As noted above, we initially identified IP3R and other Ca^{2+} signaling proteins through a proteomic search for proteins associated with Gag, but not a L-domain mutant, in a membrane-enriched subcellular fraction. The goal of that study was identification of cellular factors that participate with HIV-1 Gag and ESCRT in facilitating the budding event. Here, we show functional linkages between IP3R and Tsg101, the ESCRT-1 component recruited by the PTAP L domain motif in Gag. Compared to control cells, cells expressing Gag exhibited a higher level of cytosolic Ca^{2+} originating from the ER Ca^{2+} store, suggesting that Gag induced the change. This Gag-associated activity required an intact PTAP motif (cf. **Figure 2**), corroborating an earlier finding based on measurement of cytosolic Ca^{2+} in single cells (Ehrlich et al., 2011). Consistent with the elevated Ca^{2+} level detected in Gag-expressing cells, stable Gag association with the plasma membrane was found to require continuous IP3R function (cf. **Figure 3**). Since in almost all cell types, the IP3R channel serves as the principal means of mobilizing store Ca^{2+} (cf. **Figure 1**; Hanson et al., 2004; Patterson et al., 2004a,b; Banerjee and Hasan, 2005; Clapham, 2007; Mikoshiba, 2007), more of the WT Gag-expressing cells were in store refilling mode compared to the P7L-Gag-expressing cells (cf. **Figure 9**). Moreover, Gag resistance to 2-APB, an inhibitor of store-refilling, exhibited PTAP L domain-dependence (cf. **Figure 10**). As reported previously, adventitiously-expressed IP3R-FLAG was detected at the periphery of cells expressing Gag, but only when the PTAP motif was intact (Ehrlich et al., 2011). A similar PTAP-dependent IP3R redistribution was also observed in the current study, as

significantly more gold-tagged IP3R was detected at the periphery of cells transfected with Gag compared to mock-transfected cells (cf. **Figure 5**). Gold-tagged IP3R was also detected in association with VLPs (cf. **Figure 5**), consistent with a previous observation that IP3R is encapsidated in virions released from HIV-1-infected macrophages (Chertova et al., 2006). Interestingly, in that study, the virion-incorporated IP3R was recovered from a band in a SDS-PAG slice that exhibited a molecular mass of 100–130 kDa which is similar to the mass of the IP3R-related protein that was predominant in our study (cf. **Figures 7**, **8**). As IP3R redistribution to the cell periphery occurs in response to specific stimulus rather than to bulk changes in ER structure (Vermassen et al., 2004; Chalmers et al., 2006), the results suggest that Gag is a stimulus for IP3R translocation to the cell periphery. Together, these findings show that modulation of ER Ca^{2+} release and ER store refilling are aspects of productive Gag assembly and that these events are facilitated by recruitment of Tsg101 by Gag.

Taken together, our findings show that stable plasma membrane localization of Gag requires release of store Ca^{2+}. It is possible that some of the released Ca^{2+} maintain continuous IP3R activation itself since Ca^{2+} is an activator of PLC, the enzyme catalyzing the hydrolysis of PI(4,5)P_2 to produce the ligand (IP3) needed to activate IP3R (Rhee, 2001; Suh et al., 2008). Ca^{2+} may also play a role in the competency of Gag to bind membranes. It is known that the lipid moiety, myristate, which is co-translationally added to the second N-terminal residue in MA, contributes to Gag membrane binding (reviewed in Chukkapalli and Ono, 2011). *In vitro* membrane binding studies show two conditions that favor solvent exposure of myristate: (i) protonation of His89 in the MA domain in Gag (Fledderman et al., 2010) and (ii) Ca^{2+}-dependent binding of calmodulin to MA (Ghanam et al., 2010). IP3R-mediated Ca^{2+} store release can therefore be expected to promote the exposure of myristate, especially when it occurs at or near the plasma membrane.

Recent evidence indicates that the junction between the plasma membrane and adjacent ER allows for rapid spatially restricted Ca^{2+} signaling (reviewed in Carrasco and Meyer, 2011). We can detect Gag and ER in the TIRF field (cf. **Figure 4**); immuno-gold-labeled IP3R in VLPs (cf. **Figure 5**); IP3R-Gag fluorescence co-localization (cf. **Figure 6**); reciprocal co-IP of Gag and an IP3R-related protein (cf. **Figure 7**); and Gag-IP3R floatation to the same light membrane fraction in sucrose gradients (cf. **Figure 8**) indicating that Gag and IP3R are in proximity at the cell periphery, i.e., at ER-PM junctions. Keeping an appropriate Ca^{2+} level in this restricted space near Gag assembly sites appears to be necessary to maintain Gag on the PM (cf. **Figure 3**). Possibly, an as yet unidentified Tsg101 binding partner that is also capable of associating with IP3R links IP3R to the assembling Gag-Tsg101 complexes in this region. For example, among proteins capable of associating with the receptor, junctophilin proteins, which are essential components of ER-PM junctions, conserve a Tsg101 recognition sequence (PTXP) (Stamboulian et al., 2005). Also, members of the Src family of non-receptor tyrosine kinases have been shown to bind both IP3R and Tsg101 (Jayaraman et al., 1996; Tu et al., 2010). Early studies showed that IP3R channel activity is increased by addition of Src family kinases *in vitro* (Jayaraman et al., 1996). Tsg101 has been shown to be responsible for Src delivery to the cell periphery (Tu et al., 2010). PLC gamma also binds activated Src (Zachos et al., 2013). It has been reported that, if Src phosphorylates IP3R at Tyr353 located in the IP3R binding core, the affinity for IP3 is increased (Cui et al., 2004). The phosphorylated IP3R is more sensitive to IP3 and less sensitive to Ca^{2+}-mediated inhibition (deSouza et al., 2007). The combined effect permits the channel to be more open under a wide range of IP3 and Ca^{2+} concentrations. Thus, proteins like junctophilin or Src family members could function as tethers that permit Tsg101 to modulate IP3R gating.

In summary, we propose a model wherein Gag-recruited Tsg101 serves as agonist for redistribution of IP3R to the cell periphery. Within the confines of the ER-PM junction, close proximity between the Gag-Tsg101 complex and IP3R channels allow physical associations that alter opening, release of store Ca^{2+}, and store-refilling. The resulting elevation of Ca^{2+} level in the immediate vicinity of the plasma membrane drives events that lead to stable membrane localization of assembling Gag. In this model, Tsg101 has a novel role that relates to modulation of the gating of the ER store and store refilling. Our findings support a paradigm for HIV-1 assembly wherein both intact and hydrolyzed PI(4,5)P_2 are required. Identification of Ca^{2+} signaling machinery as a component of the PTAP L domain function in viral budding may provide new targets for development of anti-viral strategies aimed at this aspect of virus replication.

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The roles of lipids and nucleic acids in HIV-1 assembly

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During HIV-1 assembly, precursor Gag (PrGag) proteins are delivered to plasma membrane (PM) assembly sites, where they are triggered to oligomerize and bud from cells as immature virus particles. The delivery and triggering processes are coordinated by the PrGag matrix (MA) and nucleocapsid (NC) domains. Targeting of PrGag proteins to membranes enriched in cholesterol and phosphatidylinositol-4,5-bisphosphate (PI[4,5]P2) is mediated by the MA domain, which also has been shown to bind both RNA and DNA. Evidence suggests that the nucleic-acid-binding function of MA serves to inhibit PrGag binding to inappropriate intracellular membranes, prior to delivery to the PM. At the PM, MA domains putatively trade RNA ligands for PI(4,5)P2 ligands, fostering high-affinity membrane binding. Triggering of oligomerization, budding, and virus particle release results when NC domains on adjacent PrGag proteins bind to viral RNA, leading to capsid (CA) domain oligomerization. This process leads to the assembly of immature virus shells in which hexamers of membrane-bound MA trimers appear to organize above interlinked CA hexamers. Here, we review the functions of retroviral MA proteins, with an emphasis on the nucleic-acid-binding capability of the HIV-1 MA protein, and its effects on membrane binding.

Keywords: matrix, RNA, PI(4.5)P2, lipid, Gag

INTRODUCTION

FUNCTIONS OF RETROVIRAL MA PROTEINS

Retroviruses such as the human immunodeficiency virus (HIV) are membrane-enveloped viruses that bud from the plasma membranes of infected host cells (Coffin et al., 1997; Swanstrom and Wills, 1997; Freed, 1998; Goff, 2001). Retroviral genomes encode PrGag polyproteins that are necessary and sufficient for assembly and release of virus-like particles (VLP) from cells (Campbell and Vogt, 1995; Coffin et al., 1997; Swanstrom and Wills, 1997; Campbell and Rein, 1999; Gross et al., 2000; Campbell et al., 2001). The HIV-1 precursor Gag protein (PrGag) initially is synthesized on cytosolic ribosomes and becomes cotranslationally modified by the N-terminal attachment of a myristoyl group by *N*-myristoyltransferase (Mervis et al., 1988; Bryant and Ratner, 1990; Ono and Freed, 1999, 2001; Morikawa et al., 2000; Ono et al., 2000; Tritel and Resh, 2000; Resh, 2004), although myristoylation is not universal among retroviral PrGag proteins (Erdie and Wills, 1990; Provitera et al., 2000; Dalton et al., 2005). PrGag proteins associate with the inner layer of the plasma membrane (PM), where they oligomerize, assemble, and bud off from cells as immature virions. The assembly process of retroviruses appears to be triggered by the association of PrGag proteins with viral RNA (vRNA) at the plasma membrane (Rein, 1994; Spearman et al., 1994; Muriaux et al., 2001; Jouvenet et al., 2006, 2008; Ott et al., 2009). However, several retroviruses, such as mouse mammary tumor virus (MMTV) and the Mason-Pfizer monkey virus (MPMV), assemble within the cytoplasm before being transported to cell membrane (Choi et al., 1999; Stansell et al., 2007). During the maturation process, cleavage of HIV-1 PrGag by the viral protease (PR) generates the mature myristoylated matrix (MA) protein as well as capsid (CA), nucleocapsid (NC), p6 and two spacer peptides, Sp1 and Sp2 (Swanstrom and Wills, 1997; Freed, 1998).

The MA domain plays multiple roles in the viral replication cycle. One of these roles involves the incorporation of the viral envelope (Env) proteins into virus particles. Evidence indicates that HIV-1 MA interacts with the cytoplasmic tail (CT) of gp41, the transmembrane (TM) portion of the HIV-1 Env protein, to facilitate the incorporation of Env proteins into assembling virions (Yu et al., 1992; Dorfman et al., 1994; Freed and Martin, 1995, 1996; Wyma et al., 2000; Davis et al., 2006; Checkley et al., 2011). Several models have been proposed to explain the incorporation of retroviral Env protein into virus particles (reviewed by Checkley et al., 2011). One of these models is the passive model, in which membrane proteins at assembly sites are incorporated into virions as innocent bystanders. This model was based on the observation that retroviruses could incorporate foreign membrane proteins into their envelopes. When glycoproteins from heterologous viruses are assembled into a retrovirus envelope, the process is termed pseudotyping (Zavada, 1982; Lusso et al., 1990; Arthur et al., 1992; Ott, 2008; Checkley et al., 2011). For example, infectious HIV-1 particles can be produced with foreign glycoproteins such as the vesicular stomatitis virus G protein (VSV-G; Cronin et al., 2005; Checkley et al., 2011) or amphotropic murine leukemia virus (MLV) Env (Lusso et al., 1990; Wang et al., 1993). In these cases, HIV-1 cores and genomes are delivered to target cells carrying the VSV or MLV receptors (Jorgenson et al., 2009). The passive model also is supported by the finding that removal of most of HIV-1 gp41 CT has a moderate effect on Env glycoprotein incorporation into HIV-1 particles (Wilk et al., 1992; Freed and Martin, 1995, 1996; Akari et al., 2000; Murakami and Freed, 2000).

Although the passive model is consistent with observations for a number of retroviruses (Landau et al., 1991; Reiser et al., 1996; Lewis et al., 2001; Liu et al., 2004; Jorgenson et al., 2009), several lines of evidence suggest an interaction between MA and Env. For HIV-1, it has been reported that mutations in MA may decrease levels of HIV-1 Env incorporation into virions (Wang et al., 1993; Freed and Martin, 1995, 1996; Reil et al., 1998). Moreover, some mutations in MA (Freed and Martin, 1995, 1996; Reil et al., 1998) have been shown to mitigate the effects of certain Env mutations. Interestingly, for some but not all cell types, MA mutations can be compensated via full deletions of the HIV-1 Env protein cytoplasmic tail (CT; Freed and Martin, 1995, 1996; Checkley et al., 2011). These results suggest that while truncated HIV-1 Env can be incorporated passively into virions in some cell types, full-length Env requires an interaction with MA for assembly into virions (Freed and Martin, 1995, 1996; Checkley et al., 2011). Data from other experiments indicate that MA domains in immature PrGag lattices lock Env proteins into a non-fusogenic state, and that PrGag processing serves as a switch to regulate envelope protein function (Murakami et al., 2004; Wyma et al., 2004; Jiang and Aiken, 2007). *In vitro* studies have shown direct binding between MA and the CT Env in several biochemical experiments for both HIV-1 and Simian immunodeficiency virus (SIV; Cosson, 1996; Wyma et al., 2000; Manrique et al., 2008). Consistent with these observations, structural studies have shown that HIV-1 MA proteins assemble lattices on phosphatidylinositol-(4,5)-bisphosphate (PI[4,5]P2) membranes in which residues implicated in CT binding point toward lattice holes (Yu et al., 1992; Dorfman et al., 1994; Freed and Martin, 1996; Ono et al., 1997; Murakami and Freed, 2000; Davis et al., 2006; Bhatia et al., 2007; Alfadhli et al., 2009a; Checkley et al., 2011; Tedbury et al., 2013). Given this membrane organization of MA, it seems likely that membrane proteins with short cytoplasmic domains may enter Gag lattices passively, whereas proteins such as HIV-1 Env, with long cytoplasmic tails require MA interactions.

Implicit in the data described above is the assumption that MA binds to membranes, and another essential function of MA is to target PrGag proteins to their lipid raft assembly sites at the PMs of infected cells (Ehrlich et al., 1996; Spearman et al., 1997; Scarlata et al., 1998; Bouamr et al., 2003; Murray et al., 2005; Jouvenet et al., 2006; Bhatia et al., 2007; Dalton et al., 2007; Scholz et al., 2008; Hamard-Peron and Muriaux, 2011). In most mammalian retroviruses, membrane targeting is dependent on two structural features present on MA: the N-terminal myristyl group and a group of basic residues. For such viruses, the N-terminal myristyl group functions in concert with a group of conserved basic residues to promote membrane binding (Zhou et al., 1994; Tang et al., 2004; Saad et al., 2008). However, Gag proteins of some retroviruses, such as Rous sarcoma virus (RSV) and equine infectious anemia virus (EIAV), lack the myristate anchor, and Gag targeting and binding to the PM is mainly mediated by electrostatic interactions (Erdie and Wills, 1990; Parent et al., 1996; Callahan and Wills, 2000; Provitera et al., 2000; Dalton et al., 2005). Compelling evidence favors the idea that HIV assembly does not occur just anywhere at the PM, but at lipid rafts and at PI(4,5)P2-enriched areas (Ono et al., 2004; Chukkapalli et al., 2008, 2010; Chukkapalli and Ono, 2011). MA–PI(4,5)P2 interactions also have been observed for MLV, MPMV, and EIAV (Stansell et al., 2007; Chan et al., 2008; Chen et al., 2008; Hamard-Peron et al., 2010). In cell culture, decreasing the levels of cellular PI(4,5)P2 by overexpression of polyphosphoinositide 5-phosphatase IV was shown to reduce HIV-1 and MLV assembly efficiency, resulting in the delivery of viral proteins to intracellular compartments (Ono et al., 2004; Chan et al., 2008; Chukkapalli et al., 2008; Hamard-Peron et al., 2010; Inlora et al., 2011). In contrast, recent studies have shown that human T-lymphotropic virus type 1 (HTLV-1) Gag is markedly less dependent on PI(4,5)P2 for membrane binding and particle release than HIV-1 Gag (Inlora et al., 2011). For RSV, Chan et al. (2011) reported that RSV Gag bound effectively to a variety of phosphorylated phosphatidylinositols, and that reduction of cellular PI(4,5)P2 and PI(3,4,5)P3 levels did not reduce Gag PM binding or virus particle release. However, more recently, Nadaraia-Hoke et al. (2013) reported that depletion of cellular PI(4,5)P2 and PI(3,4,5)P3 yielded reductions of both RSV Gag PM binding and virus particle release. Interestingly, RSV Gag mutants that are impaired in nuclear trafficking were less sensitive to these effects, suggesting a link between RSV Gag PM targeting and nuclear trafficking (Nadaraia-Hoke et al., 2013).

In addition to Env protein and membrane binding, several reports have implicated nucleic acid binding properties to retroviral MAs. These viruses are HIV-1 (Luban and Goff, 1991; Bukrinsky et al., 1993; Von Schwedler et al., 1994;; Lochrie et al., 1997; Miller et al., 1997; Reil et al., 1998; Haffar et al., 2000; Purohit et al., 2001; Ott et al., 2005; Hearps et al., 2008; Alfadhli et al., 2009b, 2011; Cai et al., 2010; Chukkapalli et al., 2010, 2013; Monde et al., 2011), RSV (Leis et al., 1978, 1980; Steeg and Vogt, 1990), and BLV (Mansky et al., 1995; Mansky and Wisniewski, 1998; Mansky and Gajary, 2002; Wang et al., 2003). While the NC domains of retroviral PrGag proteins are essential for viral RNA (vRNA) encapsidation, experiments have shown that MA proteins may also possess binding functions and can substitute for the HIV-1 NC protein assembly function (Ott et al., 2005). [However, deletion of the NC domain dramatically reduces the assembly of MLV particles (Muriaux et al., 2004)]. It has been conjectured that such MA–nucleic acid binding might facilitate PrGag delivery to the PM, virus assembly, and/or nuclear import of viral preintegration complexes (PICs) (Bukrinsky et al., 1993; Von Schwedler et al., 1994; Miller et al., 1997; Reil et al., 1998; Haffar et al., 2000; Hearps et al., 2008; Cai et al., 2010). In this review, we focus on the role of MA binding to RNA and summarize the importance of Gag MA interactions with RNA for HIV and other retroviruses, with the hope that this comparative approach can shed more light on our understanding of the importance of this function and ways of inhibiting that role.

STRUCTURAL ASPECTS OF RETROVIRAL MATRIX PROTEINS

Matrix structures for the following retroviruses have been determined: HIV- 1 (Massiah et al., 1994; Hill et al., 1996; Tang et al., 2004; Saad et al., 2006, 2007), HIV-2 (Saad et al., 2008), SIV (Rao et al., 1995), human T-lymphotropic virus 2 (HTLV-2; Christensen et al., 1996), BLV (Matthews et al., 1996), M-PMV (Conte et al., 1997), Rous sarcoma virus (RSV; N-terminal fragment; McDonnell et al., 1998), EIAV (Hatanaka et al., 2002), and MLV

(Riffel et al., 2002). In contrast to their low sequence homology, structures of retroviral MA proteins are remarkably similar (**Figure 1**; Murray et al., 2005). They all share a globular core composed of α helices. The N-termini of the MA proteins tend to contain basic residues that appear to foster interactions of MA with acidic phospholipid head groups. Another essential element that contributes to membrane binding is the myristyl group found in most retroviral MAs, including HIV-1 (Gottlinger et al., 1989; Bryant and Ratner, 1990; Spearman et al., 1994), HIV-2 (Saad et al., 2008), MLV (Henderson et al., 1983), M-PMV (Schultz and Oroszlan, 1983), and HTLV (Ootsuyama et al., 1985). However, there are exceptions, such as RSV and EIAV viruses (Schultz and Oroszlan, 1983), which do not have myristoylated MA proteins.

A number of structural studies have been conducted on HIV-1 MA (Massiah et al., 1994; Hill et al., 1996; Tang et al., 2004; Saad et al., 2006, 2007; Alfadhli et al., 2009a). In addition to its N-terminal myristate, which is essential for efficient membrane binding (Gottlinger et al., 1989; Bryant and Ratner, 1990; Freed et al., 1994; Spearman et al., 1994; Tang et al., 2004; Saad et al., 2006, 2007), the HIV-1 MA protein is composed of six α helices and three β sheet strands (Massiah et al., 1994; Hill et al., 1996; Tang et al., 2004; Saad et al., 2006, 2007). Sedimentation equilibrium data have shown that while myristoylated HIV-1 MA exists in a monomeric–trimeric state at equilibrium, unmyristoylated MA occurs as a monomer even at high concentrations (Tang et al., 2004). NMR studies suggest that upon Gag multimerization the myristoyl group is exposed, and this fosters Gag binding to membranes (Tang et al., 2004; Saad et al., 2006, 2007).

The membrane binding face of HIV-1 MA is basic, promoting interactions with negatively charged phospholipid head groups at the inner leaflets of PMs (Massiah et al., 1994; Zhou et al., 1994; Hill et al., 1996; Tang et al., 2004; Saad et al., 2006, 2007). NMR investigations have indicated that HIV-1 MA preferentially binds to soluble PI(4,5)P2 mimics through contacts with the lipid head group and its 2′ acyl chain, and that binding promotes both exposure of the MA myristate group and protein oligomerization (Tang et al., 2004; Saad et al., 2006, 2007). Consistent with the above observations, it has been shown that HIV-1 MA and MACA proteins tend to organize as hexamers of trimers on lipid membranes containing PI(4,5)P2 (**Figure 2**; Alfadhli et al., 2009a), and that the binding specificity of MA is enhanced by cholesterol (Alfadhli et al., 2009a,b). These results suggest a model in which each MA trimer contributes to three separate hexamer rings, and MA proteins are positioned roughly above CA N-terminal domain (NTD) hexamers, which also are linked via CA C-terminal domain (CTD) contacts. This model implies that the shells of immature HIV-1 virions are stabilized by multiple Gag domain contacts, and has implications for how Env proteins assemble and fit into virus particles. Significantly, MA residues shown to be critical for incorporation of HIV-1 Env proteins orient toward the hexameric holes in the lattices (Yu et al., 1992; Dorfman et al., 1994; Freed and Martin, 1996; Ono et al., 1997; Murakami and Freed, 2000; Davis et al., 2006; Bhatia et al., 2007; Alfadhli et al., 2009a; Checkley et al., 2011; Tedbury et al., 2013).

RETROVIRAL MA BINDING TO NUCLEIC ACIDS

For a number of years, researchers have reported that retroviral MA proteins possess nucleic-acid-binding properties (Sen and Todaro, 1977; Leis et al., 1978, 1980; Steeg and Vogt, 1990; Katoh et al., 1991, 1993; Luban and Goff, 1991; Bukrinsky et al., 1993; Von Schwedler et al., 1994; Mansky et al., 1995; Lochrie et al., 1997;

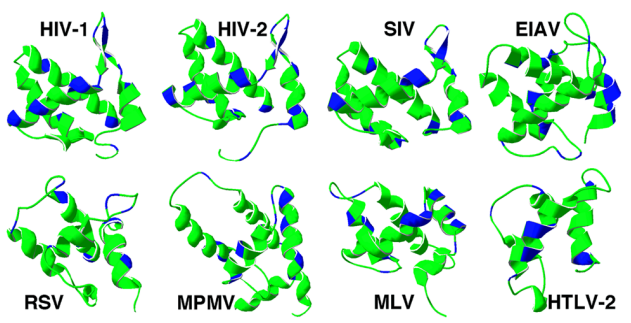

FIGURE 1 | Retrovirus matrix protein membrane binding regions. Ribbon diagrams of the membrane binding regions of four lentivirus matrix proteins (top row) and an alpharetrovirus (RSV), betaretrovirus (MPMV), gammaretrovirus (MLV), and deltaretrovirus (HTLV-2) are depicted. In each case, matrix helix one is on the right-hand side of the figure, and basic residues are indicated in blue. The PDB files for each matrix protein are as follows: HIV-1 (1UPH), HIV-2 (2K4H), SIV (1ECW), EIAV (1HEK), RSV (1A6S), MPMV (1BAX), MLV (1MN8), HTLV-2 (1JVR).

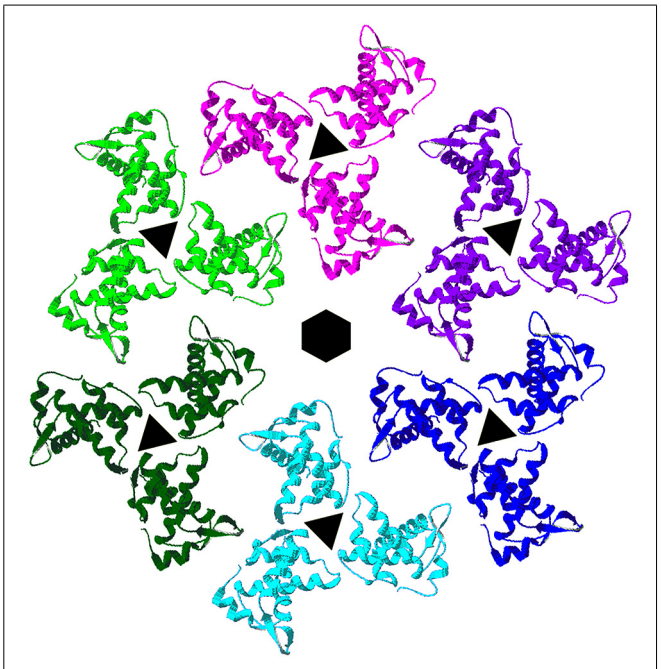

FIGURE 2 | Membrane organization of HIV-1 matrix proteins. Trimers of HIV-1 matrix proteins (PDB 1HIW) were fitted onto the electron densities of HIV-1 MA proteins assembled onto a lipid monolayer containing PI(4,5)P2 (Alfadhli et al., 2009a). As shown, trimers organize in hexameric rings around protein-free holes. MA residues that have been shown to affect HIV-1 Env protein assembly into virions locate toward the centers of the hexameric holes, implying that the cytoplasmic tails of HIV-1 Env proteins occupy these spaces.

Miller et al., 1997; Swanstrom and Wills, 1997; Mansky and Wisniewski, 1998; Reil et al., 1998; Haffar et al., 2000; Garbitt et al., 2001; Purohit et al., 2001; Mansky and Gajary, 2002; Wang et al., 2003; Hearps et al., 2008; Alfadhli et al., 2009b, 2011; Cai et al., 2010; Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011; Monde et al., 2011). In early studies on retroviruses such as avian sarcoma and leukemia viruses (ASLV) and RSV, which is closely related to ASLV, MA was reported to be associated with vRNA in virus particles (Sen and Todaro, 1977; Leis et al., 1978, 1980), although subsequent work attributed this activity to NC (Meric et al., 1984). In any event, the binding of RSV MA to RNA is not of high specificity (Steeg and Vogt, 1990), and studies have shown that RSV MA binds to vRNA, ribosomal RNA, and DNA with similar affinities (Meric and Spahr, 1986; Parent and Gudleski, 2011).

Early studies using RNA gel mobility shift assays, and radioactive cDNA hybridization and mapping studies implicated the MA domain of BLV Gag in specific binding of vRNA (Katoh et al., 1991, 1993). Although the BLV Gag NC domain contains two zinc finger domains and basic amino acids that are important for vRNA packaging (Wang et al., 2003), the mature BLV NC proteins lack selectivity for vRNA sequences containing the encapsidation signal (Katoh et al., 1991, 1993). Surprisingly, the BLV precursor MA(p15) protein binds specifically to two distinct regions of viral RNA (Mansky and Wisniewski, 1998). This observation is discussed in more detail below.

For HIV-1, *in vitro* selection experiments identified RNA aptamers that showed high-affinity binding to HIV-1 MA (Lochrie et al., 1997; Purohit et al., 2001; Ramalingam et al., 2011). Lochrie et al. (1997) identified RNA ligands that bind to two different regions within Gag, either to MA or to NC. These RNAs were 50-mer aptamers and had dissociation constants between 3 and30 nM (Lochrie et al., 1997). However, the RNA sequences identified by this screen were not related to any region on the HIV-1 vRNA (Lochrie et al., 1997). Subsequently, Purohit et al. (2001) identified high-affinity RNA ligands to HIV-1 MA that were selected by screening of random 76-mer and 31-mer RNA libraries. These investigators showed that MA binds directly to an RNA sequence that is homologous to a fragment of the *pol* sequence with an affinity of about 500 nM (Purohit et al., 2001; Alfadhli et al., 2011). The region of MA that binds to this RNA was restricted to the N-terminal basic domain, and substitution in the basic residues led to weak binding to RNA *in vitro*. Viral mutants that interfered with the MA–RNA interaction yielded a 4–5 day replication delay *in vivo* (Purohit et al., 2001). However, it is possible that mutations that affected RNA binding also affected other viral functions. In the third study (Ramalingam et al., 2011), MA-binding aptamers were found with K_ds in the range of 100–250 nM, but expression of these aptamers in cells had only minimal effects on HIV-1 functions.

Recent studies with HIV-1 MA provide corroboration of its RNA-binding capacity. In particular, bead binding experiments have indicated that fluorescently tagged RNAs and DNAs bind well to HIV-1 MA but not to control proteins (Alfadhli et al., 2009b). Interestingly, it has been shown that RNA binding enhances the binding specificity of MA to PI(4,5)P2-containing membranes. This was indicated by the fact that PI(4,5)P2-containing liposomes successfully competed with nucleic acids for MA binding, whereas other liposomes did not (**Figure 3**; Alfadhli et al., 2009b). In agreement with these results, other studies have shown that the highly basic region (HBR) on the N-terminal portion of MA not only contributes to binding of PI(4,5)P2, but also is capable of binding to RNA (Chukkapalli et al., 2008, 2010, 2013). Furthermore, RNAse treatment of *in vitro* translated Gag protein preparations decreased the binding specificity to membranes containing PI(4,5)P2, suggesting that RNA influences the membrane binding specificity of MA (Chukkapalli et al., 2008, 2010, 2013, see below).

In support of these studies, it has been shown by Burniston et al. (1999) that the basic residues of the HIV-1 MA domain contribute to Gag–Gag interactions in the presence of RNA and the absence of the NC domain, indicating that the basic residues on MA play a role in RNA binding (Burniston et al., 1999). NMR studies also have confirmed interactions of HIV-1 MA with RNA and DNA, and have implicated the nucleic-binding surfaces on MA (Cai et al., 2010; Alfadhli et al., 2011). As discussed above, over a decade ago *in vitro* selection experiments identified a 25-mer aptamer that showed high-affinity binding to HIV-1 MA (Purohit et al., 2001). More recently, MA binding to this aptamer has been characterized. MA–RNA binding was verified via gel shift assays, fluorescence anisotropy (FA) assays, analytical ultracentrifugation, and NMR methods (Alfadhli et al., 2011).

In summary, numerous studies have shown that the MA domains from different retroviruses possess nucleic-acid-binding properties. The significance of these interactions and their plausible roles are described below.

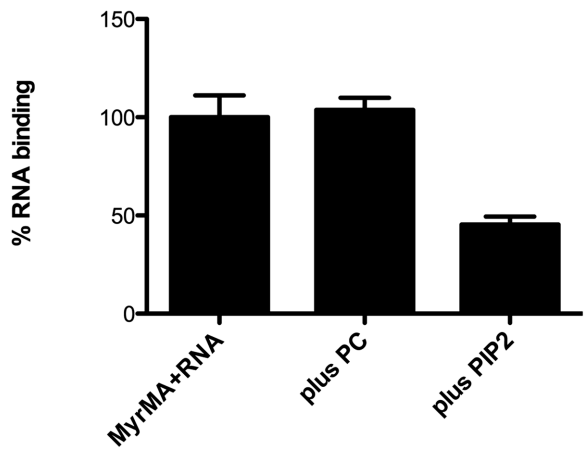

FIGURE 3 | Competition of PI(4,5)P2 liposomes for matrix protein RNA binding sites. Experiments performed by Alfadhli et al. (2011) have shown that nickel-nitrilotriacetic acid (Ni-NTA) beads coated with the myristoylated HIV-1 matrix protein (MyrMA) bind fluorescently tagged Sel25 RNA (MyrMA + RNA). Addition of liposomes composed of 80% dioleoylphosphatidyl choline + 20% cholesterol (plus PC) during binding reactions did not diminish MA–RNA binding, whereas addition of an equal amount of liposomes composed of 70% PC, 10% PI(4,5)P2, 20% cholesterol (plus PIP2) significantly reduced MA–RNA binding. These and other experiments indicate that the affinity of HIV-1 MA for RNA is less than that for membranes containing PI(4,5)P2 but greater than that for membranes without PI(4,5)P2.

THE ROLE OF MA–NUCLEIC ACID BINDING

THE ROLE OF MA IN RNA ENCAPSIDATION

The RNA encapsidation process for retroviruses involves recognition of the vRNA by the viral Gag polyprotein, and it is essential for the assembly of infectious virions. Biochemical and genetic studies have revealed that encapsidation involves the association between a stable RNA structure near the 5′ ends of viral genome called the encapsidation (Ψ, packaging) signal and, in most cases, amino acid residues in the NC domain of the Gag protein (Mansky et al., 1995; Berkowitz et al., 1996; Swanstrom and Wills, 1997; Parent and Gudleski, 2011). It has been shown that RSV NC is essential for efficient vRNA encapsidation (Dupraz and Spahr, 1992; Aronoff et al., 1993; Lee et al., 2003; Lee and Linial, 2004; Zhou et al., 2005). However, studies have shown that other regions of RSV PrGag contribute to RNA packaging (Sakalian et al., 1994; Parent and Gudleski, 2011). In particular, mutations of the N-terminal region of RSV MA have resulted in defects in RNA dimerization and encapsidation (Sakalian et al., 1994; Garbitt-Hirst et al., 2009). The RSV Gag proteins are synthesized in the cytosol, and were believed that to be targeted directly to the plasma membrane. However, genetic and biochemical studies have indicated that RSV MA and NC domains contain nuclear localization signals (NLS) for nuclear targeting (Butterfield-Gerson et al., 2006; Garbitt-Hirst et al., 2009; Gudleski et al., 2010; Baluyot et al., 2012). Studies by Parent and co-workers have indicated that RSV MA influences vRNA encapsidation indirectly, and have proposed a working model for RSV MA role in vRNA packaging (Scheifele et al., 2002; Butterfield-Gerson et al., 2006; Garbitt-Hirst et al., 2009; Gudleski et al., 2010). According to this model, the NLS on MA binds directly to importin-11 and/or the NC NLS binds to the importin-alpha/importin-beta complex, and Gag nuclear import is directed by the importins. Once in the nucleus, Gag is released from import factors and binds to vRNA, primarily through an interaction of the NC domain with the packaging signal. RSV Gag–RNA binding may induce conformational changes in RSV Gag that expose a nuclear export signal (NES) in the Gag p10 domain (Garbitt-Hirst et al., 2009; Gudleski et al., 2010). This proposed conformational change appears to promote binding of the Gag p10 NES directly to CRM-1/RanGTP, a major exporter of RNA-binding proteins from the nucleus (Scheifele et al., 2002; Garbitt-Hirst et al., 2009; Gudleski et al., 2010). The Gag–RNA complex is then exported through the nuclear pore and travels to the plasma membrane where Gag undergoes multimerization and budding. However, it should be noted that a chimeric protein with the HIV-1 MA domain fused to the remainder of RSV Gag was able to replicate in a single round infectivity assay even though nuclear trafficking of the HIV/RSV chimeric protein was not readily detected by fluorescence microscopy (Baluyot et al., 2012).

In contrast to RSV, the BLV MA appears to play a direct role in vRNA encapsidation. While the NC domain of BLV plays a major role in genome recognition and RNA encapsidation, evidence in the literature implicates the MA protein of BLV in these events (Katoh et al., 1991, 1993; Mansky and Wisniewski, 1998). In particular, the MA domain of BLV PrGag is involved in the specific selection and packaging of vRNAs (Katoh et al., 1991, 1993; Parent and Gudleski, 2011). As one line of evidence, RNA gel mobility shift assays have shown that BLV MA binds specifically to RNAs representing the 5′ region of the BLV vRNA (Katoh et al., 1991, 1993). Furthermore, cDNA hybridization and mapping studies demonstrated that the BLV MA specifically binds to two different segments of the vRNA (Katoh et al., 1991, 1993). The first RNA region contains the vRNA dimerization domain, while the second RNA region is at the 5′ end of the *gag* gene, which is the location of the encapsidation signal for BLV (Mansky and Wisniewski, 1998). Interestingly, it is the BLV precursor MA (p15) protein and not the mature MA (p10) that binds specifically to the vRNA dimerization element and the encapsidation signal (Mansky and Wisniewski, 1998). Moreover, studies conducted by Katoh et al. (1991) showed that the BLV NC protein possesses only a non-specific RNA-binding activity, with little selectivity for the vRNA encapsidation signal. However, studies conducted by Mansky and co-workers provide genetic evidence that both the MA and NC domains of BLV PrGag are needed for efficient RNA packaging (Mansky et al., 1995; Mansky and Wisniewski, 1998; Mansky and Gajary, 2002; Wang et al., 2003). Mutational analysis of MA and NC showed that charged residues within both of these regions of Gag are needed for optimal genome packaging (Wang et al., 2003). In particular, mutation of residues K41 and H45 in MA, and of basic and zinc finger residues on NC resulted in BLV vRNA encapsidation defects (Wang et al., 2003). Thus, BLV provides one example in which the MA–RNA binding function is directly employed in the viral replication strategy.

THE ROLE OF HIV-1 MA IN NUCLEAR IMPORT

Historically, HIV-1 MA was the first protein implicated in directing the nuclear import of pre-integration complexes (PICs) early in infection (Bukrinsky et al., 1993; Von Schwedler et al., 1994). Reports indicated that HIV-1 MA contains an NLS that maps to the basic residues 25–33 (Bukrinsky et al., 1993; Von Schwedler et al., 1994; Depienne et al., 2000), and that mature MA enters infected cells along with vRNA and other viral proteins. Moreover, some MA molecules were found to be localized to PICs (Bukrinsky et al., 1993; Von Schwedler et al., 1994), and it thus was originally proposed that the MA NLS facilitates nuclear translocation of PICs prior to proviral integration (Bukrinsky et al., 1993; Von Schwedler et al., 1994; Miller et al., 1997; Reil et al., 1998; Haffar et al., 2000).

However, a number of reports challenged the role of HIV-1 MA in directing the nuclear import of PICs (Freed et al., 1994; Fouchier et al., 1997; Reil et al., 1998; Hearps et al., 2008). Notably, Gottlinger and co-workers showed that viruses lacking most of MA were capable of infecting non-dividing cells, suggesting that the putative MA NLS is not essential for HIV-1 replication (Reil et al., 1998; Depienne et al., 2000). Hearps et al. (2008) assessed the nuclear import properties of GFP-tagged MA, and concluded that MA is excluded from the nuclei of transfected cells. MA also failed to enter the nuclei of cells in *in vitro* transport assays using cells with perforated PMs but intact nuclear membranes (Hearps et al., 2008). Nevertheless, MA mutants have been shown to affect proviral DNA circularization and integration (Mannioui et al., 2005), and MA binding to DNA was demonstrated using *in vitro* DNA gel shift analysis (Hearps et al., 2008). Instead of a nuclear localization

role for HIV-1 MA, these observations suggest that MA associates with PICs and augments integration. Recent NMR studies showing that MA residues R22, K27, Q28, K30, and K32 mediate binding to dsDNA (Cai et al., 2010) are consistent with this notion.

THE ROLE OF HIV-1 MA BINDING TO RNA: REGULATION OF MEMBRANE BINDING

Studies by Alfadhli et al. (2009b, 2011) demonstrated that MA binds to nucleic acids, and that PI(4,5)P2-containing liposomes successfully compete with nucleic acids for MA binding, whereas other liposomes do not (Alfadhli et al., 2009b, 2011; **Figure 3**). Complementary studies by Ono and co-workers indicated that RNase treatment of Gag *in vitro* translation extracts reduced the selectivity of Gag binding for PI(4,5)P2 (Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011). These studies imply that MA and NC domains of HIV-1 PrGag bind to RNA in the cytoplasm of infected cells until PrGag reaches PI(4,5)P2-rich domains at the plasma membrane. By this scenario, MA–RNA binding increases the specificity of PrGag for PI(4,5)P2. This could be plausible if the MA affinity for RNA were between its affinity for PI(4,5)P2 and that for other phospholipids, so that RNA binding could protect MA from binding to inappropriate membranes (Alfadhli et al., 2009b, 2011; Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011). Consistent observations by Jones et al. (2011), showed that both MA and NC PrGag domains can bind nucleic acids, and that binding of MA to inositol phosphate (IP) derivatives, which resemble the PI(4,5)P2 head group, alters the association of PrGag to nucleic acids. Notably, experiments demonstrated that *in vitro* tRNA annealing to vRNA catalyzed by PrGag is enhanced over 10-fold by the addition of IPs to the reaction (Jones et al., 2011). In contrast, the IPs had no effect on the annealing induced by NC alone or CA–NC proteins. These results show that MA and NC can bind to nucleic acids, and that MA–RNA binding reduces tRNA annealing. By this model, IPs compete with vRNAs for MA binding, allowing NC to perform its encapsidation and annealing functions (Rein, 2010; Jones et al., 2011).

Another line of investigation based on biochemical and structural studies using hydrodynamic and small angle neutron scattering (SANS) methods showed that the Gag protein adopts a compact bent shaped conformation in solution. When only RNA is added, the Gag proteins assemble very small VLPs, suggesting that both MA and NC domains bind to RNA. However, in the presence of both RNA and IP membrane mimics, Gag undergoes a conformational switch to an extended rod-shaped form (Datta et al., 2007, 2011). Overall, the data above suggest that RNA provides a chaperone function in preventing HIV-1 Gag proteins from binding to membranes until they reach PI(4,5)P2-rich plasma membranes. Such a model is depicted in **Figure 4**, which illustrates the binding of PrGag MA and NC domains to vRNA, followed by an MA switch to membrane binding at PM assembly sites.

To test the MA–RNA chaperone model, Ono and co-workers measured cellular RNA levels and found that they are sufficient for blocking PrGag binding to phosphatidylserine (PS; Chukkapalli et al., 2013). These results provide cell-based evidence supporting the notion that RNA regulates membrane binding, and prevents PrGag from binding promiscuously to PS-containing membranes

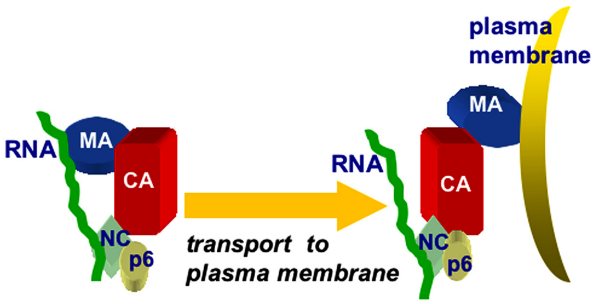

FIGURE 4 | Chaperone model for MA–RNA binding. The chaperone model for MA-RNA binding posits that both the MA and NC domains of PrGag proteins bind to RNA in the cytoplasm of infected cells, and that the MA–RNA binding protects MA from binding to inappropriate intracellular membranes, lacking PI(4,5)P2. Once at PI(4,5)P2-rich sites at the plasma membrane, MA binding switches from RNA to PI(4,5)P2, facilitating the trafficking and assembly of PrGag proteins.

(Chukkapalli et al., 2013). However, recent studies conducted by Dick et alindicate that RNA regulation of PrGag membrane binding is not universal among retroviruses (Dick et al., 2013). In contrast to HIV-1 Gag, RNAse treatment of reticulocyte lysates containing *in vitro*-translated RSV Gag did not alter the protein's membrane-binding characteristics. Potentially, this is because the interactions of RSV MA with RNA are weaker than those of HIV MA (Dick et al., 2013).

Given the implicated chaperone role for HIV-1 MA-nucleic acid binding (Chukkapalli et al., 2008, 2010, 2013; Alfadhli et al., 2009b, 2011), some aspects of HIV-1 MA–RNA binding have been examined further. One basic question relating to these observations concerns the nature of MA–RNA interactions. Previously, *in vitro* selection experiments identified a 25-mer RNA aptamer called Sel25 (GGACA GGAAU UAAUA GUAGC UGUCC) which demonstrated high-affinity binding to HIV-1 MA (Lochrie et al., 1997; Purohit et al., 2001). The central fifteen nucleotides (Sel15) also showed high-affinity binding to the protein. As a step toward characterization of MA–RNA interactions, MA binding to Sel25, Sel15, and their random sequence counterparts (Ran25, Ran15) was tracked via gel shift assays, fluorescence anisotropy (FA) assays, and analytical ultracentrifugation methods (Alfadhli et al., 2011). These investigations confirmed the specificity of MA binding to Sel15 and Sel25 RNAs. In addition, these studies identified RNA as a competitor for membrane binding, and assays indicated that PI(4,5)P2-containing liposomes significantly reduced RNA binding to MA. *In vitro* competition binding experiments also showed that a soluble PI(4,5)P2 mimic (PIPC8) reduced Sel25 binding to MA, whereas a soluble PS mimic did not; while FA competition data indicated that PIPC8 reduced MA–RNA binding levels to a greater extent than did the PS mimic (Alfadhli et al., 2011). These results are consistent with the notion that RNA increases the ability of MA to distinguish between phospholipid head groups.

What MA surfaces are sensitive to RNA binding? In an attempt to address this question, NMR binding studies were performed. Using this approach, MA residues at the putative RNA binding site were identified by their chemical shift perturbations upon

titration. In particular, significant NMR shifts were observed for residues located to the matrix protein β-II-V cleft corresponding to residues Gln-28, His-33, Glu-40, Glu-42, Ile-60, Leu-68, Thr-70, Glu-73, Arg-76, Ser-77, Tyr-79, and Asn-80 (**Figure 5**; Alfadhli et al., 2011). Some of these residues (residues 33, 73, 76, and 79) previously were shown to contribute to the PI(4,5)P2 binding site (**Figure 5**; Saad et al., 2006). This observed overlap of PI(4,5)P2-MA and RNA–MA binding sites reinforces a chaperone function hypothesis. These results also are in agreement with other NMR studies which implied that MA residues 28–33 and, to a lesser extent, residues 70–79 contribute to MA–DNA binding in preintegration complexes (Cai et al., 2010). It also is pertinent to note that NMR titrations indicated residues 94, 97, 103, and 104 were affected by RNA titrations (Alfadhli et al., 2011). These residues are located on MA helix VI and may involve a conformational change of MA upon RNA binding that also could affect binding specificity. While the sum of the above results support a hypothesis in which MA–RNA binding is utilized by HIV-1 to regulate virus assembly, the identity of the RNA(s) that bind to MA *in vivo* remains to be determined. In this regard, it is noteworthy that a nearly exact match of the Sel15 RNA sequence is located in the *pol* coding region of HIV-1, but while mutations of consensus nucleotides involved in MA–Sel RNA binding reduced binding *in vitro*, they only modestly reduced viral infectivity *in vivo* (Purohit et al., 2001). Thus, it is likely that MA can bind to other sequences on viral or cellular RNAs to effect its chaperone functions.

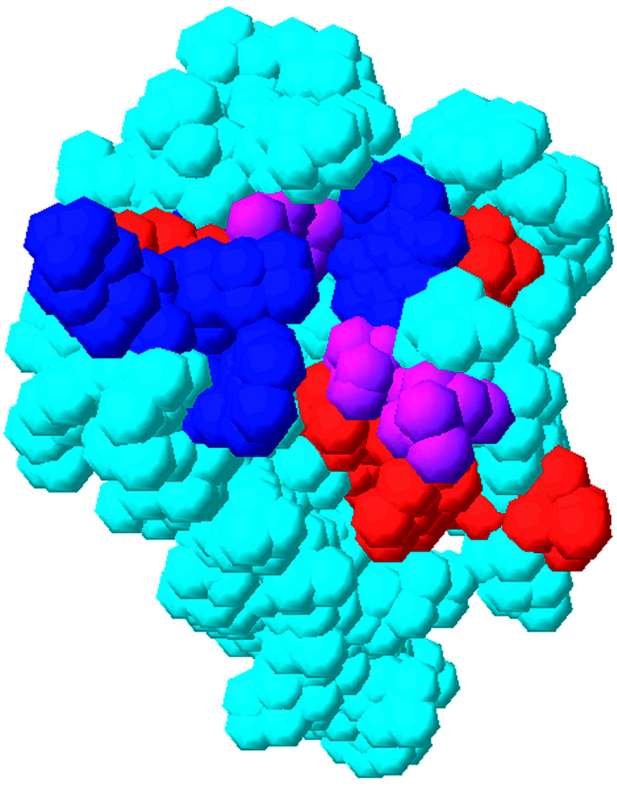

FIGURE 5 | Overlap of matrix RNA and PI(4,5)P2 binding sites. The membrane-binding surface of the HIV-1 matrix protein (PDB 1UPH) is illustrated as a space-filling model. Residues that have been implicated in PI(4,5)P2 binding are indicated in blue, residues that have been implicated in RNA binding (Alfadhli et al., 2011) are indicated in red, and residues that have been implicated in both PI(4,5)P2 and RNA binding are indicated in purple.

INHIBITION OF HIV-1 MA/RNA BINDING

Despite the effectiveness of the current highly active antiretroviral therapy (HAART) in the treatment of AIDS, development of novel anti-viral strategies is dictated by the medical significance of the AIDS epidemic, side effects of current drugs, and the possible development of drug-resistant HIV strains (Larder and Kemp, 1989; Richman et al., 1991; Moreno et al., 2010). The process of virus assembly, controlled by the HIV-1 Gag proteins, represents an attractive target for such therapies. Findings on the interplay between MA and RNA lay a foundation for determining how HIV-1 MA matrix binds RNA, and the role of MA–RNA interactions in HIV replication. Furthermore, these findings pave the way for efforts to use the MA–RNA interaction as a potential target for a new class of HIV assembly inhibitors. These interactions can be monitored with *in vitro* techniques, making them suitable for screening purposes. Consequently, assays that facilitate the identification of potential inhibitors of MA–RNA interactions have been developed. The reasoning here is that molecules that interfere with the binding of RNAs to MA may impair either an essential MA–RNA binding function, the overlapping MA-PI(4,5)P2 binding function, or both. Based on this, we have designed novel high throughput screens (HTS) in which small-molecule competitors to MA–RNA binding may be identified. The basic assay involves binding of C-terminally His-tagged MyrMA to 96-well nickel-NTA plates, incubation of the plates with biotin-Sel15 RNA in the presence or absence of potential competitors, and colorimetric determination of bound biotin-Sel15 (Alfadhli et al., 2013). Using the MA–RNA binding assay, a library of 14,000 compounds was screened for inhibition of MyrMA–Sel15 RNA binding, looking for candidates that significantly reduced Sel15 RNA binding to MyrMA. The robustness of the assay was indicated by the consistently large difference between samples containing no inhibitor versus those using untagged Sel15 RNA as an inhibitor control, and a favorable Z screening window coefficient (Zhang et al., 1999) of 0.69 for the screen. Using this assay, a small group of compounds that compete with RNA for MA binding was identified. Interestingly, three of the four best inhibitor candidates were thiadiazolanes. These potential inhibitors were characterized with respect to MA binding by NMR, FA, and electrophoretic mobility shift assays (EMSA). Importantly, results showed that MA–thiadiazolanes binding sites do overlap the MA–RNA binding site, validating the concept of such a screening effort. The thiadiazolanes also were shown to inhibit HIV-1 replication in cell culture, but unfortunately also demonstrated cytotoxicity in the 10–20 mM range (Alfadhli et al., 2013). Despite this, such efforts should open the door to the development of new classes of HIV antivirals that target MA and its nucleic-acid-binding pocket.

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New insights into retroviral Gag–Gag and Gag–membrane interactions

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A critical aspect of viral replication is the assembly of virus particles, which are subsequently released as progeny virus. While a great deal of attention has been focused on better understanding this phase of the viral life cycle, many aspects of the molecular details remain poorly understood. This is certainly true for retroviruses, including that of the human immunodeficiency virus type 1 (HIV-1; a lentivirus) as well as for human T-cell leukemia virus type 1 (HTLV-1; a deltaretrovirus). This review discusses the retroviral Gag protein and its interactions with itself, the plasma membrane and the role of lipids in targeting Gag to virus assembly sites. Recent progress using sophisticated biophysical approaches to investigate – in a comparative manner – retroviral Gag–Gag and Gag–membrane interactions are discussed. Differences among retroviruses in Gag–Gag and Gag–membrane interactions imply dissimilar molecular aspects of the viral assembly pathway, including the interactions of Gag with lipids at the membrane.

Keywords: plasma membrane, oligomerization, multimerization, lentivirus, deltaretrovirus, spectroscopy

The assembly of virus particles is a key aspect of viral replication that is still poorly understood at the molecular level. Retroviral assembly has been extensively investigated, though detailed information is lacking for many aspects of the process. This is true for human immunodeficiency virus type 1 (HIV-1), and particularly true for human T-cell leukemia virus type 1 (HTLV-1). In this review, we review the retroviral Gag protein, its translocation to the plasma membrane (PM), as well as Gag–Gag and Gag–PM interactions. We then highlight recent progress made using sophisticated biophysical approaches to investigate Gag–Gag and Gag–PM interactions, which represent key early events in the virus assembly pathway, including that of interacting with lipids at the PM.

THE Gag POLYPROTEIN

Gag is the primary retroviral structural protein responsible for orchestrating the majority of steps in viral assembly. Most of these assembly steps occur via interactions with three Gag subdomains – matrix (MA), capsid (CA), and nucleocapsid (NC; **Figure 1**). These three regions have a low level of sequence conservation among the different retroviral genera, which belies the observed high level of structural conservation. Outside of these three domains, Gag proteins can vary widely. For example, HIV-1 Gag additionally codes for a C-terminal p6 protein as well as two spacer proteins, SP1 and SP2, which demarcate the CA–NC and NC–p6 junctions, but HTLV-1 contains no additional sequences outside of MA, CA, and NC (Oroszlan and Copeland, 1985; Henderson et al., 1992).

The Gag subdomains are structurally discrete but have functionally overlapping roles in the viral assembly process. The N-terminus of Gag begins with MA, which contains key residues responsible for the recruitment of Gag to the PM via an N-terminal myristoyl moiety and a highly basic region (Bryant and Ratner, 1990; Zhou et al., 1994; Ono et al., 2000; Stansell et al., 2007; Hamard-Peron et al., 2010). CA is divided into two structurally distinct domains – the N-terminal domain (NTD) and C-terminal domain (CTD) – and contains the majority of the residues responsible for Gag–Gag interactions. While the primary amino acids for HIV-1 Gag oligomerization are located in the CA CTD (Dorfman et al., 1994; Gamble et al., 1997; Alfadhli et al., 2005; Dalton et al., 2007; Datta et al., 2007), there are additional residues located throughout CA, NC, and SP1 that are responsible for laterally stabilizing Gag–Gag interactions (Krausslich et al., 1995; von Schwedler et al., 2003; Li et al., 2007). Functionally distinct from the CTD, in HIV-1 the CA NTD is not necessary for viral assembly (Borsetti et al., 1998; Accola et al., 2000). Finally, NC possesses two zinc finger domains along with several key amino acids that function to bind and package viral RNA into particles (Gorelick et al., 1988; Cimarelli et al., 2000; Muriaux et al., 2004). It is important to note that the functions of these domains are not exclusive, and there is much overlap. For example, HIV-1 NC has recently been implicated in facilitating the budding process, which was believed to be driven solely by

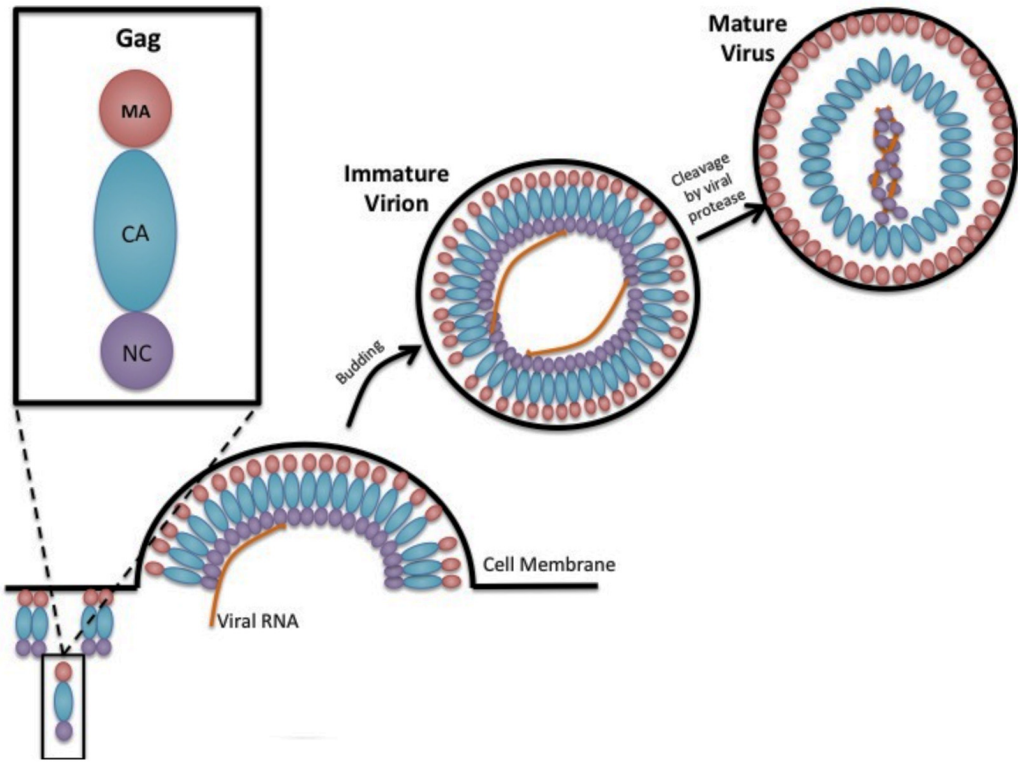

FIGURE 1 | Gag and retrovirus particle assembly. A cartoon depiction is shown in cross section of the assembly of a prototypic retrovirus particle, emphasizing the oligomerization of Gag along the inner leaflet of the plasma membrane, incorporation of two copies of the viral RNA, the budding of immature virus particles, and the conversion of the immature virus particle to mature infectious virus particle that is catalyzed by the viral-encoded protease. Gag is shown as being composed of the matrix domain (red circle), the capsid domain (blue oval), and the nucleocapsid domain (purple circle). Two copies of the viral RNA (two orange lines inside the viral particle) are shown packaged into the virus particle.

motifs within the p6 domain (Dussupt et al., 2011; Bello et al., 2012).

Once a cell has been infected by a retrovirus, full-length Gag polyproteins assemble to form immature virions. The reported numbers of Gag incorporated in each particle varies greatly from ∼750–5000 (Yeager et al., 1998; Vogt and Simon, 1999; Briggs et al., 2004; Chen et al., 2009). It is theorized that the preparation of the particles can affect this number as well as the genus of retrovirus. Upon the assembly of these Gag proteins into a lattice shell, the viral protease will cleave the Gag polyprotein into its respective domains to form a mature virus. This cleavage event changes the virus morphology, yielding a CA core, which is conical in the case of HIV-1 (Ganser et al., 1999; Briggs and Krausslich, 2011). In addition to the change in morphology, the individual Gag domain proteins no longer exhibit the same roles as the full-length Gag polyprotein.

Gag TRAFFICKING TO THE PLASMA MEMBRANE

Previously, retroviral research posited that Gag proteins trafficked to endosomal compartments and assembled there. This model was based on studies that found Gag in intracellular endosomal compartments (Raposo et al., 2002; Nydegger et al., 2003; Pelchen-Matthews et al., 2003) as well as studies that found virions associated with the mannose receptor CD63 (Nguyen and Hildreth, 2003; Ono et al., 2004; Gousset et al., 2008). Over the past decade, this idea has been challenged by studies showing that the vacuolar structures bearing retroviral virions were actually invaginations of the PM (Welsch et al., 2007; Finzi et al., 2013). Additionally, it was found that HIV-1 Gag is only found in endosomes at late time points and preventing endosomal function with drugs does not inhibit the release of viral particles (Jouvenet et al., 2006; Finzi et al., 2007). Based upon these and other studies, it is generally agreed that the productive site of retroviral particle assembly is the PM.

Before particle assembly can occur at the PM, Gag must traffic from the site of synthesis through the cytoplasm. While this process is not well-understood, it is increasingly thought that Gag must interact with a large amount of host cell machinery, including microtubule networks, motor proteins, and vacuolar transport complexes.

Microtubules are highly dynamic structures that make up the cytoskeleton of the cell. They serve as the road-like structures that motor proteins such as kinesins use to traffic cellular cargo to various cytoplasmic locations (Vale, 2003). It has been shown that HIV-1 Gag interacts with kinesin superfamily member KIF4, a protein that traffics cellular cargo from the perinucleolar region to the membrane (Hirokawa and Noda, 2008). Downregulating KIF4 not only slows Gag trafficking

and reduces particle production but also seemingly increases intracellular Gag degradation, indicating that KIF4 has some sort of stabilizing action. Additionally, suppressor of cytokine signaling 1 (SOCS1) has been shown to colocalize with Gag along the cytoskeleton and promote microtubule stability (Nishi et al., 2009). These studies would indicate that Gag binds to host cell proteins to stabilize its transport via cytoskeletal networks.

The idea that Gag utilizes the microtubule network to directly traffic to the PM is not without controversy (Naghavi and Goff, 2007). Other studies have shown that kinesin family members that direct membranous organelles such as endosomes and lysosomes to the PM are involved in HIV-1 replication (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008). Studies have yet to reconcile these different pathways of Gag transport. It is likely that there are multiple trafficking pathways that lead to Gag expression at the PM, but further analyses need to be done to clarify the mechanisms.

DETERMINANTS OF Gag–Gag INTERACTIONS

The formation of Gag–Gag oligomers appears to be a complex and multifactorial process involving several Gag domains, host proteins, and environmental factors. Examples of important host proteins include the molecular motors and microtubule networks mentioned above, which can concentrate Gag in particular locations of the cell (Naghavi and Goff, 2007). Gag concentration levels as well as subcellular location of Gag are some of the important environmental factors that can contribute to Gag oligomerization.

It is known that CA is the primary region involved in oligomerization (Gamble et al., 1997). In HIV-1, it has been shown that mutations specifically in the CA CTD affect Gag–Gag interactions and severely impede viral particle production (von Schwedler et al., 2003). Based on studies using chimeric Gag molecules with different CA domains, it appears that this is the case for other retroviruses as well (Ako-Adjei et al., 2005). In HTLV-1, the CA NTD is required in addition to the CTD to form Gag–Gag interactions (Rayne et al., 2001).

While CA is typically thought to be the primary site of Gag–Gag interactions, other Gag domains are necessary for stabilizing these interactions. NC has been shown to be an important factor in the formation of Gag–Gag interactions, likely due to its RNA-binding capacities. However, it has also been shown that the HIV-1 MA NTD is capable of binding to the RNA through an electrostatic interaction (Purohit et al., 2001). RNA may serve as a binding platform for Gag assembly, as it may promote Gag oligomerization and expose domains necessary for interactions with the PM (**Figures 2A,B**; Khorchid et al., 2002; Hogue et al., 2009; Rein et al., 2011).

Myristoylation appears to be another requirement for Gag–Gag multimerization, at least in the case of HIV-1 (Li et al., 2007; O'Carroll et al., 2012). Fluorescence resonance energy transfer (FRET) studies found that without a myristic acid moiety, there was a significant decrease in Gag–Gag interactions. The simplest explanation for this phenomenon is that myristoylation concentrates Gag molecules at the PM, bringing the CA domains into contact and facilitating oligomerization.

The requirement for the CA interface, nucleic acid scaffolding, and myristic moiety have repeatedly been confirmed (Li et al., 2007; O'Carroll et al., 2012, 2013). It appears that this "functional redundancy" allows for some give in the ability of HIV-1 to assemble – only two of the three components are necessary for VLP production – but when more than one function is ablated, no particles can be produced (O'Carroll et al., 2012).

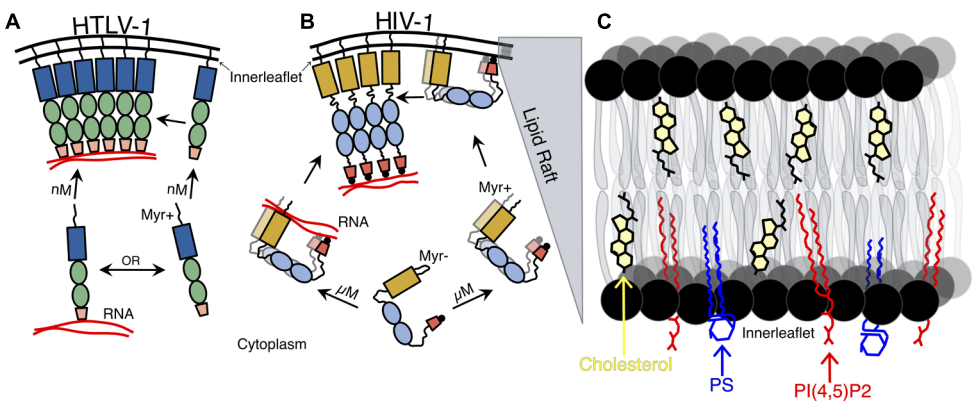

FIGURE 2 | Schematic representation of HTLV-1 and HIV-1 Gag–membrane association. (A) HTLV-1 Gag associates with the plasma membrane (PM) as a monomer and is found in the PM at nM concentrations. HTLV-1 Gag is shown as recruiting viral RNA after its association with the inner leaflet of the PM (though monomeric Gag may recruit the viral RNA in the cytoplasm and are transported together to the PM). **(B)** Concentration-dependent HIV-1 Gag dimerization and translocation to the PM. HIV-1 Gag must reach a critical cytoplasmic concentration (~0.5 μM) in order for Gag–Gag dimers to form and subsequent Gag–membrane association to occur. The myristoyl moiety of the HIV-1 Gag is exposed, allowing for association with the PM of Gag dimers (including Gag dimers associated with dimeric viral RNA). Both HIV-1 Gag N- and CTD interact with the inner leaflet of the PM and to NA, but is not until HIV-1 Gag interacts with the PM, other HIV-1 Gag molecules, and to NA that the protein becomes extended. **(C)** Expanded view of lipid raft that HIV-1 Gag associates with at the PM. Shown are three key constituents of lipid rafts: cholesterol (yellow), phosphatidylserine (PS; blue), and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2; red].

Gag OLIGOMERIZATION

Eventually, Gag must form higher order oligomers (e.g., dimers, trimers) in order to form the lattice structure seen in immature virions and virus-like particles. It is unlikely that these large-scale interactions form in the cytosol, as Gag is translated as a soluble protein. Gag–Gag interactions have been primarily studied using HIV-1 as a model system. This system has been thought to be useful due to the structural similarities between retroviruses, specifically the MA domain. Initial studies used crosslinking to show that HIV-1 Gag mainly exists as a dimer in the cytoplasm and does not form trimers or hexamers until it reaches the PM (Kutluay and Bieniasz, 2010). This observation was recently confirmed using dual-color z-scan fluorescence fluctuation spectroscopy (dczFFS), which quantifies fluorescent proteins inside of living cells (Fogarty et al., 2011, 2013).

It has typically been thought that other retroviruses, such as HTLV-1, trafficked to and assembled at the PM in a similar fashion to that of HIV-1. Using dcz-FFS together with total internal reflection fluorescence and conventional, epi-illumination imaging, it was recently reported that HIV-1 requires micromolar concentrations of Gag in order for it to target and associate with the PM (**Figure 2B**), while HTLV-1 only requires nanomolar concentrations of Gag to become associated with the PM (**Figure 2A**). These results correlate with previous observations that HTLV-1 Gag–Gag interactions were absent in the cytoplasm (Fogarty et al., 2011). This data also supports the hypothesis that HTLV-1 reaches the PM as a monomer where it then forms higher order oligomers (**Figure 2A**), which is in contrast to HIV-1 Gag–Gag interactions, which traffics as lower order oligomers in the cytoplasm prior to targeting the PM (**Figure 2B**; Lindwasser and Resh, 2001; Perez-Caballero et al., 2004). Therefore, HTLV-1 Gag monomers must translocate to the same area at the inner leaflet of the PM to form Gag–Gag oligomers (**Figure 2A**).

The facts that HTLV-1 NC is a bad chaperone (Qualley et al., 2010) and that HTLV-1 Gag–membrane interaction is independent of viral RNA binding (Inlora et al., 2011), taken together with the finding that HTLV-1 Gag targets the PM at low cytoplasmic concentrations (Fogarty et al., 2013), suggest that the viral RNA interacts with Gag at the PM, acting as a scaffold for Gag–Gag interactions (**Figure 2A**). However, it is possible that HTLV-1 monomers interact and traffic the viral RNA to the PM. In the case of HIV-1 Gag, it has been suggested that only a few Gag molecules are needed to recruit the viral RNA to the cytoplasm, which play an important role in initiating the assembly of HIV-1 virions at the PM (**Figure 2B**; Jouvenet et al., 2009). This indicates that different retroviruses may have different determinants of Gag–Gag interactions and Gag trafficking pathways, despite similarities in viral particle morphology.

Gag–MEMBRANE INTERACTIONS

In order for Gag molecules to initiate particle assembly, it must recognize a site of assembly at the PM where it oligomerizes into higher-order multimers. It has been shown that Gag targets and assembles at specific PM microdomains known as lipid rafts, which are dense, ordered groups of tightly packed saturated lipids stabilized by cholesterol (**Figure 2C**). The molecular composition of these lipid rafts is different than that of the surrounding membrane (Lingwood et al., 2009; Ono, 2009; Sonnino and Prinetti, 2013).

MA is responsible for the binding of Gag to the inner leaflet of the PM, which is mediated by the MA NTD that contains multiple membrane binding signals necessary for membrane interactions. A hydrophobic myristic acid moiety found in MA of most retroviruses, such as HIV-1 and HTLV-1, plays an important role in targeting and inserting Gag to the inner leaflet of the PM (Ootsuyama et al., 1985; Zhou and Resh, 1996; Resh, 1999, 2004; Tang et al., 2004). MA also contains a highly basic region, mostly arginines and lysines, that interacts electrostatically with the inner leaflet of the PM. Furthermore, it has been shown that HIV-1 Gag is flexible and can adopt a closed conformation, which brings the MA and NC terminal domains in close proximity, allowing these to interact with the anionic inner leaflet of the PM (**Figure 2B**; Datta et al., 2011). The inner leaflet of the PM is rich in acidic phospholipids, such as phosphatidylserine (PS) and acidic phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], which is important for efficient membrane binding and targeting to the PM (**Figure 2C**; Murray et al., 2005; Chukkapalli et al., 2008).

CHARACTERISTICS ALONG THE INNER LEAFLET OF THE PLASMA MEMBRANE THAT ARE SITE OF Gag ASSEMBLY

The PM is composed of different transmembrane proteins and a wide variety of lipids. These include cholesterol and multiple phospholipids such as phosphatidylinositol phosphates (PIPs), phosphatidyl glycerol (PG), and PS; however, the most abundant lipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The inner leaflet of the PM is mostly composed of PE, PC, PS, and $PI(4,5)P_2$, which makes it acidic and better suited for MA to interact with the PM (van Meer et al., 2008; Chukkapalli and Ono, 2011). HIV-1 Gag uses each one of these lipids as a signal to recognize the PM. However, as one of the main lipids in the inner leaflet, it has been suggested that HIV-1 Gag primarily interacts with $PI(4,5)P_2$ which promotes the HIV-1 Gag to preferentially target the PM (Ono et al., 2004). Further studies have contradicted these findings and suggest that $PI(4,5)P_2$ is not the most important site-specific acidic signal in the PM for HIV-1 Gag, HTLV-1 and other retroviral Gag proteins (e.g., RSV Gag) that do not have a $PI(4,5)P_2$ binding signal, but rather can strongly interact with other acidic phospholipids (Chan et al., 2011; Inlora et al., 2011). However, contradictory results show that RSV Gag does interact with $PI(4,5)P_2$ at the PM (Nadaraia-Hoke et al., 2013). Furthermore, HIV-1 can differentiate membranes with multiple compositions of fatty acids and cholesterol (Dick et al., 2012).

Gag MOVEMENT AND TRAFFICKING ALONG THE INNER LEAFLET

A new assembly model for HIV-1 suggests that the viral genome is recruited by Gag and then directed and anchored to the PM (Jouvenet et al., 2009). In the latter location, the RNA–Gag complex functions as a scaffold to form large Gag oligomers by recruiting other Gag molecules (**Figures 2A,B**). The lateral

movement of the viral RNA in the PM and the progressive accumulation of Gag molecules over time, suggest that after the RNA–Gag complex attaches to the PM, it moves in the PM plane to recruit more Gag molecules. Furthermore, it has been shown that HIV-1 Gag translocate from internal compartments towards virological synapses, which are the contact sites that allow the virus to be transferred, between infected macrophages and uninfected T cells (Gousset et al., 2008). Therefore, it is possible that the Gag anchored to the PM can also traffic to these virological synapses. Further studies need to be done to fully understand Gag movement along the infer leaflet of the PM.

CONCLUDING REMARKS

Recent observations have demonstrated differences in the form and concentration of Gag that is associated with translocation to the PM. While HIV-1 Gag dimers are the primary form of Gag that is thought to translocate to the PM at μM cytoplasmic concentrations (i.e., concentration dependent translocation), HTLV-1 Gag has been found to translocate to the PM as a monomer at nM cytoplasmic concentrations. These observations indicate that fundamental differences may exist in the association of different Gag proteins with the PM, including interactions with lipids. Furthermore, such differences suggest that the movement of Gag along the inner leaflet of the PM may also be distinct among different retroviruses. Taken together, these observations argue for the importance of comparative studies of retroviruses in order to provide the greatest insights into the diversity of strategies associated with the virus assembly pathway.

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Role of Gag and lipids during HIV-1 assembly in CD4$^+$ T cells and macrophages

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HIV-1 is an RNA enveloped virus that preferentially infects CD4$^+$ T lymphocytes and also macrophages. In CD4$^+$ T cells, HIV-1 mainly buds from the host cell plasma membrane. The viral Gag polyprotein targets the plasma membrane and is the orchestrator of the HIV assembly as its expression is sufficient to promote the formation of virus-like particles carrying a lipidic envelope derived from the host cell membrane. Certain lipids are enriched in the viral membrane and are thought to play a key role in the assembly process and the envelop composition. A large body of work performed on infected CD4$^+$ T cells has provided important knowledge about the assembly process and the membrane virus lipid composition. While HIV assembly and budding in macrophages is thought to follow the same general Gag-driven mechanism as in T-lymphocytes, the HIV cycle in macrophage exhibits specific features. In these cells, new virions bud from the limiting membrane of seemingly intracellular compartments, where they accumulate while remaining infectious. These structures are now often referred to as Virus Containing Compartments (VCCs). Recent studies suggest that VCCs represent intracellularly sequestered regions of the plasma membrane, but their precise nature remains elusive. The proteomic and lipidomic characterization of virions produced by T cells or macrophages has highlighted the similarity between their composition and that of the plasma membrane of producer cells, as well as their enrichment in acidic lipids, some components of raft lipids and in tetraspanin-enriched microdomains. It is likely that Gag promotes the coalescence of these components into an assembly platform from which viral budding takes place. How Gag exactly interacts with membrane lipids and what are the mechanisms involved in the interaction between the different membrane nanodomains within the assembly platform remains unclear. Here we review recent literature regarding the role of Gag and lipids on HIV-1 assembly in CD4$^+$ T cells and macrophages.

Keywords: HIV-1, lipids, assembly, Gag, CD4$^+$ T cells, macrophages

THE ROLE OF GAG IN HIV ASSEMBLY

For proper assembly of newly synthesized virions, the different viral and cellular components of HIV have to be addressed to the assembly site. The polyprotein Gag is the major structural orchestrator of the assembly process (Cimarelli and Darlix, 2002). Beside Gag itself, GagPol polyproteins, the envelop (Env) glycoprotein (Checkley et al., 2011) and the viral genomic RNA (gRNA) are recruited to the assembly site (Muriaux and Darlix, 2010; O'Carroll et al., 2013). In addition, host cell factors are required for proper trafficking of the viral constituents, as well as for virus assembly and budding. The nature of the host cell factors and their incorporation into new virions can vary depending on the producer cell and thus may impact HIV-1 infectivity (reviewed in Iordanskiy et al., 2013). Nevertheless, Gag expression alone is sufficient for virus-like particle (VLP) production. Gag is synthesized in the cytosol as a 55 kDa polyprotein comprising several domains that are cleaved into independent proteins after budding: the Matrix (MA), the Capsid (CA), the Nucleocapsid (NC) and the p6 domain (**Figure 1**). Gag is targeted to the site of budding where it interacts with the membrane and multimerizes. Viral assembly requires Gag–Gag interactions that can occur at different levels: MA–MA interactions upon MA-membrane interactions, CA–CA interactions and NC–NC interactions via the genomic RNA (gRNA) (**Figure 1**). The gRNA is recruited to the nascent viral particle via a selective interaction between its Psi encapsidation sequence and the NC domain of Gag (Muriaux and Darlix, 2010; Lu et al., 2011). The formation of VLPs at the plasma membrane (PM) of infected cells requires the myristoylation of Gag (Bryant and Ratner, 1990; Resh, 2005) and the presence of a highly basic region (HBR) in the N-term of MA for Gag anchoring into the cell membrane lipid bilayer (Chukkapalli and Ono, 2011). Other amino acids in the vicinity of MA/membrane interface could also be involved but have not yet been described. In this review, we will focus on the Gag-driven viral assembly

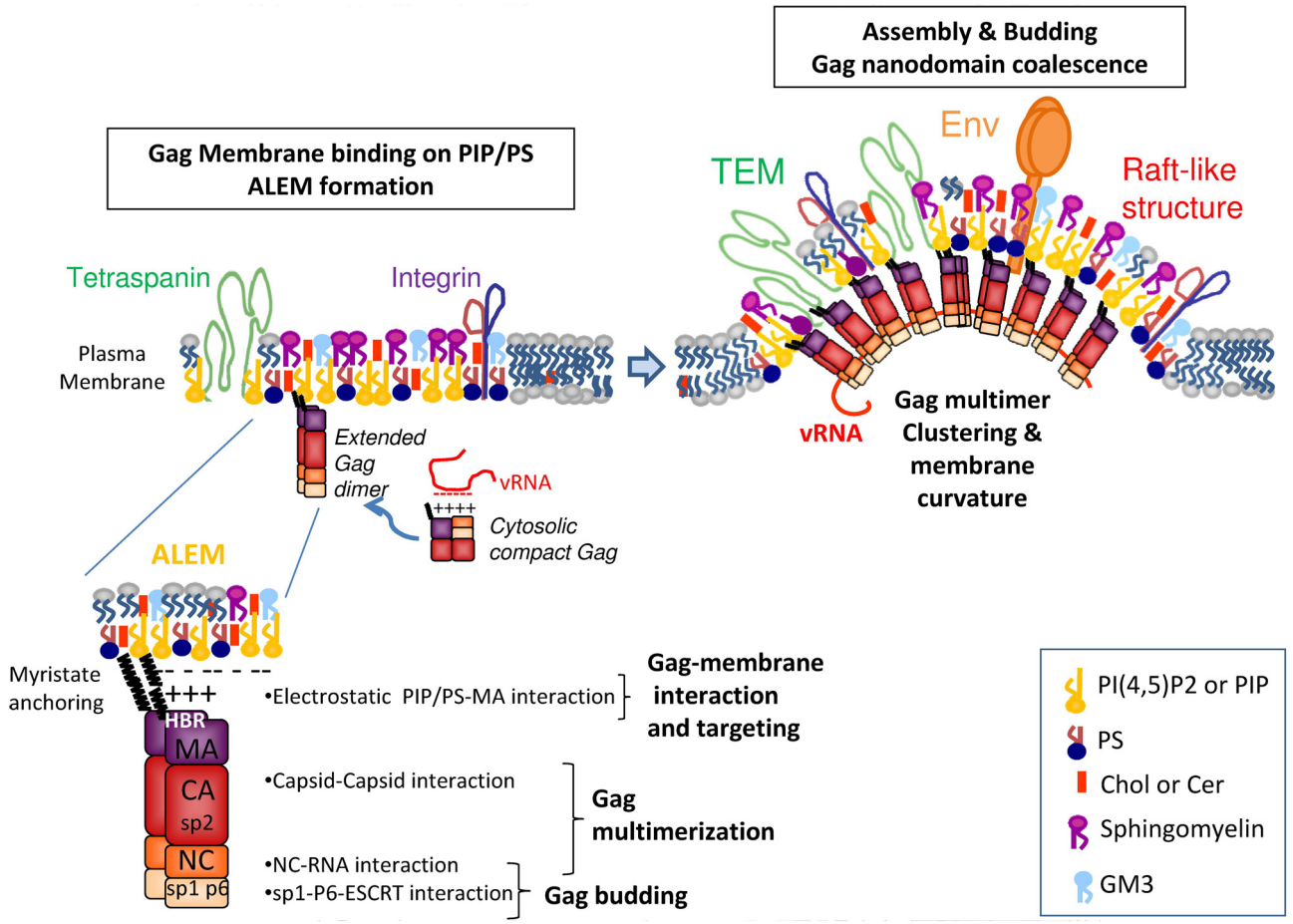

FIGURE 1 | HIV Gag assembly at the molecular level. The viral HIV-1 Gag protein is composed of several domains: the matrix MA is myristoylated in its N-term and contains a highly basic region (HBR) that binds the lipidic membrane or the viral genomic RNA, the capsid CA that favors Gag–Gag multimerization, the NC domain that selects the viral genome and favors Gag multimerization, its C-term p6 domain that binds ESCRT proteins requires for retroviral budding and particle release and 2 spacer peptides, sp2 and sp1 required for HIV assembly and morphogenesis. In the cytosol, Gag is a dimer and adopts a compact shape in which the viral genomic RNA is in interaction with the NC and MA domains. At the cell membrane, the MA domain of Gag targets, via its HBR, the inositol head of the $PI(4,5)P_2$ located at the inner leaflet of the PM and its myristate anchors the lipid bilayer. The MA domain of Gag can recruit several $PI(4,5)P_2$, and PS, thanks to its basic residues. This should allow the recruitment of other Gag molecules due to Gag electrostatic membrane interaction concomitant with Gag multimerisation, via CA, and NC on the viral RNA. This process should trigger the formation of ALEM (acidic lipid enriched microdomains) in the cell membrane. These ALEM containing Gag could now join together to create higher ordered microdomains of Gag and/or microdomains containing other proteins such as Env, or tetraspanins, integrins or other cell factors found in the viral envelope. The coalescence of these microdomains containing several Gag multimers creates the HIV assembly platform.

process in HIV-1-infected T cells and macrophages. The recognition between Gag preassembling complexes and the Env will not be developed here and has been reviewed by others (Checkley et al., 2011).

HIV PRODUCING CELLS: CD4$^+$ T-CELLS AND MACROPHAGES

HIV-1 mainly infects activated CD4$^+$ helper T cells *in vivo* (Freed and Martin, 2001). Most infected activated T cells produce large amounts of new virions and die of apoptosis. A minority acquire a memory phenotype and progress to latently infected cells, able to survive for decades in the absence of virus production (Kuritzkes D.R., 2007). Upon arrest of therapy, these cells can be activated and participate in the rebound in HIV-1 titers, and are therefore important reservoirs of the virus. HIV-1 assembly, budding and release from CD4$^+$ T cells occurs mainly at the plasma membrane (PM). In polarized CD4$^+$ T-cells, HIV components for assembly, such as Gag and the genomic RNA are localized at the uropod membrane as shown by live cell fluorescence microscopy (Hatch et al., 2013; Llewellyn et al., 2013). An exception among T cells are the chronically-infected MOLT lymphoblasts in which infectious HIV-1 are found in intracellular compartments (Grigorov et al., 2006).

Macrophages are the other main cellular target of HIV. They play important roles during HIV infection and AIDS progression due to their specific features (Koppensteiner et al., 2012). Infected macrophages have been found in all tissues such as the vaginal mucosa, the brain and the lung (Gartner et al., 1986; Shen et al., 2009; Jambo et al., 2014), where they remain infectious for long periods of time (Sharova et al., 2005). In contrast with T cells, macrophages resist HIV-induced cytopathic effects for months

in vitro as well as *in vivo* (Koppensteiner et al., 2012). They accumulate virions in large intracytoplasmic vacuoles (Orenstein et al., 1988), that are often referred to as VCCs (Virus-Containing Compartments) (**Figure 2C**) or IPMCS (Intracellular Plasma Membrane-Connected Compartments) (Mlcochova et al., 2013). Electron microscopy studies of infected macrophages revealed budding profiles at the limiting membrane of the VCC and the presence of immature virion in the lumen of the compartments, demonstrating that VCCs represent the site of HIV assembly in macrophages (Orenstein, 1998; Raposo et al., 2002; Pelchen-Matthews, 2003; Jouve et al., 2007). Newly formed viral particles accumulate in the VCC lumen and VCCs tend therefore to fill up with time (Gaudin et al., 2013). Early on, VCCs were classified as late endosomes or multivesicular bodies because of their endosomal markers and their morphology (Raposo et al., 2002; Pelchen-Matthews, 2003). However, they were later shown to be inaccessible to BSA-gold, devoid of EEA1 (Deneka et al., 2007; Jouve et al., 2007) and to possess a neutral luminal pH (Jouve et al., 2007), suggesting that they were not true endosomes. The lumen of the VCCs can be transiently accessible to extracellular molecules (Deneka et al., 2007; Berre et al., 2013; Gaudin et al., 2013) possibly through direct tubular connections to the PM (Welsch et al., 2007; Bennett et al., 2009). VCCs are now regarded as specialized, intracellular sequestered portions of the PM, possibly with dynamic connections to the extracellular milieu (Gaudin et al., 2013; Mlcochova et al., 2013), for reviews see Benaroch et al. (2010) and Tan and Sattentau (2013).

The two main cellular targets of HIV, $CD4^+$ T cells and macrophages, exhibit very different morphology, function and physiology. The assembly site of HIV in both cell types appears different : the PM of small round T-lymphocytes versus the internal convoluted membrane meshwork of large macrophages (**Figure 2**). This raises the question of the cell specificity for the mechanism(s) underlying virus assembly and for the lipid

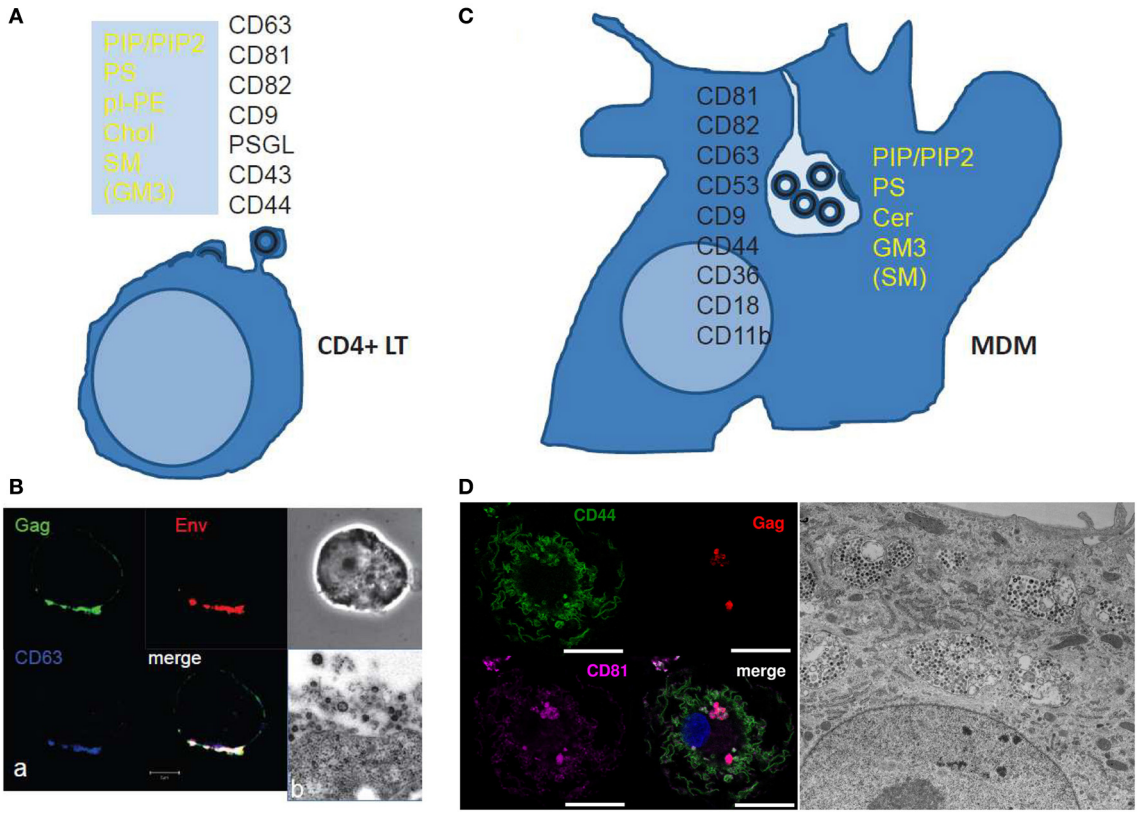

FIGURE 2 | Scheme and images of HIV assembly at the cellular level. (A) Membrane cell proteins and lipid composition that are found in the virus membrane issued from $CD4^+$ T cell lines or at polarized T cell uropods. From Brügger et al. (2006); Chan et al. (2008); and Lorizate et al. (2013) for the virus lipid membrane composition. From Grigorov et al. (2006, 2009); and Nydegger et al. (2006) for the virus membrane protein composition. From Llewellyn et al. (2013) for the Gag containing T cell uropod microdomains. **(B)** HIV-1 assembly in chronically infected MOLT cells as shown by immunofluorescence confocal for Gag, Env and the cell CD63 tetraspanin (a) and electron(b) microscopy. (a) HIV-1 infected MOLT cells were fixed in 3% PFA and stained for Gag with an anti-MAp17, anti-gp120, and anti-CD63 for immunofluorescence confocal imaging. (From D. Muriaux lab.) (b) HIV-1 infected MOLT cells were fixed with 2.5% glutaraldéhyde, embedded and thin-sectioned for electron microscopy imaging, as in Grigorov et al. (2009) (From P. Roingeard lab.). **(C)** The scheme shows the cell membrane proteins and lipids found associated with the viral membrane of HIV-1 issued from monocyte derived macrophages. From Chertova et al. (2006); Chan et al. (2008); Lorizate et al. (2013). **(D)** HIV assembly in VCC in MDM as shown by immunofluorescence confocal microscopy and electron microscopy. MDM 4 days post-infection with WT HIV-1 NL-AD8, stained by immunofluorescence for CD44 (green), Gag (red), and CD81 (magenta). The nucleus is stained with DAPI. Bar: 20 μm. CD44 and CD81 are present at the plasma membrane but also in intracellular compartments were they co-localize with Gag in infected MDM. (From P.Benaroch lab). For electron microscopy, kindly provided by Mabel Jouve, MDM 15 days post-infection with WT HIV-1 NL-AD8 were fixed, embedded in epon, and ultrathin sections were contrasted with uranyl acetate and lead citrate. Bar: 2 μm.

membrane composition of produced virions. Most studies pertaining to the role of Gag and lipids in HIV assembly in live cells used T-cell lines or adherent epithelial cell lines, with only few results confirmed in human primary T-cells or monocyte-derived macrophages (MDM), the classical culture model for macrophages. However, we will see below that most observations and mechanistic hypotheses regarding the role of membrane lipids in HIV assembly can apply for T-cells and macrophages.

COMPOSITION OF HIV ASSEMBLY SITES IN T CELLS AND MACROPHAGES

TETRASPANIN-ENRICHED MICRODOMAINS

In primary T-cells and T-cell lines, Gag accumulates in specific domains of the PM containing the tetraspanins CD9, CD63, CD81, and CD82 that are referred to as Tetraspanin Enriched Microdomains (TEM) (Booth et al., 2006; Grigorov et al., 2006; Nydegger et al., 2006). These tetraspanins are present in subdomains of the PM of HIV-infected T cells together with Gag and Env as shown by immunofluorescence analysis and immunoprecipitation (Jolly and Sattentau, 2007; Grigorov et al., 2009) (**Figures 2A,B**). In addition, virions released and purified from these cells are associated with the same tetraspanins (Grigorov et al., 2009). HIV-infected and polarized T-cells possess "specific uropod-directed microdomains" that contain PSGL-1 (P-Selectin Glyco- protein Ligand 1), CD43 and CD44 (**Figure 2A**) where Gag localizes due to its NC and MA regions (Llewellyn et al., 2013).

In infected macrophages, TEMs are also a component of the assembly site of HIV and the same tetraspanins are enriched at the VCC limiting membrane as revealed by immunofluorescence and electron microscopy (**Figure 2C**) (Deneka et al., 2007; Gaudin et al., 2013; Mlcochova et al., 2013). This is also reflected by the composition of virions produced by macrophages, which harbor several tetraspanins (CD9, CD81, CD82, CD53, CD63) as assessed by mass spectrometry (Chertova et al., 2006). These virions also contain other proteins enriched in the VCCs, such as CD44, CD18, and CD11b, further highlighting that the envelope of the virus derives from the membrane of the VCC (**Figures 2C,D**). Interestingly, the limiting membrane of the VCC as well as the virions present in the VCC of infected macrophages contains the scavenger receptor CD36, which represents so far the first macrophage specific marker of the viral assembly site as compared to T lymphocytes (Berre et al., 2013).

VIRUS MEMBRANE LIPID COMPOSITION

Lipidomic analysis of HIV virions has provided important information regarding the nature of the membrane domains where the viral budding takes place (Aloia et al., 1993; Brügger et al., 2006; Chan et al., 2008; Lorizate et al., 2013). Early work revealed that HIV-1 and HIV-2 virions were enriched in cholesterol when compared to the PM of producer cells H9 T-cells, suggesting that HIV buds from specific domains of the PM (Aloia et al., 1993). Later, Chan et al. showed that virions from T cells were mainly enriched in acidic lipids (PI, PIP_2, PS), GM3, cholesterol and SM (Chan et al., 2008), a composition close to that of "rafts-like" domains. However, the HIV assembly site exhibits some differences with these domains since the GM1 raft lipid and the CD55 raft protein were only transiently found trapped in viral assembly sites, at least in adherent cell lines (Krementsov et al., 2010). The dynamics of other lipids have not been studied during Gag assembly in living primary host cells and should be investigated in order to validate the role of lipids in HIV assembly and budding or the role of Gag in the formation of lipidic domains. A recent study done on MT4 T cells showed an enrichment in PS, PI, pl-PE, PG, and hexCer but not Cholesterol in the virus particles when compared to the PM (Lorizate et al., 2013). This suggested that the HIV envelope composition can vary as a function of the membrane composition of the producer cell or depends on experimental conditions, such as techniques used to purify the PM (Lorizate et al., 2013) or to separate viral particles from microvesicles shed by the cells (Chertova et al., 2006; Chan et al., 2008; Coren et al., 2008). However, a constant observation is the enrichment in glycosphingolipids (Cer or SM) and/or Cholesterol that could locally order the virion bud membrane and in an acidic phospholipid (PI, PIP_2, or PS) that is required for Gag membrane recruitment (see below). **Figure 2A** summarizes the lipidic and proteic enrichment of HIV envelope composition in T-lymphocytes and MDM.

Only one study so far investigated the lipidomic composition of HIV virions produced by macrophages (Chan et al., 2008). It showed that, when compared to the PM of the macrophages, viral particles are enriched in phosphorylated derivatives of phosphatidylinositol (PIP, PIP_2), the glycosphingolipid GM3 and to a lesser extend sphingomyelin but not in cholesterol. HIV virions from macrophages are also enriched in ceramide, which may be a sign of coalescence of other lipid nanodomains into bigger domains at the level of the assembly platform. Importantly, Chan et al. compared HIV virions produced by T-cells and macrophages. Both types of virions exhibited a very similar lipidic composition while the global PM composition was different in macrophages as compared to T-cells (Chan et al., 2008). These data suggest that HIV is able to reach or to create a favorable lipidic environment containing the components necessary for its assembly and budding in both cell types.

Thus, in macrophages as in T cells, HIV assembles at and buds from specific membranes enriched in acidic lipids, sterols and/or glycosphingolipids (reviewed in Kerviel et al., 2013). Nevertheless, the fine mechanism underlying Gag targeting to such PM domains remains unclear.

GAG TARGETING TO THE VIRAL BUDDING SITE

Gag synthesis occurs in the cytoplasm where it is myristoylated (Bryant and Ratner, 1990). Gag selectively interacts with the genomic RNA from which it has been translated (de Breyne et al., 2013). Then Gag is targeted to the cell PM (Kutluay and Bieniasz, 2010) either by transport as minimal Gag-RNA complexes or simply by diffusion. At this point, cytosolic Gag is probably in a low dimerization state (Kutluay and Bieniasz, 2010) and in a compact conformation likely in interaction with the viral RNA (**Figure 1**) (Datta et al., 2007; Munro et al., 2014) and should reach the plasma membrane for assembly.

GAG INTERACTION WITH THE PLASMA MEMBRANE: A ROLE FOR PI(4,5)P_2

The MA domain of Gag can bind lipids at the inner surface of the cell PM where Gag multimerizes, thanks to its myristate

(Bryant and Ratner, 1990) and its Highly Basic Region (HBR) (Ono, 2009). Based on biophysical experiments, conformations of a monomeric form of HIV-1 Gag were analyzed in solution, and the results revealed that Gag can adopt a compact conformation (Datta et al., 2007). Single molecule FRET experiments and FCS revealed that upon assembly into VLP, Gag undergoes a conformational transition from compact to an extended form (Munro et al., 2014). It has been proposed that upon its interaction with membrane phospholipids and during its multimerization, Gag adopts an extended conformation, similar to the one observed in immature virus. This process is triggered by the interaction of the HBR at the N-ter of MA with the PM $PI(4,5)P_2$ phospholipid (Ono et al., 2004a; Saad et al., 2006; Chukkapalli et al., 2008). In addition, the genomic RNA and the $PI(4,5)P_2$ are probably in competition for the binding of the MA HBR (Chukkapalli et al., 2010). This suggests that under its compact conformation, Gag is able to interact with the genomic RNA through the MA HBR as well as through the NC and as soon as Gag is in the vicinity of a $PI(4,5)P_2$ containing membrane, it switches to the extended conformation which the HBR interacts with $PI(4,5)P_2$. Thus, it appears that MA-membrane binding requires the anchoring of the N-term MA myristate in the membrane lipid bilayer, a change of Gag conformation from a compact to an extended rod-like shape, and the electrostatic interaction between the charged sugar head of phospholipids (such as $PI(4,5)P_2$ and PS) and the MA HBR (**Figure 1**).

$PI(4,5)P_2$ facilitates Gag-membrane binding to the plasma membrane in T cells and is required for efficient virus release (Monde et al., 2011). However, in T cells engineered to express low levels of $PI(4,5)P_2$, the virus particle production can still occur, suggesting that the MA domain of Gag can probably interact with other charged acidic lipids, such as PS, as reported by lipidomic analysis of HIV virions (Lorizate et al., 2013). Interestingly, in these T cells, the virus adapted to compensate the lack of $PI(4,5)P_2$ by a "charged" mutation in MA (L74R) that enhances virus infectivity (Monde et al., 2011). The role for $PI(4,5)P_2$ dependence for virus release remains unclear and may involve other cellular lipids or proteins or a cell signaling cascade. This interaction regulates the proper targeting of the viral components to the assembly site. Indeed, mutations of the HBR MA domain induce re-localization of Gag from the PM to CD63+ intracellular compartments in HeLa and in T cells (Ono et al., 2004b).

In macrophages, both WT and MA-mutant Gag co-localize with a subpopulation of $CD63^+$ compartments (Ono et al., 2004b; Gousset et al., 2008). However, it is unclear whether these $CD63^+$ compartments represented true VCCs, since few other VCC specific markers were used in these studies (Ono et al., 2004a; Gousset et al., 2008). Importantly, $PI(4,5)P_2$ was detected at the limiting membrane of intracellular CD81+, CD44+, or Gag+ compartments and at the PM in infected macrophages (Mlcochova et al., 2013). Together, these results suggest that the basic region of the MA domain may contribute to Gag targeting to the VCC through phospholipid binding, in MDM as in T-cells. In addition, other unknown factors may be required to regulate this targeting (Ono et al., 2004b; Gousset et al., 2008; Mlcochova et al., 2013).

OTHER RETROVIRUSES

The mechanism by which the HBR of retroviral MA (Murray et al., 2005) binds the PM $PI(4,5)P_2$ phospholipid is likely to be general for many retroviruses such as HIV-1 (Ono et al., 2004a; Saad et al., 2006; Chukkapalli et al., 2008), HIV-2 (Saad et al., 2008), EIAV (Chen et al., 2008), MLV (Hamard-Peron et al., 2010), and MPMV (Prchal et al., 2012). It is controversial for Rous Sarcoma Virus (RSV) as some authors have found that *in vitro* RSV MA binding to membranes requires acidic lipids like PS but not $PI(4,5)P_2$ (Chan et al., 2011). In addition, no effect of the phosphatase-mediated depletion of $PI(4,5)P_2$ was reported on RSV Gag cellular localization and VLP production. In contrary, another study described, in a cellular context, that the presence of $PI(4,5)P_2$, and/or $PI(3,4,5)P_3$, is required for RSV Gag targeting to the PM and for virus release in a NC-dependent Gag multimerization manner (Nadaraia-Hoke et al., 2013). In the case of EIAV, MA has greater affinity for PI(3)P than $PI(4,5)P_2$, as shown *in vitro* by NMR lipid titration. Interfering with the metabolism of PI(3)P, but not of $PI(4,5)P_2$, prevents EIAV assembly and release, indicating a slight difference of PI recognition for this retrovirus compared to others (Fernandes et al., 2011). Only one retroviral Gag seems to make an exception, as HTLV-1 MA does not require $PI(4,5)P_2$ interaction to trigger Gag targeting and membrane binding (Inlora et al., 2011).

GAG-DRIVEN CONSTRUCTION OF THE ASSEMBLY PLATFORM

Differences in lipid composition between HIV envelop and the host cell PM suggest, as mentioned earlier, that the virus buds from a specific Gag-containing membrane domain. Gag could either bind these pre-formed lipidic and proteic microdomains at the PM, or induce their formation by segregating the components through electrostatic interactions and Gag multimerization.

ACIDIC LIPID ENRICHED MICRODOMAINS (ALEM)

We hypothesize that Gag alone or minimal Gag-RNA complexes are targeted to the PM not only by the electrostatic interaction of the HBR region of MA with the $PI(4,5)P_2$/PS lipids, but also by Gag multimerization via its CA/CA interaction and NC/RNA interaction. The stabilization of the MA—membrane interaction appears to rely on the insertion of the myristate into the lipid bilayer (Charlier et al., 2014), as well on the interaction of the HBR motif with the sugar head of $PI(4,5)P_2$ and consequently on Gag multimerization. Other cellular cofactors of membrane microdomain formation such as cortical actin (unpublished data) or tetraspanin web (Thali, 2009) could contribute to the stabilization of Gag multimers at the cell PM. We propose that upon Gag—membrane interaction, Gag multimerization triggers the formation of acidic lipid enriched microdomains (ALEM) at the inner leaflet of the cell membrane (Kerviel et al., 2013) as it has been shown by dynamic coarse grained modeling of HIV-1 MA anchoring in a lipidic membrane (Charlier et al., 2014). The generation of ALEM by Gag itself is likely due to lipid sequestering induced by electrostatic interaction as reported before for other cellular proteins (van den Bogaart et al., 2011). This early event certainly generates nanodomains smaller than the size of a virus bud and different from rafts. Then viral assembly could propagate

either by expansion of these nanodomains or by coalescence of several Gag-enriched membrane nanodomains to form the final viral bud.

MICRODOMAINS COALESCENCE

The formation of ALEM by higher order Gag multimer formation could then drain other microdomains of the external leaflet of the cell PM such as tetraspanin-enriched microdomains (TEM) or rafts, that contain other lipids like Ceramide, Cholesterol, Sphingomyelin and proteins such as tetraspanins (Yanez-Mo et al., 2009) or the embedded Env glycoproteins (Checkley et al., 2011). Coalescence of nanodomains has been reported for Gag with "rafts-like" membrane domains and TEM in Hela cells (Krementsov et al., 2010; Hogue et al., 2011). This model is supported by the work of Ono and co-workers, with antibody-mediated copatching and FRET assays (Hogue et al., 2011) conducted on adherent Hela cells expressing HIV-1. Interactions between Gag and the inner leaflet of PM appear to induce the coalescence of TEM and lipid raft at the viral assembly site. This is also in line with a model where Gag does not associate with pre-existing virus-sized microdomains organized by cellular factors, but rather functions as a microdomain-organizing factor to create novel virus-induced microdomains in a stepwise manner during the course of assembly (Krementsov et al., 2010; Hogue et al., 2011; Kerviel et al., 2013).

In this model (**Figure 1**), Gag recruits $PI(4,5)P_2$ upon its binding to the membrane, then its multimerization induces the formation of ALEM in the inner leaflet of the membrane, which further triggers the coalescence of other nanodomains of the outer leaflet of the membrane, such as lipid rafts and TEM (Kerviel et al., 2013; Charlier et al., 2014).

In polarized $CD4^+$ T cells, such advanced studies have not been done, but Ono and co-workers have shown that HIV-1 Gag assembly occurs in uropod-specific microdomains and that this Gag localization depends on higher order NC dependent multimerization of Gag (Llewellyn et al., 2010), in agreement with the model proposed above. In macrophages, studies that pertain to the molecular interactions driving the construction of the assembly platform are still lacking. Considering that Gag assembly is a sequential process and several molecules are required to form higher ordered microdomains, Gag-acidic lipid nanodomains certainly fuse together. In addition, they can get enriched in other lipids via the coalescence with other surrounding nanodomains containing cell membrane proteins such as CD81 tetraspanin (Nydegger et al., 2006; Grigorov et al., 2009) or other lipids from the upper bilayer, such as SM and GM3 (Chan et al., 2008).

A ROLE FOR CHOLESTEROL?

Gag could directly sense other lipids than PIP_2, like cholesterol (Dick et al., 2012). Depletion of cellular cholesterol markedly and specifically reduced HIV-1 particle production (Ono and Freed, 2001). Drug-induced redistribution of the cholestorol from the PM to endosomes led to relocation of Gag from the PM to intracellular MVB-like $CD63^+$ compartments (Lindwasser and Resh, 2004), suggesting a possible role for cholesterol in Gag targeting to assembly sites. However, Gag contains no cholesterol binding motif, unlike other viral proteins with roles in entry or morphogenesis (Schroeder, 2010). Several studies have proposed that HIV-1 regulates the levels of cellular cholesterol, presumably to achieve efficient viral egress. A Nef-induced decrease of the ABCA1-dependent efflux of cholesterol to the Apolipoprotein A1 has been reported (Mujawar et al., 2006; Morrow et al., 2010), leading to increased cholesterol in lipid rafts (Cui et al., 2012). This mechanism is likely involved in HIV-associated dyslipidemia as observed in macaques infected by SIV (Asztalos et al., 2010). However, Nef had no impact on virus content in cholesterol in MT4 T cells (Brügger et al., 2007). Thus, the role of cholesterol in virus budding and infectivity remains to be assessed.

MODULATION OF CALCIUM SIGNALING IN HIV-1 ASSEMBLY AND RELEASE

The $PI(4,5)P_2$ represents 1–2% of the total phospholipids at the inner leaflet of the cell PM, and it is a key regulator of several cellular processes taking place at the cell PM, such as endocytosis, exocytosis, cytoskeleton attachment and activation of enzymes (reviewed in McLaughlin and Murray, 2005). $PI(4,5)P_2$ anchors cellular proteins to the PM through Pleckstrin Homology domains that can activate different ion channels located at the PM (Suh and Hille, 2005). $PI(4,5)P_2$ is also the precursor of second messengers such as inositol(1,4,5)triphosphate (IP_3) or diacylglycerol (DAG). IP_3 can regulate release of Ca^{2+} from internal storages; DAG can activate the protein kinase C (PKC). In the course of HIV assembly and budding, it was suggested a role for intracellular Ca^{2+} increase in boosting virus release (Grigorov et al., 2006; Perlman and Resh, 2006). In addition, calmodulin, CaM, a regulator of intracellular Ca^{2+} concentration, interacts with the N-terminal domain of HIV-1 MA as shown, *in vitro*, by NMR and biochemical experiments (Samal et al., 2011; Vlach et al., 2014). This interaction depends on Ca^{2+} and is able to induce the MA myristate exposure and an extended MA conformational change (Chow et al., 2010; Ghanam et al., 2010; Taylor et al., 2012). In cells, Carter and co-workers have shown that HIV-1 Gag induces Ca^{2+} release from intracellular compartments, probably from the endoplasmic reticulum, by activating the cascade $PI(4,5)P_2$-PLC-IP_3-IP_3R (Ehrlich et al., 2010; Ehrlich and Carter, 2012). IP_3 receptor and Sprouty2, also regulators of Ca^{2+} signaling, are required for HIV Gag particle release (Ehrlich et al., 2011). Thus, it appears that in addition to the requirement of the $PI(4,5)P_2$ as a co-factor for HIV-1 Gag targeting to the PM, related lipid metabolism and second messengers or Ca^{2+} signaling are involved in virus particle release. These findings open a new door in the regulation of HIV-1 particle production.

CONCLUSION

$CD4^+$ T lymphocytes and macrophages are very different in size, shape and immune functions. The HIV-1 virions produced by $CD4^+$ T cells or macrophages carry specific lipids and cellular proteins, incorporated during virus assembly. The cellular components identified so far appear quite similar in both cell types, suggesting that, at the molecular level, Gag-membrane interaction with lipids (and proteins) occurs in a similar way during assembly. This is in line with a model where Gag contains most of the determinants for selecting or creating the best lipidic environment for viral bud formation. We propose that

Gag multimerization will generate an assembly platform containing specific lipid membrane nanodomains through its interaction with selective lipids. The creation of this assembly platform in the inner layer of the PM, probably recruits the nanodomains enriched in other proteo-lipid domains present in the upper layer.

Finally, all the components involved in the PM structure and signaling (i.e., lipid metabolism, calcium mobilization and/or actin cytoskeletal reorganization) may contribute to the assembly process of enveloped viruses. Deciphering the complex process of assembly, will require the identification of additional cellular proteins that have yet to be discovered.

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Structural and molecular determinants of HIV-1 Gag binding to the plasma membrane

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Targeting of the Gag polyprotein to the plasma membrane (PM) for assembly is a critical event in the late phase of immunodeficiency virus type-1 (HIV-1) infection. Gag binding to the PM is mediated by interactions between the myristoylated matrix (MA) domain and PM lipids. Despite the extensive biochemical and *in vitro* studies of Gag and MA binding to membranes over the last two decades, the discovery of the role of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] in Gag binding to the PM has sparked a string of studies aimed at elucidating the molecular mechanism of retroviral Gag–PM binding. Electrostatic interactions between a highly conserved basic region of MA and acidic phospholipids have long been thought to be the main driving force for Gag–membrane interactions. However, recent studies suggest that the mechanism is rather complex since other factors such as the hydrophobicity of the membrane interior represented by the acyl chains and cholesterol also play important roles. Here we summarize the current understanding of HIV-1 Gag–membrane interactions at the molecular and structural levels and briefly discuss the underlying forces governing interactions of other retroviral MA proteins with the PM.

Keywords: HIV-1, Gag, matrix, myristoyl, NMR, plasma membrane, PI(4,5)P_2

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Introduction

Prior to assembly on the plasma membrane (PM), the human immunodeficiency virus type-1 (HIV-1) Gag polyprotein adopts a compact "folded over" conformation and exists in the monomeric or low-order oligomeric states (Datta et al., 2007a, 2011; Kutluay and Bieniasz, 2010; Kutluay et al., 2014). Whereas it is established that the nucleocapsid (NC) domain of Gag specifically recognizes motifs in the viral RNA genome for packaging (Jouvenet et al., 2009; Kutluay et al., 2014), there is compelling evidence that the matrix (MA) domain also binds to cellular RNA to prevent premature Gag targeting to intracellular membranes (**Figure 1**; Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011; Hogue et al., 2012; Inlora et al., 2014; Kutluay et al., 2014; Olety and Ono, 2014). Upon transport of Gag to the PM, the interaction of MA with RNA is exchanged for an interaction of MA with PM components (**Figure 1**; Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011; Hogue et al., 2012; Inlora et al., 2014; Olety and Ono, 2014). This molecular switch induces an extended conformation of Gag, leading to formation of high-order Gag oligomers on the PM (Datta et al., 2007a,b, 2011; Munro et al., 2014). The key to understanding this essential switch is elucidating at the molecular level the interaction of MA with specific PM components. Our current understanding

Abbreviations: MA, matrix protein; NMR, nuclear magnetic resonance; PS, phosphatidylserine; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate.

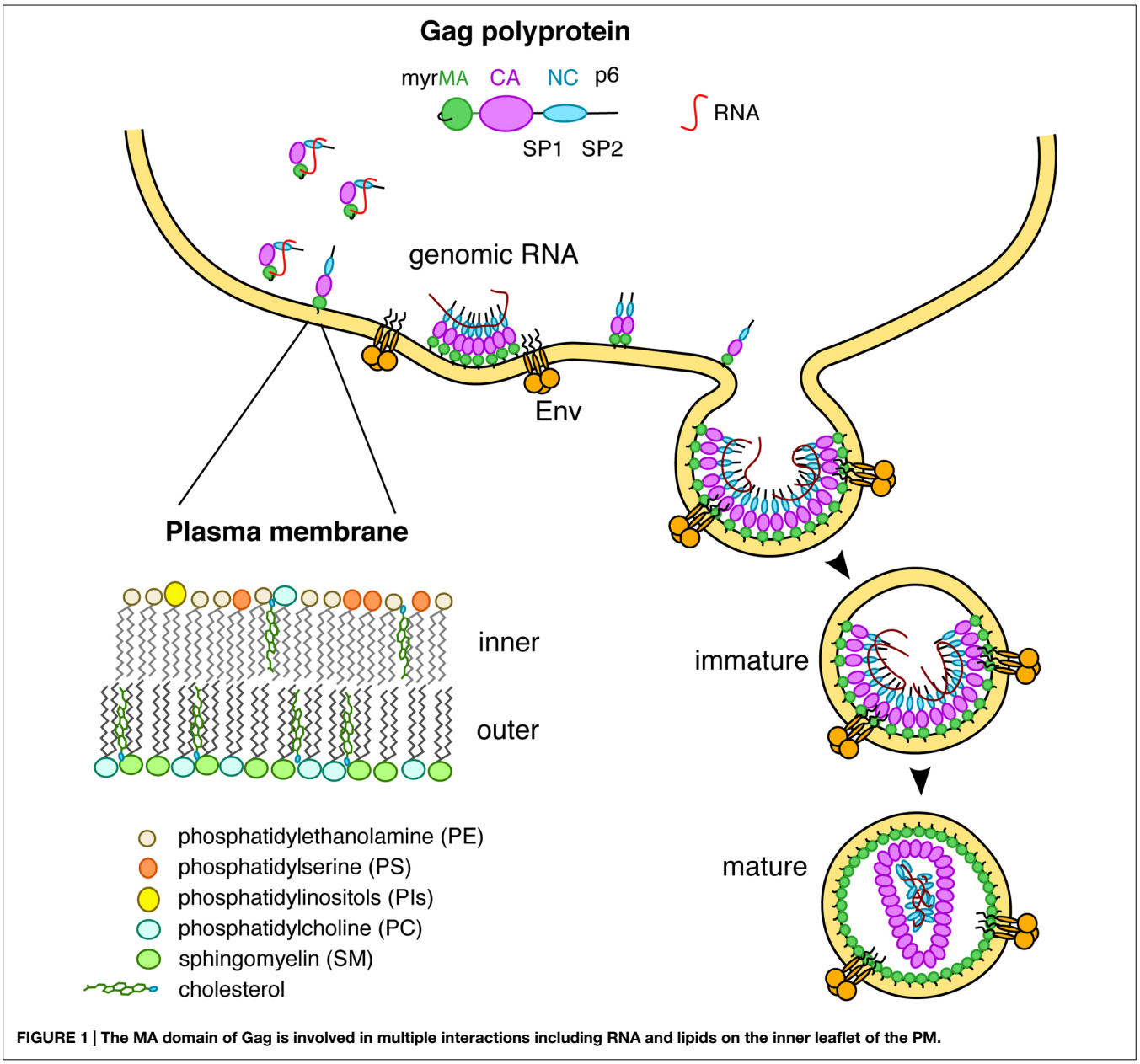

FIGURE 1 | The MA domain of Gag is involved in multiple interactions including RNA and lipids on the inner leaflet of the PM.

of Gag–PM interaction is incomplete because of the lack of molecular details on how various membrane components contribute to the overall binding and how they control this molecular switch.

Factors that Control HIV-1 Gag Assembly on the PM

For most retroviruses, assembly of the Gag proteins occurs on the PM of the infected cell (Ono et al., 2004; Jouvenet et al., 2006, 2008; Finzi et al., 2007; Welsch et al., 2007; Chukkapalli et al., 2008, 2010; Chu et al., 2010; Chukkapalli and Ono, 2011). The role of MA domain in Gag–PM binding is indispensable. Several factors can influence Gag–membrane binding including the myristoyl (myr) group, a conserved basic region in MA, protein multimerization, cellular RNA, and phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] (Ono and Freed, 1999; Ono et al., 2004; Jouvenet et al., 2006; Dalton et al., 2007; Chukkapalli et al., 2008, 2010, 2013; Alfadhli et al., 2011; Chukkapalli and Ono, 2011; Ghanam et al., 2012). The finding that Gag binds to membranes more efficiently than the isolated MA protein led to the hypothesis that the myr group is exposed in Gag and sequestered in the MA protein, which has become known as "the myr switch mechanism" (Zhou and Resh, 1996; Spearman et al., 1997; Hermida-Matsumoto and Resh, 1999; Ono and Freed, 1999; Paillart and Gottlinger, 1999). For over two decades, biochemical, *in vivo*, *in vitro*, and genetic data have provided invaluable insights on multiple factors that modulate Gag–membrane binding. However, only recently the molecular and structural determinants of this interaction have begun to emerge (Saad et al., 2006, 2007b, 2008; Shkriabai et al.,

2006; Chukkapalli et al., 2008, 2010, 2013; Chukkapalli and Ono, 2011; Prchal et al., 2012; Vlach and Saad, 2013). Nuclear magnetic resonance (NMR) and analytical ultracentrifugation studies revealed that the myr group can adopt sequestered and exposed conformations in the MA protein, that the MA protein resides in monomer-trimer equilibrium, and that myr exposure is coupled with protein trimerization (Tang et al., 2004; Saad et al., 2007b). Exposure of the myr group is also modulated by other factors including the solution pH, inclusion of the CA domain, and binding of calmodulin (Tang et al., 2004; Fledderman et al., 2010; Ghanam et al., 2010). There is now convincing evidence that binding of RNA to MA prevents Gag from interacting with intracellular membranes (Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011; Inlora et al., 2014). As a consequence, RNA is considered as a negative regulator of Gag–membrane binding. Recent studies have shown that the MA domain binds almost exclusively to specific tRNAs in the cytosol (Kutluay et al., 2014). Incorporation of $PI(4,5)P_2$ in membranes inhibits the interaction between MA and cellular RNA (Chukkapalli et al., 2010, 2013; Chukkapalli and Ono, 2011). Binding of Gag to membranes induces an extended conformation in the absence (Datta et al., 2011) or presence (Datta et al., 2007b) of inositol phosphates. Altogether, these studies indicate that particle assembly is regulated by coordinated interactions between the MA and NC domains of Gag with RNA and membrane lipids.

Structural Studies of HIV-1 MA Binding to PM Lipids

Proper targeting of HIV-1 Gag to the PM is dependent on specific interactions between the MA domain and $PI(4,5)P_2$ (Ono et al., 2004; Chukkapalli et al., 2008, 2010; Chukkapalli and Ono, 2011; Inlora et al., 2014). The most abundant form of $PI(4,5)P_2$ contains saturated 18-carbon 1′ and 20-carbon unsaturated 2′ fatty acid chains (Dudley and Spector, 1986), which promote micelle formation in aqueous solution (Janmey et al., 1987). Interactions of HIV-1 Gag and MA with $PI(4,5)P_2$ have been detected by mass spectrometric protein footprinting (Shkriabai et al., 2006). Titration of native $PI(4,5)P_2$ into MA samples led to severe broadening and loss of NMR signals. Therefore, soluble analogs of $PI(4,5)P_2$ with truncated 1′- and 2′-acyl chains (C_4 or C_8) have been used (Saad et al., 2006, 2007a, 2008). NMR studies have shown that soluble analogs of $PI(4,5)P_2$ bind directly to HIV-1 MA, inducing a conformational change that promotes myr exposure (Saad et al., 2006). The solution structure of the MA–$PI(4,5)P_2$ complex revealed that the 2′-acyl chain is inserted in a hydrophobic cleft, whereas the inositol group is packed against a highly basic region of MA (**Figure 2A**). The 1′-acyl chain, however, is not involved in binding and is exposed to solvent. It was suggested that $PI(4,5)P_2$ can function as both an allosteric trigger for myr exposure and as a direct membrane anchor (Saad et al., 2006). The involvement of the acyl chain of $PI(4,5)P_2$ in MA and Gag binding has been confirmed by surface plasmon resonance methods (Anraku et al., 2010). Based on the NMR studies, a structural model for Gag bound to PM has been proposed. In this model, MA is anchored to the membrane by the myr group and 1′-acyl chain of $PI(4,5)P_2$, which bracket a patch of conserved basic residues that can interact with the negatively charged surface of PM (Saad et al., 2006). A molecular model of MA bound to native $PI(4,5)P_2$ shows that a longer 2′-acyl chain (18 carbons) can be accommodated into the hydrophobic cleft (Saad et al., 2006). Structural studies have yet to determine the precise mode of MA binding to native $PI(4,5)P_2$ within the context of a membrane bilayer.

In addition to $PI(4,5)P_2$, other lipids have been implicated in HIV-1 Gag–membrane binding (Dalton et al., 2007; Ono, 2010a; Waheed and Freed, 2010; Chan et al., 2011; Chukkapalli and Ono, 2011; Chukkapalli et al., 2013). The affinity of Gag and MA to membranes is increased upon increasing the stoichiometry of phosphatidylserine (PS; Ehrlich et al., 1996; Zhou and Resh, 1996; Scarlata et al., 1998; Dalton et al., 2007; Chukkapalli et al., 2008, 2010, 2013; Alfadhli et al., 2009; Chan et al., 2011). NMR studies on HIV-1 MA binding to soluble analogs of PM lipids revealed that PS, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) bind directly to MA via a distinct site that is adjacent to the $PI(4,5)P_2$ binding site (Vlach and Saad, 2013). All three phospholipids interact with MA via sequestration of the 2′-acyl chain into a cavity formed by hydrophobic residues in helices α2 and α3, leaving the 1′-acyl exposed (**Figure 2B**). Surprisingly, the polar heads of PE, PS, or PC do not appear to play a major role in stabilizing the complex. Structural studies have been conducted with single lipids. It is likely that in a membrane environment the lipid polar heads will orient to make favorable electrostatic contacts with the basic domain of MA. Consistent with the hydrophobic nature of binding, the affinities of all three phospholipids to MA increased by two orders of magnitude upon extending the acyl chains from hexanoyl to octanoyl (Vlach and Saad, 2013). Intriguingly, the myr group is readily exposed when MA is bound to membrane mimetics such as micelles and bicelles in the absence of $PI(4,5)P_2$. Exposure of the myr group does not appear to be triggered by an allosteric mechanism as in the case of $PI(4,5)P_2$ binding, but rather by the mere presence of a lipid agglomerate. Based on these studies, we have proposed a trio engagement model by which HIV-1 Gag is anchored to the PM via the 1′-acyl chains of $PI(4,5)P_2$ and PS/PE/PC lipids, and the myr group, which collectively bracket a basic patch projecting toward the polar leaflet of the membrane (Vlach and Saad, 2013). Exposure of the myr group by a membrane-like environment, independent of $PI(4,5)P_2$ binding, was also observed in a reverse micellar system (Valentine et al., 2010). Spontaneous exposure of the myr group prior to association to membranes has also been observed by using coarse-grained simulation approaches (Charlier et al., 2014). The authors reported that insertion of the myr group into the bilayer is necessary for the orientation of MA to allow for the experimentally identified region of MA to interact with the $PI(4,5)P_2$ head group. Flipping of the lipid acyl chain out of the membrane environment to the hydrophobic groove in MA was, however, not observed. Further studies are needed to answer yet unresolved questions: What is the actual trigger and mechanism of myr exposure *in vivo*? Are acyl chains of either $PI(4,5)P_2$ or other phospholipids involved in MA binding to the PM? The Vogt lab has recently shown that Gag and MA strongly preferred lipids with both acyl chains unsaturated over those with one chain unsaturated (Dick et al., 2012). Furthermore, Gag can sense cholesterol in the membrane bilayer allowing for a more efficient

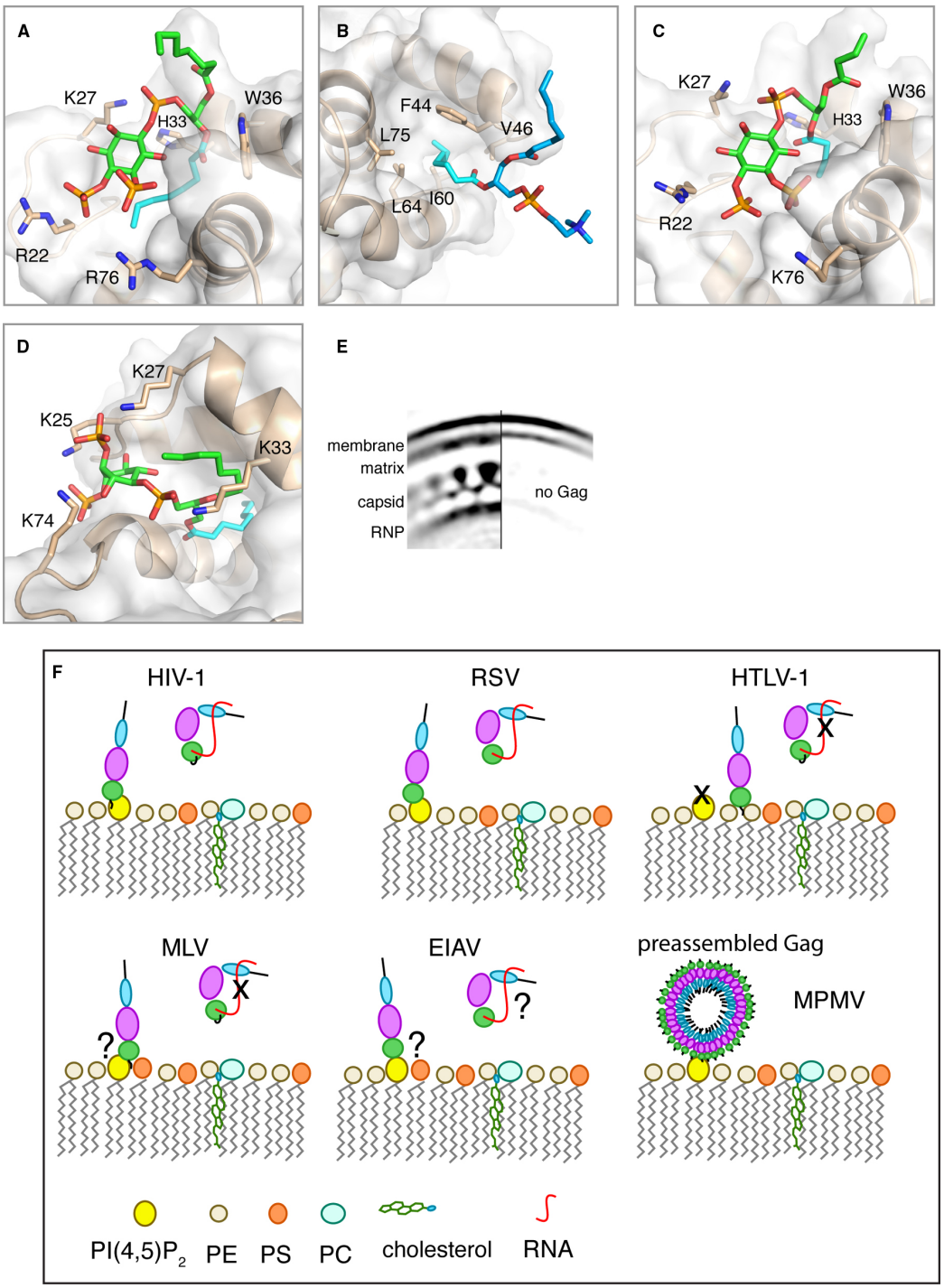

FIGURE 2 | (A–D) Close-up views of structures of HIV-1, HIV-2, and M-PMV MA proteins bound to lipids. MA proteins are shown in ribbon and surface representations with residues implicated in binding shown as sticks. Phospholipids are shown as green [$PI(4,5)P_2$] or blue (PC) sticks and their acyl chains involved in binding are shown in cyan. **(A)** HIV-1 myr(−)MA bound to di-C_8-$PI(4,5)P_2$ (PDB ID: 2H3V). **(B)** HIV-1 myr(−)MA bound to di-C_8-PC (PDB ID: 2LYA). **(C)** HIV-2 MA bound di-C_4-$PI(4,5)P_2$ (PDB ID: 2K4I). **(D)** M-PMV MA bound to di-C_8-$PI(4,5)P_2$ structure provided by Prchal et al. (2012). **(E)** Cryoelectron microscopy reconstruction of HIV-1 immature particle in a section with (left) or without (right) Gag polyprotein present. Used with permission [Copyright (2009) National Academy of Sciences, USA (Briggs et al., 2009)]. **(F)** A schematic representation of potential mechanisms for Gag binding to the PM for different retroviruses. Gag binding to membranes is regulated by $PI(4,5)P_2$ and RNA for some but not all retroviruses. Binding of Gag to membranes via the MA domain displaces RNA, which binds non-specifically to the basic region of MA. Data are not in agreement on the role of $PI(4,5)P_2$ in MLV Gag binding to the PM. For EIAV, Gag binding to membranes appears to have no specific requirement for $PI(4,5)P_2$ since other phosphoinositides may also play a role. For M-PMV, Gag assembly occurs in the cytoplasm prior to transport to the PM where MA specifically recognizes $PI(4,5)P_2$.

Gag binding to membranes (Dick et al., 2012). However, it is not yet known whether cholesterol can bind directly to Gag. Recent studies have also suggested that interactions can occur between the isolated NC and p6 domains of Gag with membrane models (Solbak et al., 2013; Kempf et al., 2015). However, the biological significance of these interactions has yet to be established. Further studies are needed to determine the synergy and interplay between PM components and their role in modulating the conformational switch of HIV-1 Gag during assembly (Ono, 2010b).

Other Retroviral Gag and MA Interactions with Lipids and Membranes

Like HIV-1, the site of HIV-2 assembly *in vivo* is also dependent on $PI(4,5)P_2$ (Saad et al., 2008). Structural studies show that the HIV-1 and HIV-2 MA structures are very similar and that $PI(4,5)P_2$ binds to both proteins in an identical manner (**Figures 2A,C**; Saad et al., 2008). However, in contrast to HIV-1 MA the position of the myr group in HIV-2 MA is less sensitive to factors that modulate myr exposure in HIV-1 MA such as protein concentration and binding of di-C_4-$PI(4,5)P_2$ (Saad et al., 2008). Data are not in agreement on the role of $PI(4,5)P_2$ in other retroviruses. Previous studies have shown that murine leukemia virus (MLV) Gag targeting to the PM is mediated by $PI(4,5)P_2$ and PS, suggesting a synergy between these two lipids to modulate Gag–PM binding (Hamard-Peron et al., 2010). The Ono laboratory, however, has recently shown that MLV Gag–PM binding is not dependent on $PI(4,5)P_2$ (Inlora et al., 2014). The Gag proteins of Rous sarcoma virus (RSV) and equine infectious anemia virus (EIAV) lack the myr group and it is thought that Gag–membrane interaction is driven mainly by electrostatic interactions (Erdie and Wills, 1990; Provitera et al., 2000; Dalton et al., 2005; Chen et al., 2008; Fernandes et al., 2011). Association of RSV MA and Gag with liposomes of defined composition is dependent on the presence of a biologically relevant concentration of negatively charged lipids like PS (Dalton et al., 2005). The Vogt laboratory has shown that RSV Gag has no specific requirement for $PI(4,5)P_2$ for PM association *in vivo* or liposome interaction *in vitro* (Chan et al., 2011). Parent and colleagues, however, have shown that depletion of intracellular $PI(4,5)P_2$ and phosphatidylinositol-(3,4,5)-trisphosphate [$PI(3,4,5)P_3$] levels impaired RSV Gag PM localization (Nadaraia-Hoke et al., 2013). It was suggested that differences are partially attributed to the sensitivity of assays utilized in these studies (Dick and Vogt, 2014). Most recent *in vitro* and *in vivo* studies from the Ono laboratory have shown that Gag–membrane binding via RSV MA is both $PI(4,5)P_2$ dependent and is susceptible to RNA-mediated inhibition (Inlora et al., 2014). No structural studies are currently available on how RSV MA interacts with PM lipids.

The Carter laboratory has reported that a soluble analog of $PI(4,5)P_2$ interacts directly with EIAV MA (Chen et al., 2008). More recently, it was shown that EIAV Gag is not only present on the PM but also in compartments enriched in phosphatidylinositol 3,5-biphosphate [$PI(3,5)P_2$] (Fernandes et al., 2011). In contrast to HIV-1, release of EIAV particles is not significantly diminished by co-expression with 5-phosphatase IV, which may suggest that $PI(4,5)P_2$ does not play a critical role in EIAV Gag assembly on the PM. Additional NMR studies have shown that phosphatidylinositol 3-phosphate [PI(3)P] binds to EIAV MA tighter than that of $PI(4,5)P_2$ (Fernandes et al., 2011). Altogether, it is likely that EIAV Gag membrane targeting proceeds via a mechanism that is different from that of HIV-1, HIV-2, MLV, or RSV.

The Gag precursor of deltaretrovirus human T-lymphotropic virus type 1 (HTLV-1), Pr53Gag, localizes at the cell surface and intracellular compartments in HeLa cells (Le Blanc et al., 1999, 2002; Heidecker et al., 2004). It has been shown that subcellular localization of HTLV-1 Gag and VLP release are minimally or not sensitive to depletion of $PI(4,5)P_2$ from the PM, suggesting that the interaction of HTLV-1 MA with $PI(4,5)P_2$ is not essential for HTLV-1 Gag–membrane binding and particle assembly (Inlora et al., 2011, 2014). Single amino acid substitutions that confer a large basic patch rendered HTLV-1 MA susceptible to the RNA-mediated block, a phenotype similar to that observed for HIV-1 and RSV. It remains unknown whether other PM lipids play important roles in HTLV-1 Gag assembly as further studies are needed to identify the precise molecular requirements for HTLV-1 Gag binding to membranes.

Assembly and particle production of Mason-Pfizer monkey virus (M-PMV) is perhaps the most distinct from all cases discussed above. M-PMV is thought to catalyze the membrane envelopment of a preassembled spherical capsid shell to release infectious virions (Sfakianos and Hunter, 2003). Assembly of M-PMV capsids occurs in a pericentriolar region of the cytoplasm prior to transport to the PM for budding. It is thought that both MA and CA domains of M-PMV Gag interact with the PM (Sfakianos and Hunter, 2003). Initial evidence for a potential role of $PI(4,5)P_2$ in M-PMV Gag–PM binding has been reported by Hunter and colleagues (Stansell et al., 2007). Depletion of $PI(4,5)P_2$ from the PM led to dramatic decrease of particle release from M-PMV infected cells. Direct interaction between M-PMV MA and a soluble analog of $PI(4,5)P_2$ has been detected (Prchal et al., 2012). The solution structure of M-PMV MA protein and its binding mode to di-C_8-$PI(4,5)P_2$ have been determined by NMR and molecular docking methods (for more details, see Prchal et al., 2012, 2014). The hallmark of the binding mode is the deep penetration of one of the C_8 chains into a hydrophobic pocket formed between helices α1, α2, and α4 (**Figure 2D**). The position of the myr group of M-PMV MA was not affected by di-C_8-$PI(4,5)P_2$ binding and remained sequestered in the protein core. Interestingly, no binding was detected between the unmyristoylated MA protein of M-PMV and soluble analogs of $PI(4,5)P_2$ (Prchal et al., 2012). The authors attributed this observation to the structural differences between MA and unmyristoylated MA, in which the hydrophobic cavity is absent in the latter. Altogether, the mechanisms of retroviral Gag assembly appear to be complex and require more detailed investigation at the molecular level (**Figure 2F**).

Gaps in Our Understanding of Gag Binding to the PM and Future Directions

Structural studies of HIV-1 MA interactions with $PI(4,5)P_2$, PS, PE, and PC have provided novel insights into the molecular mechanism of Gag assembly on the PM. These studies have utilized

lipids with truncated acyl chains. The precise mode of binding of native lipids with longer chains to MA has yet to be elucidated. The lack of an atomic snapshot for MA when bound to membranes containing physiologically relevant lipid composition remains a major gap in our understanding of virus assembly. Investigation of the molecular rearrangements of Gag in the immature and mature HIV particles have heavily relied on cryoelectron microscopy (cEM) data. Although details of the hexameric CA lattice exist, cEM studies have not provided a clear picture of the membrane-associated MA domain of Gag in either of the immature or mature particles (Fuller et al., 1997; Briggs et al., 2006, 2009; Wright et al., 2007). cEM data show that the membrane bilayer is thicker in regions where the Gag lattice is present and that MA density appears to "penetrate" the membrane bilayer (**Figure 2E**; Briggs et al., 2009). Earlier cEM tomography studies have suggested that the membrane-bound MA domain lacks periodicity (Wright et al., 2007). We believe that future studies may benefit from hybrid structural and biophysical methods to construct a model of Gag and/or MA bound to membranes. These may include small-angle x-ray scattering, atomic force microscopy and high-resolution cEM. Understanding the molecular basis of Gag assembly will not only shed light on the assembly of HIV-1 particles but will likely provide insight into the control of assembly in other retroviruses that assemble at the PM. Such studies are of great importance as these interactions may serve as pharmacological targets to inhibit HIV assembly. Structure-based studies have identified small molecule inhibitors that bind to the $PI(4,5)P_2$ site of HIV-1 MA and possess modest potency in reducing virus production (Zentner et al., 2013a,b; LaPlante et al., 2014). Although considered weak binders, these inhibitors may serve as a template for future studies aimed at the development of novel inhibitors of retroviral assembly.

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Membrane binding and bending in Ebola VP40 assembly and egress

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Lipid-enveloped viruses contain a lipid bilayer coat that protects their genome and helps to facilitate entry into the host cell. Filoviruses are lipid-enveloped viruses that have up to 90% clinical fatality and include Marbug (MARV) and Ebola (EBOV). These pleomorphic filamentous viruses enter the host cell through their membrane-embedded glycoprotein and then replicate using just seven genes encoded in their negative-sense RNA genome. EBOV budding occurs from the inner leaflet of the plasma membrane (PM) and is driven by the matrix protein VP40, which is the most abundantly expressed protein of the virus. VP40 expressed in mammalian cells alone can trigger budding of filamentous virus-like particles (VLPs) that are nearly indistinguishable from authentic EBOV. VP40, such as matrix proteins from other viruses, has been shown to bind anionic lipid membranes. However, how VP40 selectively interacts with the inner leaflet of the PM and assembles into a filamentous lipid enveloped particle is mostly unknown. This article describes what is known regarding VP40 membrane interactions and what answers will fill the gaps.

Keywords: Ebola, filovirus, membrane binding, plasma membrane, phosphatidylserine, phosphoinositides, VP40

INTRODUCTION

The discovery of filoviruses, Marburg virus (MARV) and Ebola virus (EBOV) in 1967 and 1976, respectively, spread fear of new pandemics that could spread globally and kill millions of people (Dowdle, 1976; Johnson et al., 1977). While outbreaks have been smaller for filoviruses, the fatality can be as high as 90% and there is some concern that a more significant pandemic could be looming. EBOV and MARV are also classified as category A pathogens by the NIH, a designation indicating they pose the highest risk to public safety and national security. In addition, filoviruses may be found outside of sub-Saharan Africa (Marsh et al., 2011; Negredo et al., 2011; Yuan et al., 2012), further underscoring the need for new treatment options. To date, therapeutics or vaccines have yet to be approved by the FDA for EBOV, but great strides have been made toward this goal (Geisbert et al., 2010; Blaney et al., 2013; Johansen et al., 2013; Pettitt et al., 2013; Warren et al., 2014).

Filoviruses are lipid-enveloped, filamentous in shape, and harbor a negative-sense RNA genome. The genome encodes seven proteins including nucleoprotein (NP), VP24, VP30, VP35, and L protein, which constitute the nucleocapsid (NC; Olejnik et al., 2011; Booth et al., 2013). The transmembrane glycoprotein (GP) is rooted in the lipid envelope of the virus and is responsible for entry of virions into the host cell (Lee et al., 2008; Cote et al., 2011; Olejnik et al., 2011). VP40 (viral protein 40 kDa) is the EBOV matrix protein, which regulates viral budding and NC recruitment as well as virus structure and stability. Sole expression of VP40 in mammalian cells is enough to assemble and form virus-like particles (VLPs) that are similar in size, shape, and nearly indistinguishable from the authentic virus (Jasenosky et al., 2001; Timmins et al., 2001, 2003; Noda et al., 2002, 2006; Licata et al., 2004). VP40 consists of 326 amino acids and has been shown to be a peripheral protein (Jasenosky et al., 2001), which localizes to the inner leaflet of the plasma membrane (PM) of human cells. Here, VP40 oligomers guide formation of new viral particles (Timmins et al., 2003; Adu-Gyamfi et al., 2012a, 2013; Bornholdt et al., 2013). Although the molecular basis of VP40 membrane binding is not well understood, mutations of VP40 that abrogate PM localization (McCarthy et al., 2007; Adu-Gyamfi et al., 2013; Bornholdt et al., 2013) or PM insertion (Adu-Gyamfi et al., 2013; Soni et al., 2013) inhibit viral budding.

PLASMA MEMBRANE LIPIDS

The PM is an asymmetric bilayer that is approximately 30 Å thick and contains ∼15% of its mass as transmembrane protein. A hallmark of PM asymmetry is an outer leaflet composed mainly of phosphatidylcholine (PC), sphingomyelin (SM), and glycosphingolipids and an inner leaflet enriched with phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphoinositides (PIPs; Schick et al., 1976; Higgins and Evans, 1978; Venien and Le Grimellec, 1988; van Meer et al., 2008; see **Figure 1**). Additionally, the PM is enriched in cholesterol and by some accounts may contain as much as 50% of cholesterol (van Meer et al., 2008) although the distribution of cholesterol among the outer and inner leaflets is not as well understood. The PM inner leaflet contains ∼20–30 mol% anionic lipid (McLaughlin et al., 2002; McLaughlin and Murray, 2005; Vance and Steenbergen, 2005; Vance and Tasseva, 2013), which can attract peripheral proteins with cationic patches and selective binding domains. The enrichment of PIPs, including $PI(4,5)P_2$ (McLaughlin et al., 2002; McLaughlin and Murray, 2005; Balla, 2013), PI(4)P (Hammond et al., 2012), and $PI(3,4,5)P_3$ (Heo

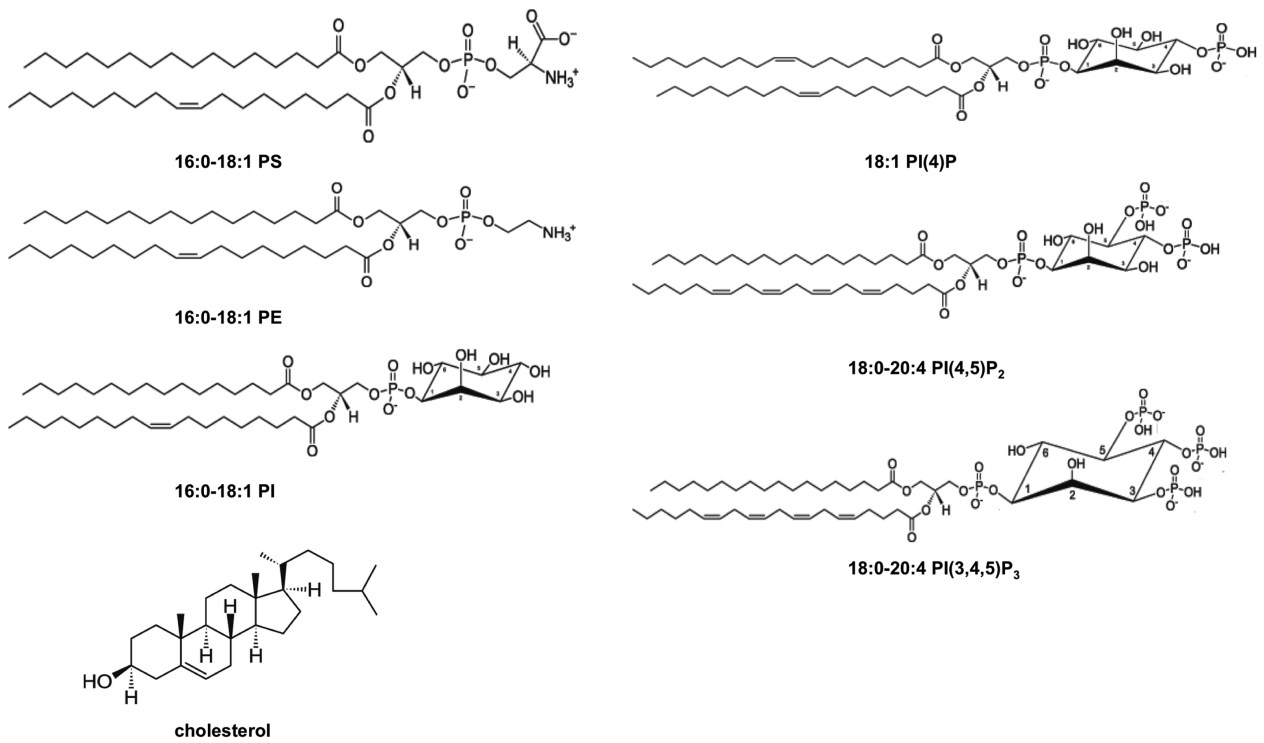

FIGURE 1 | Chemical structures of lipids found in the PM cytoplasmic leaflet. The inner leaflet of the PM has a relatively high concentration of PE and PS and it is known to be enriched in cholesterol and PI. Additionally, PIPs including PI(4)P, PI(4,5)P_2, and PI(3,4,5)P_3 are found in low abundance in the PM inner leaflet but have crucial roles in regulating peripheral protein localization and function at the membrane interface. Structures are shown at physiological pH.

et al., 2006) make up a small fraction of phospholipids on the inner leaflet but play an important role in recruiting high-affinity targets and mediating electrostatic interactions.

PS is the most abundant anionic lipid in the cytosolic leaflet at ∼15–20% (Vance and Steenbergen, 2005; Yeung et al., 2008; Vance and Tasseva, 2013) and contributes to the recruitment of polycationic proteins as well as proteins containing specific PS binding domains (Cho and Stahelin, 2005, 2006). PS has also been shown to interact with several viral matrix proteins (Zakowski et al., 1981; Dick et al., 2012) as well as VP40 (Ruigrok et al., 2000; Scianimanico et al., 2000; Adu-Gyamfi et al., 2013; Soni et al., 2013). The coexistence of PS and PIPs in the same membrane provides a mode of regulation for peripheral proteins that bind weakly to just one anionic lipid. Coincidence detection, which refers to recognition of two or more distinct membrane lipids (Balla, 2005; Lemmon, 2008; Moravcevic et al., 2012; Scott et al., 2012; Stahelin et al., 2014) has recently become a popular model of peripheral protein recruitment to biological membranes including that of HIV-1 Gag (Vlach and Saad, 2013). Additive and synergistic effects of either the anionic charge at the PM, the presence of two distinct binding sites (e.g., PI(4,5)P_2 and PS), or binding to a lipid in a region of positive curvature can mediate the selective PM localization of some peripheral proteins.

Membrane rafts are a common and controversial model of membrane-related research. Rafts are enriched in cholesterol and ordered membrane lipids with high-melting (T_m) temperatures (Komura and Andelman, 2013; Sonnino and Prinetti, 2013). Lipids are dynamic in bilayers and can move and rotate in the plane of the membrane, which can change with variation in lipid composition and temperature. Membrane phase behavior describes these lipid properties and is defined as having the state of liquid-ordered (L_o), liquid-disordered (L_d), or solid-gel (L_β). The rafts in membranes refer to the coexistence of the L_o and L_d phases. L_o phases are enriched with cholesterol and lipids with saturated acyl chains or a mixture of saturated and unsaturated acyl chains (Sonnino and Prinetti, 2013). L_d phases, on the other hand, harbor unsaturated acyl chains and lower concentrations of cholesterol. The unsaturated acyl chains are kinked and more spread out, leading to a more loosely packed bilayer. The loose packing is not as favorable to shield cholesterol when compared to that of an ordered and saturated membrane.

Several viruses including HIV (Ono and Freed, 2001; Brugger et al., 2006; Waheed and Freed, 2009) and EBOV (Panchal et al., 2003) have been suggested to bud from raft regions of the PM. This is because viruses have been shown to be enriched with cholesterol and sphingomyelin, be dependent upon membrane cholesterol levels for budding (Ono et al., 2007), and associate with the detergent-resistant fraction (Panchal et al., 2003) of cellular membranes commonly associated with rafts. The PM outer leaflet can harbor coexistence of L_o and L_d phases but as recently and elegantly discussed in the context of HIV-1 Gag (Dick et al.,

2012), it is important to note that the coexistence of $L_d + L_o$ phases has not been detected in bilayer models that more closely resemble the PM inner leaflet (Wang and Silvius, 2001). Admittedly, *in vitro* models are limited to more chemically simple lipid mixtures than biological membranes but there are still many questions in this field including whether viral proteins themselves can induce raft-like properties in the membrane.

VP40 INTERACTIONS WITH MEMBRANES

THE VP40 C-TERMINAL DOMAIN ASSOCIATES WITH PS-CONTAINING MEMBRANES

The first VP40 X-ray structure (Dessen et al., 2000) revealed a matrix protein with an N-terminal domain that is important for oligomerization (Timmins et al., 2003; Bornholdt et al., 2013) and a C-terminal domain now shown to be essential to membrane binding (Ruigrok et al., 2000; Scianimanico et al., 2000; Jasenosky et al., 2001; Adu-Gyamfi et al., 2013; Bornholdt et al., 2013; Soni et al., 2013). Ruigrok et al. (2000) first demonstrated that VP40 associated with lipid vesicles composed of 25% PC, 50% PS, and 25% cholesterol using a VP40 construct containing residues 31-326 (see also **Table 1**). They also demonstrated that the VP40 C-terminal domain was an important determinant of membrane binding as a truncated construct (residues 31-212) has greatly reduced binding to the same PS-containing vesicles. In these studies, 1 M NaCl was also able to reduce association with the vesicles containing 50% PS. Additionally, vesicles containing only 5% PS did not support significant VP40 binding. Taken together, C-terminal domain electrostatic interacts seem to be a key determinant of anionic membrane association.

Lipid vesicles containing PS have also been shown to induce a conformational change in VP40 that results in hexamerization (Scianimanico et al., 2000). This study used the same lipid composition noted above (25% PC, 50% PS, and 25% cholesterol) and two VP40 constructs (31-319 and 31-326). Cross-linking demonstrated dimer, trimer, tetramer, and hexamers formed for the VP40 31-319 construct. Cross-linking with PS liposomes exhibited no detectable monomer but rather extensive oligomerization including the large hexamer as the predominant band. It is also important to note that they found 1% β-octyl-glucoside was needed to avoid extensive VP40 aggregation in these assays. Hexamers were also found exclusively localized in filopodia-like projections emanating from the PM of human cells (Adu-Gyamfi et al., 2012a). In these studies, total internal reflection fluorescence (TIRF) microscopy was used to selectively excite fluorophores on or near the PM interface. VP40 assembly and oligomerization into hexamers and larger oligomers was clearly dependent on association with the PM interface.

VP40 C-TERMINAL DOMAIN HYDROPHOBIC INTERACTIONS ARE IMPORTANT FOR VLP FORMATION

In 2001, it was shown that VP40 alone formed VLPs from cells (Jasenosky et al., 2001). These studies investigated VP40 and respective truncation constructs' ability to associate with cellular membranes. Triton X-114 detergent extraction was done in order to separate the aqueous and detergent phase to investigate the mode of VP40 PM association. Integral membrane proteins and lipidated proteins are found in the detergent phase, while peripheral proteins reside in the aqueous phase of these extractions. VP40 was found to be a peripheral protein as it was almost exclusively extracted in the aqueous phase. Notably, these authors propose the important role of hydrophobic interactions of VP40 in membrane binding as they find 1 M NaCl did not release VP40 from the membrane bilayer. A hydropathy plot demonstrated that the majority of VP40 hydrophobic residues (Jasenosky et al., 2001) were in the last 50 residues of the C-terminal domain, which was further supported with a VP40 1-276 truncation. The low-to-moderate PM association of this truncation (VP40 1-276) was perturbed by an increase in the salt concentration. This is in contrast to the study discussed above that demonstrated VP40 association with membranes was driven by electrostatic interactions (Ruigrok et al., 2000). Note, however, that those studies were done with PS-containing liposomes and VP40 was not completely displaced from the vesicles. Additionally, the PM bilayer is much more complex than PC/PS/cholesterol liposomes and hydrophobic interactions may play a more prominent role in locking VP40 into the PM interface, where VP40 stays until the virions infect a new round of cells.

VP40 OLIGOMERS ASSOCIATE WITH PLASMA MEMBRANE RAFTS

Membrane rafts have also been implicated in VP40 assembly and oligomerization at the PM (Panchal et al., 2003). VP40 oligomers were found exclusively associated in the membrane fraction, while the soluble cytosolic fraction contained mostly monomeric VP40. The raft fractions, which are also referred to as detergent-resistant membranes (DRM), showed enrichment of VP40 oligomers. Several truncations of VP40 were prepared including a 9-residue C-terminal deletion (1-317) and an 18-residue C-terminal deletion (1-308). The authors proposed that the C-terminal 9 amino acids likely contribute to the membrane binding as this truncation had less PM localization than wild type (WT). The 18-residue C-terminal deletion had undetectable PM localization and formed globules in the cytoplasm, which may be a sign of octameric ring formation. Octamer formation has been shown to occur in some mutations of the C-terminal domain, resulting in perinuclear globules (Bornholdt et al., 2013). This 18-residue truncation additionally was shown to be oligomeric, associated with the detergent soluble fraction, and was unable to make VLPs (Panchal et al., 2003).

The authors identified proline 283 and proline 286 akin to an SH3 domain-binding site so they were mutated to leucine (Panchal et al., 2003). This resulted in oligomers in the cell lysate and the detergent soluble fraction but exclusion from DRM. The proline double mutant formed large aggregates in the cellular cytoplasm as well. Additionally, no VLPs were generated for P283L/P286L. Panchal et al. proposed that association with DRM is required for release of VP40 VLPs and that residues 309–317 as well as Pro^{283} and Pro^{286} are crucial for VP40 association with the PM microdomains. It still is unclear how rafts, which are relatively small in size compared to the EBOV virions, regulate budding of filamentous particles that are a micron or more in length. Moreover, the PM inner leaflet has not been shown to harbor a raft-like composition further confounding how VP40 may assemble

Table 1 | Summary of VP40 mutations and truncations that have been assessed in the context of membrane binding or plasma membrane association.

VP40 mutation or truncation	*In vitro* membrane-binding effects	Cellular 'effects	Reference
VP40 31-326	Associated with liposomes containing 50% PS and only weakly with liposomes containing 5% PS; 1 M NaCl significantly reduced association with liposomes containing 50% PS	Not measured	Ruigrok et al. (2000)
VP40 31-326	Liposome association induces hexamerization	Not measured	Scianimanico et al. (2000)
VP40 31-319	*In vitro* liposome binding was enhanced and induced hexamerization	Not measured	Scianimanico et al. (2000)
VP40 1-326	Not measured	A peripheral protein in the aqueous phase of a TX-114 extraction; 1 M NaCl did not perturb cellular membrane interactions indicating hydrophobic interactions are important; oligomers associated with membrane rafts	Jasenosky et al. (2001), Panchal et al. (2003)
VP40 1-276	Not measured	Greatly reduced association with cellular membranes and no VLPs; membrane association was sensitive to 1 M NaCl	Jasenosky et al. (2001)
VP40 1-318	Not measured	Less PM association and VLP formation	Panchal et al. (2003)
VP40 1-308	Not measured	Globular oligomeric cytosolic structures observed; no VLP formation	Panchal et al. (2003)
AAXY (P10A/P11A)	Not measured	Associates with membrane the same as WT but cannot form VLPs	Jasenosky et al. (2001)
W95A	Not measured	Reduces oligomerization and budding	Hoenen et al. (2010)
T112R	Abolishes dimerization, forms monomers and rings	Undetectable PM localization and budding	Bornholdt et al. (2013)
L117R	Abolishes dimerization, forms monomers and rings	Undetectable PM localization and budding	Bornholdt et al. (2013)
R134A	Abolishes RNA binding	Similar budding and PM localization; abolishes RNA binding	Bornholdt et al. (2013)
212-KLR-214 mutations	Not measured	Altered cellular localization and oligomerization; greatly reduced budding	McCarthy et al. (2007)
L213A	Reduced penetration into plasma membrane mimetic	Reduced PM localization and oligomerization	McCarthy et al. (2007), Adu-Gyamfi et al. (2013)
Δ221-229	Not measured	Abolished budding and PM localization	Bornholdt et al. (2013)
K224E/K225E	Not measured	Abolished budding and PM localization	Bornholdt et al. (2013)
K224M/K225M	Not measured	Abolished budding and PM localization	Bornholdt et al. (2013)
K224R/K225R	Not measured	Restored budding and PM localization	Bornholdt et al. (2013)
M241R	Twisted hexamer is formed	PM localization and membrane ruffling but no VLP formation	Bornholdt et al. (2013)
K274A/275A	Not measured	Reduced budding but retains PM localization	Bornholdt et al. (2013)
K274R/275R	Not measured	Restores budding	Bornholdt et al. (2013)
P283L/P286L	Not measured	Cytosolic oligomers in the detergent soluble fraction; no VLP formation	Panchal et al. (2003)

(Continued)

Table 1 | Continued

VP40 mutation or truncation	*In vitro* membrane-binding effects	Cellular 'effects	Reference
I293A	Reduced penetration into PM mimetic	Greatly reduced PM localization, oligomerization and budding	Adu-Gyamfi et al. (2013)
L295A	Reduced penetration into PM mimetic	Greatly reduced PM localization, oligomerization and budding	Adu-Gyamfi et al. (2013)
V298A	Reduced penetration into PM mimetic	Greatly reduced PM localization, oligomerization, and budding	Adu-Gyamfi et al. (2013)
A299W	Used to assess VP40 depth of membrane penetration (8.1 Å) and binds membranes similar to WT	Localizes and buds similar to WT	Soni et al. (2013)
I293A/A299W	Significantly reduced membrane penetration	Reduced PM localization and abolished budding	Soni et al. (2013)
L295A/A299W	Significantly reduced membrane penetration	Reduced PM localization and abolished budding	Soni et al. (2013)
I307R	Induces ring formation	No budding or PM localization. Globular perinuclear structures observed.	Bornholdt et al. (2013)

and bud. One interesting and somewhat related possibility is the location of PS on the PM inner leaflet. Recently, it was shown using the robust PS sensor lactadherin C2 that PS is clustered and associated with caveolae in the PM inner leaflet (Fairn et al., 2011), which may explain VP40 oligomers being localized to DRM. A careful examination of the EBOV lipidome would help resolve some of these questions.

VP40 CAN PENETRATE INTO THE PLASMA MEMBRANE HYDROCARBON CORE

To investigate if hydrophobic interactions are important for VP40 association with membranes *in vitro,* a lipid monolayer assay was employed (Adu-Gyamfi et al., 2013). Lipid monolayers are a useful platform for assessing the ability of peripheral proteins to penetrate into membranes of a lipid composition the experimentalist chooses (Cho et al., 2001). Biological membranes and lipid vesicles have surface pressures in the 30–35 mN/m range (Demel et al., 1975; Marsh, 1996), so proteins that are thought to penetrate under physiological conditions usually have a critical pressure (π_c) above 30 mN/m. The ability of VP40 to penetrate into monolayers that served as a mimetic of the cytoplasmic face of the PM (PC:PE:PS:PI:cholesterol (12:35:22:9:22)) or the nuclear envelope (PC:PE:PS:PE:cholesterol (61:21:4:7:7); Stahelin et al., 2003b) was assessed. In brief, VP40 was able to penetrate significantly into the PM mimetic ($\pi_c \sim 34$ mN/m) compared to that of the nuclear membrane mimetic ($\pi_c \sim 25$ mN/m; Adu-Gyamfi et al., 2013).

To investigate the molecular basis of VP40-mediated penetration, the authors used the rationale that the C-terminal domain undergoes hydrophobic interactions with the membrane bilayer within the last 50 amino acids (Jasenosky et al., 2001). Examining the structure of the VP40 C-terminal domain revealed a loop region rich in hydrophobic residues (Ile^{293}, Leu^{295}, and Val^{298}) conserved among the EBOV strains. A control mutation of Leu^{303} that was previously shown to interact with Sec24C (Yamayoshi et al., 2008) was also prepared. It was also noted that Leu^{213}, which was previously shown to be important for VP40 PM localization and oligomerization (McCarthy et al., 2007), was on the same interface as Ile^{293}, Leu^{295}, and Val^{298}. Thus, L213A was also prepared. L213A, I293A, L295A, and V298A all exhibited greatly reduced penetration into the PM mimetic indicating these residues are important for docking into lipid membranes. L303A, however, did not influence the ability of VP40 to penetrate into the PM mimetic. The effects of these hydrophobic mutations on VP40 PM localization, oligomerization in cells, and the ability to form VLPs were examined. In consonance with *in vitro* penetration data L213A, I293A, L295A, and V298A all had significantly reduced PM localization. This indicated that membrane penetration by this hydrophobic interface is important for the localization and latching on of VP40 to the PM bilayer. Single-molecule imaging using TIRF demonstrated that membrane penetration is an important step in VP40 oligomerization as these mutations abolished detectable hexamerization of VP40. Finally, these mutations also had greatly diminished VLP formation signifying the importance of this region in filamentous particle formation. The control mutation, L303A, behaved similar to WT VP40 in all cellular experiments. Importantly, hydrophobic mutations did not exhibit globular cytosolic structures, which are usually indicative of octameric ring formation (Bornholdt et al., 2013).

VP40 CAN DEEPLY PENETRATE THE PLASMA MEMBRANE AND INDUCE MEMBRANE CURVATURE CHANGES

To dig deeper into the mechanism of VP40-mediated membrane penetration, a Trp residue was introduced at Ala^{299} within the hydrophobic loop (Soni et al., 2013). Trp is advantageous as its ability to fluoresce can be used in combination with brominated lipids to determine the depth of penetration of peripheral

proteins. Brominated lipids are available with bromine on different positions of the acyl chain so their ability to quench Trp in the lipid-binding site can be compared using depth-dependent fluorescence quenching profiles (DFQP). Since the depth of bromine positions have been calibrated using X-ray diffraction (McIntosh and Holloway, 1987; Kleinschmidt and Tamm, 1999), the depth of Trp was determined using the distribution analysis (DA) method (Ladokhin, 1997, 1999). It is important to note that VP40 and A299W had similar affinity for PS liposomes, PM localization, oligomerization, and A299W retained the ability to form VLPs. Lipids with bromine on the 9th and 10th carbons were most efficient at quenching A299W indicating their proximity to the residues in this hydrophobic loop region. The DA calculation determined the mean depth of VP40 penetration to be 8.1 Å, which is more than halfway through one leaflet of the bilayer. *In vitro* and cell experiments demonstrated that introducing less hydrophobic mutations into the A299W construct (I293A/A299W and L295A/A299W) lead to poor penetration into the hydrocarbon core, reduced PM localization, and very low levels of VLP formation (Soni et al., 2013). These data indicated that loss of perhaps even one key hydrophobic contact would reduce viral egress. Electron microscopy (EM) and giant unilamellar vesicle (GUV) imaging demonstrated that VP40 induced negative membrane curvature changes in a PS-dependent manner. This type of curvature generation is consistent with VP40 pushing the inner leaflet of the PM out of the cell to form a VLP. As expected from the other assays, hydrophobic mutations reduced the ability of VP40 to induce membrane curvature changes. These studies suggested VP40 plays a major role in remodeling the PM shape to form the filamentous VLP.

VP40 MEMBRANE INTERACTION SUMMARY

These cellular and *in vitro* membrane-binding studies on VP40 have revealed some of the important processes that regulate VP40 PM localization and VLP formation. VP40 has both electrostatic and hydrophobic components to its membrane interaction that are important to VLP formation. Notably, the C-terminal domain of VP40 deeply penetrates the PM, which can induce negative curvature changes in synthetic membranes (Soni et al., 2013). Membrane association of VP40 also appears to be a prerequisite to inducing hexamerization (Scianimanico et al., 2000; Adu-Gyamfi et al., 2012a, 2013; Bornholdt et al., 2013). On the other hand, taken together these studies demonstrate there is still dearth of information on how VP40 associates with the PM inner leaflet. Remarkably, it appears VP40 lipid selectivity has not been systematically and carefully investigated. This is of utmost importance when the complex chemical composition of the PM inner leaflet is considered. Moreover, many peripheral proteins have been shown to undergo a complex set of interactions with membrane bilayers in order to achieve membrane docking and membrane curvature generation (Balla, 2005; Lemmon, 2008; Moravcevic et al., 2012; Scott et al., 2012; Mim and Unger, 2012; Stahelin et al., 2014). Thus, more detailed biochemical and biophysical investigation at the membrane interface is warranted to elucidate how VP40–lipid interactions mediate filamentous particle formation.

VP40 STRUCTURES REVEAL THE BASIS OF OLIGOMERIZATION AND MEMBRANE BINDING

Recently, Bornholdt et al. (2013) solved several EBOV VP40 high-resolution structures that demonstrated VP40 can transform into multiple arrangements. These distinct VP40 structures have separate but critical roles in the life cycle of the virus (Bornholdt et al., 2013). VP40 was revealed to be a dimer (see **Figure 2**) where each dimer serves as a building block that can further assemble into a flexible filamentous matrix protein structure (Bornholdt et al., 2013). VP40 dimerization is mediated by a hydrophobic N-terminal domain (NTD) interface, which when mutated, leads to abolishment of PM localization and VLP formation (Bornholdt et al., 2013). VP40 dimers, which are oblong, form linear filaments that have a hydrophobic C-terminal domain (CTD) interface (Bornholdt et al., 2013). These hexamers may further multimerize into a lattice or filamentous structure (Bornholdt et al., 2013) that regulates EBOV budding and virion shape. A third interface containing Trp^{95} was revealed following incubation with dextran sulfate, which was used as an anionic membrane mimetic (Bornholdt et al., 2013). This third interface is also important to assembly and budding as previous mutation of Trp^{95} diminished oligomerization and egress (Hoenen et al., 2010). VP40 dimers and hexamers may interact laterally through this third interface (Trp^{95}), which would be more accessible after membrane binding, to form a highly dense filament (Bornholdt et al., 2013). VP40 can also form an octameric ring, which binds RNA and functions in viral transcription inside the cell (Bornholdt et al., 2013). The octameric ring is not recruited into VLPs or infectious virions and if or how it interacts with cellular membranes is still unknown.

VP40 DIMERS INTERACT THROUGH A HYDROPHOBIC NTD INTERFACE

The interface in the NTD of VP40 buries ∼1500–1700 $Å^2$ in the dimer (Bornholdt et al., 2013). This involves α-helices containing residues 52–65 and 108–117 (Bornholdt et al., 2013). The NTD interactions are primarily hydrophobic and have few H-bonds. This interaction network specifically involves Ala^{55}, His^{61}, Phe^{108}, Thr^{112}, Ala^{113}, Met^{116}, and Leu^{117} (Bornholdt et al., 2013). Leu^{117} was found to be a key residue in the NTD interaction. This Leu extends into a hydrophobic pocket (Ala^{55}, His^{61}, Met^{116}, and Phe^{108}) to lock the dimer together (Bornholdt et al., 2013). This NTD interface is also much different than the interface that mediates the RNA-binding octameric ring (Gomis-Rüth et al., 2003). In order to form the octameric ring from the VP40 dimer, large conformational changes are necessary including movement of the C-terminal domain as well as extensive unraveling of the N-terminal 69-amino acids (Bornholdt et al., 2013). T112R and L117R abrogate the dimer formation and form monomers and octameric ring structures (Bornholdt et al., 2013). Neither mutant is released as VLPs and PM localization for the most part is undetectable (Bornholdt et al., 2013).

VP40 DIMERS FORM FILAMENTOUS HEXAMERS THROUGH A HYDROPHOBIC CTD INTERFACE

In all crystal structures of VP40 solved with the CTD visible, the CTD formed CTD-to-CTD interfaces that are hydrophobic and involved residues Leu^{203}, Ile^{237}, Met^{241}, Met^{305}, and Ile^{307}

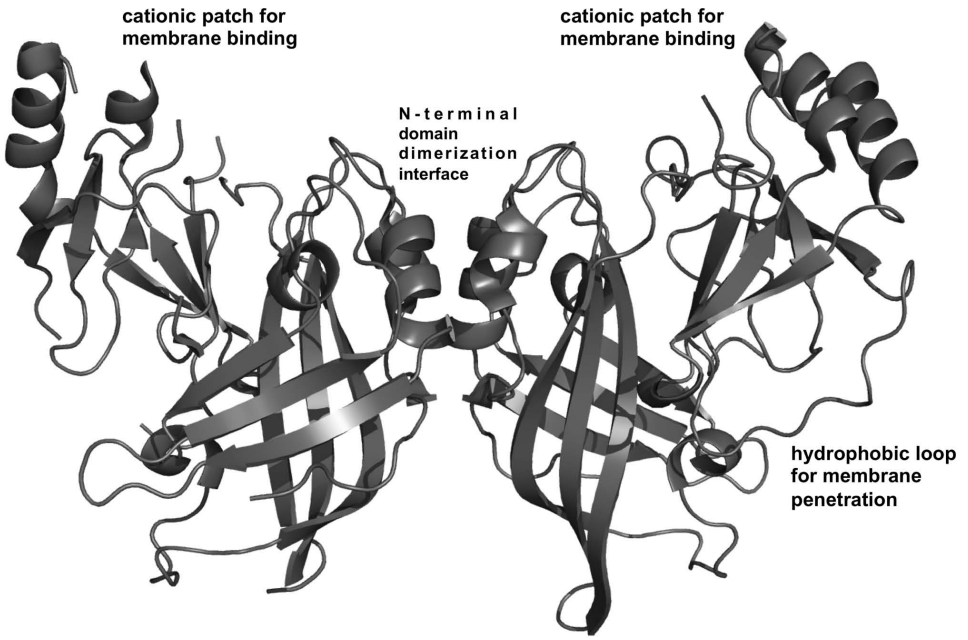

FIGURE 2 | VP40 structural analysis. VP40 is a dimer (Bornholdt et al., 2013) that is the building block for VP40 filament formation. Dimerization is mediated by a hydrophobic N-terminal domain interface (Bornholdt et al., 2013). The dimeric structure revealed a robust cationic patch exposed on one face of the dimer (Bornholdt et al., 2013) Mutation of Lys residues in this C-terminal domain cationic patch greatly reduced PM localization and budding in cells and is surmised to mediate membrane binding (Bornholdt et al., 2013). Adjacent to the cationic patch is a hydrophobic loop in the C-terminal domain that mediates membrane insertion of VP40, an important step for VP40 PM binding and membrane curvature generation (Adu-Gyamfi et al., 2013; Soni et al., 2013).

(Bornholdt et al., 2013). These interactions allowed VP40 dimers to form filamentous structures. The structure revealed this interface to have torsional motion, perhaps an important property in forming a flexible and filamentous structure from PM lipids (Bornholdt et al., 2013). Met^{241} was mutated as it resides at the center of the interface but is not essential to the fold of the CTD. M241R was generated, is dimeric in solution, and does not bud VLPs (Bornholdt et al., 2013). However, membrane ruffling was observed for M241R indicating the dimer is the building block to induce membrane curvature changes (Bornholdt et al., 2013). The M241R structure was solved to 4.15 Å resolution, which further demonstrated that this mutation did not disrupt the fold of the dimer but forced packing of dimers with a twisted interface relative to that of WT (Bornholdt et al., 2013). In the twisted M241R structure, the CTD interface harbored residues Leu^{203} and Ile^{307} at the center of the interface rather than Met^{241} (Bornholdt et al., 2013). M241R also prevented rotation about the CTD–CTD interface (Bornholdt et al., 2013) suggesting that a fairly precise hexamer with proper torsional motion is necessary to form filamentous particles via PM bending.

VP40 OCTAMERIC RINGS CAN BE INDUCED BY THE I307R MUTATION

I307R was made to investigate the CTD interface further but demonstrated extensive formation of octameric rings that are bound to nucleic acid (Bornholdt et al., 2013). In cells, I307R is not detectable at the PM but instead forms globular structures that are perinuclear (Bornholdt et al., 2013). Octameric ring formation thus blocked budding of VLPs (Bornholdt et al., 2013). Gln^{309} was mutated as a control for Ile^{307} and behaved similar to WT (Bornholdt et al., 2013) while R134A was prepared to knockout RNA binding (Gomis-Rüth et al., 2003). R134A behaved similar to WT in PM localization and budding and formed a dimer in solution (Bornholdt et al., 2013). The double mutant, R134A/I307R, was made to prevent CTD-mediated filament assembly where the R134A component would prevent RNA binding and thus octameric ring formation. As expected, R134A/I307R is a nucleic-acid-free dimer in solution and no longer forms globular structures that are perinuclear in cells (Bornholdt et al., 2013). Instead, this double mutant translocates to the PM but does not bud or form VLPs as it cannot form effective hexamers (Bornholdt et al., 2013). While PM localization is evident for R134A/I307R, membrane ruffling is not like that of the M241R mutation (Bornholdt et al., 2013). Overall, their modeling studies suggested that I307R prohibits CTD-to-CTD interactions while M241R permits them but with twisted and defective interactions (Bornholdt et al., 2013).

A CATIONIC C-TERMINAL DOMAIN INTERFACE IS REVEALED

These new VP40 structures also revealed a disordered loop in the C-terminal domain that contains several positively charged residues (Bornholdt et al., 2013). A cationic patch in the CTD is electrostatically important for binding the PM and forming VLPs (Bornholdt et al., 2013). The basic patch in the CTD is exposed and made up of six Lys residues (Lys^{221}, Lys^{224}, Lys^{225}, Lys^{270}, Lys^{274}, and Lys^{275}; Bornholdt et al., 2013) some of which are in

a loop region not previously observed in the original structure (Dessen et al., 2000). This patch is also conserved across the five species of EBOV. A 10-amino-acid loop deletion ($\Delta^{221-229}$) that included removal of Lys^{221}, Lys^{224}, and Lys^{225} lead to abrogation of budding but the dimeric structure was still maintained (Bornholdt et al., 2013). Mutations of Lys^{224} and Lys^{225} to Met or Glu abrogated PM localization and budding but when mutated to Arg retained WT function (Bornholdt et al., 2013). K274E/K275E exhibited little formation of VLPs but some translocation to the PM interface (Bornholdt et al., 2013). Restoration of positive charge to this region (K274R/K275R) was able to recover VLP formation at WT levels (Bornholdt et al., 2013). These C-terminal domain basic charges are necessary for budding and it is important to note that these cationic residues are not essential to the structure or any known protein–protein interfaces. It should also be noted that these loop regions harbor several Asn and Ser residues that could H-bond with PM lipid headgroups. The cationic residues are exposed on one side of the dimer and also adjacent to the hydrophobic residues previously shown to penetrate into the PM hydrocarbon core (**Figure 2**). Together they may form a robust interface for PM docking (Stahelin, 2014).

CONCLUSION AND FUTURE PERSPECTIVES

VP40 MEMBRANE BINDING MODEL

VP40 may be somewhat similar to the VSV matrix protein, which has been shown to engage in both electrostatic and hydrophobic interactions with lipid membranes (Ye et al., 1994; Gaudier et al., 2002). Taking into account the lipid-binding studies performed on VP40 and the new structural information that has become available, a rough membrane-binding model can be proposed (see **Figure 3**). In the first step of PM binding, the electrostatic patch in the C-terminal domain likely mediates the membrane association step with the highly anionic PM interface. The VP40 dimer would be advantageous in this regard as it would have enhanced avidity for the membrane compared to a VP40 monomer with only one cationic patch. Whether or not this patch directly coordinates specific lipid headgroups is unknown, but in the case of PS binding would induce VP40 hexamerization (Scianimanico et al., 2000). This structural rearrangement would allow the penetration and docking of the C-terminal hydrophobic loop into the PM, so VP40 could lock in the membrane for the ride out of the cell.

How does PM binding induce VP40 hydrophobic penetration? This is still unknown but some peripheral proteins have been shown to coordinate a lipid headgroup to decrease the desolvation penalty associated with hydrophobic membrane insertion (Stahelin et al., 2002, 2003a). The VP40 hexamer or longer filament that concatenates may then provide the energetic force necessary to induce negative membrane curvature generation from the PM interface. The studies by Bornholdt et al. (2013) provided a very attractive model for understanding this process as the M241R mutation that alters torsional motion of the VP40 hexamer demonstrates extensive membrane protrusions from the PM. However, this mutant lacked effective VLP formation suggesting an inability to undergo scission (Bornholdt et al., 2013). Thus, the VP40 hexamer may be the driving force for negative membrane curvature generation and formation of PM protrusions but without proper torsional motion across the CTD interface VP40 may not effectively further multimerize longitudinally or laterally to either drive effective VLP formation or alter interactions with the ESCRT complex, which may be needed for efficient VLP release. The elegant proposal by Bornholdt et al. (2013) which accounted for the precise location of VP40 between the viral lipid envelope and nucleocapsid, proposed that VP40 C-termini point out from both planes of a VP40 lattice engaging both the lipid envelope and viral RNA. This model may also help explain how VP40 achieves initial membrane curvature generation as some C-termini would be deeply engaged in the membrane hydrophobic core while the C-termini in the opposite plane would interact with RNA (Bornholdt et al., 2013). As VP40 interacts both laterally and longitudinally beneath the PM (Bornholdt et al., 2013), the dual engagement of membrane and RNA by two C-terminal interfaces may provide the driving force for PM protrusion and virus structure.

Protein–protein interactions may also play an important role in VP40 assembly and egress and cannot be discounted in these models. VP40 may associate with actin (Han and Harty, 2005), IQGAP1 (Lu et al., 2013), Sec24C (Yamayoshi et al., 2008), and microtubules (Ruthel et al., 2005). Additionally, actin has been shown to regulate ballistic motion of VP40, where inhibition of actin polymerization lead to constrained diffusion of VP40 molecules on the PM (Adu-Gyamfi et al., 2012b). The roles these interactions play are only beginning to be unraveled and a comprehensive view of VP40–protein and VP40–lipid interactions could help resolve the molecular details of EBOV assembly and budding.

IS VP40 AN ANIONIC CHARGE SENSOR OR DOES IT SPECIFICALLY BIND LIPID HEADGROUPS?

To date, the lipid selectivity and membrane affinity of VP40 is still unknown. Several studies, as outlined in this review, have shown that VP40 can associate with, penetrate, and hexamerize in response to interactions with PS-containing liposomes. However, many peripheral proteins have distinct lipid selectivity and binding sites for lipid headgroups including those of PS and PIPs (Cho and Stahelin, 2005; Lemmon, 2008; Huang et al., 2011; Lucas and Cho, 2011). Additionally, many lipid-binding proteins are coincidence detectors and interact with two distinct lipid headgroups or a lipid and membrane physical property such as charge or curvature (Balla, 2005; Lemmon, 2008; Mim and Unger, 2012; Moravcevic et al., 2012; Scott et al., 2012; Stahelin et al., 2014). More systematic analysis of VP40 lipid selectivity is needed to discover the optimal lipid composition that VP40 interacts with. This is especially important considering the complex chemistry of the PM lipid environment including important PM lipids, such as PS, PE, PI, cholesterol, and PIPs. Additionally, lipid acyl chain length and saturation can vary at the PM, which may also play an important role in VP40 PM detection and membrane curvature generation. As a VP40 budding particle is formed relatively flat membrane curvature is generated along the particle trajectory, while the neck region extending from the PM contains a region of negative curvature. Perhaps, the VP40 hexamers and filaments that form becomes activated in the sense they assemble on the more negatively curved membrane neck region. In other words, perhaps distinct VP40 structures have selectivity for the shape of

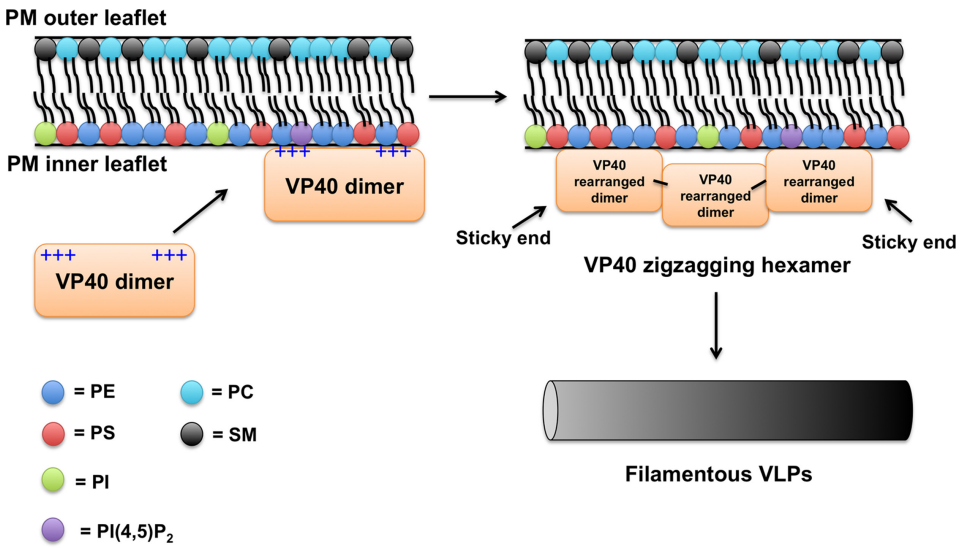

FIGURE 3 | Plasma membrane binding model for VP40. Considering previous studies and available data on VP40 structure and membrane-binding properties a simplified model of VP40 association with the PM is proposed. The VP40 dimer, which contains a large cationic solvent exposed patch in the C-terminal domain likely mediates the association of VP40 with the highly anionic interface of the PM. VP40 interactions with PS (Ruigrok et al., 2000; Scianimanico et al., 2000; Adu-Gyamfi et al., 2013; Soni et al., 2013) and perhaps other lipids such as PIPs induce a conformational change of VP40 rearranging the dimers into a zigzagging hexamer (Bornholdt et al., 2013). The VP40 dimers can interconnect through the CTD to form linear hexamers as well (Bornholdt et al., 2013). These hexamers have "sticky" ends that can further interact to form long VP40 filaments (Bornholdt et al., 2013). Lateral interactions between VP40 filaments are also possible and can occur between dimers and hexamers (Bornholdt et al., 2013). Note, in this model a VP40 monomer would not robustly interact with the PM (Bornholdt et al., 2013) presumably due to weak affinity or lack of transport to the PM. Hydrophobic interactions between the more liberated (Bornholdt et al., 2013) C-terminal domain hydrophobic loop (Jasenosky et al., 2001; Adu-Gyamfi et al., 2013; Soni et al., 2013) and the PM hydrophobic core act to increase the membrane residence time of VP40. As VP40 assembles on the inner leaflet of the PM, the C-terminal hydrophobic residues are able to penetrate ~8.1Å. VP40 oligomers are able to induce budding (negative membrane curvature generation; Soni et al., 2013) of a filamentous particle that eventually will undergo scission and be released.

the PM. In time these questions will likely be answered but will require robust biochemical and biophysical investigation of VP40 lipid-binding and -bending properties. These analyses should also include the lipid-binding determinants of VP40 zigzagging hexamer formation.

For the sake of comparison, it should be noted that the matrix domain of HIV-1, which has been known to associate with PM $PI(4,5)P_2$ (Ono et al., 2004) has more recently been shown to undergo a complex set of interactions with the PM that includes roles for lipid acyl chain saturation (Dick et al., 2012), lipid headgroup structure (Vlach and Saad, 2013), and protein oligomerization (Dick et al., 2013). Notably, PS/PE/PC have been shown to bind the HIV-1 matrix domain at a different site than that of $PI(4,5)P_2$ (Vlach and Saad, 2013). HIV-1 matrix has also been shown to be sensitive to the saturation on the acyl chains of PS and cholesterol content in lipid vesicles (Dick et al., 2012). Thus, viral matrix proteins likely use a complex set of chemical interactions with the membrane to regulate the spatial and temporal assembly of viral particles. This may also be important when considering formation of long and flexible EBOV filamentous particles.

CAN VP40 INDUCE FLIPPING OF PS?

A number of viruses including EBOV have now been shown to utilize PS exposed on the outer membrane envelope of the virus to facilitate entry into target cells (Soares et al., 2008; Jemielity et al., 2013; Morizono and Chen, 2014). PS interacts with the TIM-1 receptor on human cells and inhibition of PS exposure or PS availability to interact with the TIM-1 receptor, may be of great therapeutic value in a number of viral infections (Jemielity et al., 2013; Moller-Tank et al., 2013; Morizono and Chen, 2014). Strikingly, VP40 VLPs lacking the GP are able to enter target cells in a PS-dependent manner (Jemielity et al., 2013; Moller-Tank et al., 2013). This suggests that the information encoding PS exposure in VLPs may be encoded in VP40 alone. The PM asymmetry in eukaryotic cells is kept intact by ATP-dependent aminophospholipid flippases (Daleke, 2007; Baldridge and Graham, 2012), which keep PE and PS concentrated on the PM inner leaflet. Studies on erythrocytes have shown that >96% of PS is on the inner leaflet (Zachowski, 1993) meaning only 4% or less is exposed to the outer leaflet. While the percentage of PS exposure natively on most cell types is not known, it is not expected to be high. Is 4% PS on the outer filovirus envelope enough molecules of PS to facilitate entry? Perhaps, however, how, when, and how much PS is exposed on viral particles is unknown. This leads the author to speculate that PS may become more significantly exposed on VP40 VLPs or EBOV virions. Several possibilities exist: (i) Curvature generation in vesicular transport from the Golgi has been shown to expose (flip) PS in budding vesicles (Xu et al., 2013), which may also occur as the VP40 bud site is formed. (ii) VP40 VLPs may restrict or limit incorporation of flippases and along with lack of ATP in the VLPs leads to some loss of asymmetry. (iii) Calcium-dependent scramblases may become active in the plasma membrane (Zhou et al.,

1997) or in VLPs. Lipid scramblases are still a controversial subject but in some studies have been shown to randomize the lipid distribution across the PM. Given the important nature of PS in viral entry, these are some potential lines of investigation to explore. We still have a lot to learn regarding VP40-mediated assembly of the EBOV envelope but there are many exciting endeavors to pursue in the lipid field along the way.

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