Relationships between plasma membrane microdomains and HIV-1 assembly

Akira Ono[1]

Department of Microbiology and Immunology, University of Michigan Medical School, 5736 Medical Science Building II, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0620, U.S.A.

Advances in cell biology and biophysics revealed that cellular membranes consist of multiple microdomains with specific sets of components such as lipid rafts and TEMs (tetraspanin-enriched microdomains). An increasing number of enveloped viruses have been shown to utilize these microdomains during their assembly. Among them, association of HIV-1 (HIV type 1) and other retroviruses with lipid rafts and TEMs within the PM (plasma membrane) is well documented. In this review, I describe our current knowledge on interrelationships between PM microdomain organization and the HIV-1 particle assembly process. Microdomain association during virus particle assembly may also modulate subsequent virus spread. Potential roles played by microdomains will be discussed with regard to two post-assembly events, i.e., inhibition of virus release by a raft-associated protein BST-2/tetherin and cell-to-cell HIV-1 transmission at virological synapses.

Introduction

In most cell types, including natural hosts such as T cells, HIV-1 (HIV type 1) assembles at the PM (plasma membrane). HIV-1 particle formation is a multi-step process driven by the viral structural protein Gag (Adamson and Freed, 2007) (Figure 1). This process includes (i) targeting of Gag to the PM, (ii) Gag binding to membrane, (iii) Gag multimerization, (iv) encapsidation of viral genomic RNA, (v) incorporation of the viral Env glycoprotein and (vi) budding and releasing of virus particles. Although the order of some of these steps remains to be determined, regions in Gag involved in each step are well defined. Gag is a multidomain protein consisting of four major domains, MA (matrix), CA (capsid), NC (nucleocapsid) and p6, as well as spacer peptides, SP1 and SP2. MA mediates Gag target-ing and binding to the PM and Env incorporation, whereas the CA-CTD (C-terminal domain) and NC promote Gag multimerization. The CA-CTD contains a dimerization interface, while NC, an RNA-binding domain, is thought to promote higher-order multimerization as scaffolding through NC-RNA-NC binding. NC also contains zinc-finger motifs that mediate specific recognition of viral RNA for packaging of the genome. p6 recruits cellular ESCRT (endosomal sorting complex required for transport) complexes that facilitate fission of virions from the PM.

Advances in cell biology and biophysics have revealed that the PM is heterogeneous, consisting of multiple microdomains that contain specific sets of lipids and proteins. These microdomains may have various lifetimes, sizes and dynamics and may coalesce or dissociate from each other, thereby modulating cellular functions. Among them, lipid rafts and TEMs (tetraspanin-enriched microdomains) have been implicated in various aspects of the HIV-1 life cycle. In this review, I focus on interrelationships between these two specific types of microdomains and HIV-1 assembly. I also discuss potential roles of these microdomains in two post-assembly events currently under intense scrutiny, i.e., BST-2/tetherin-mediated virion
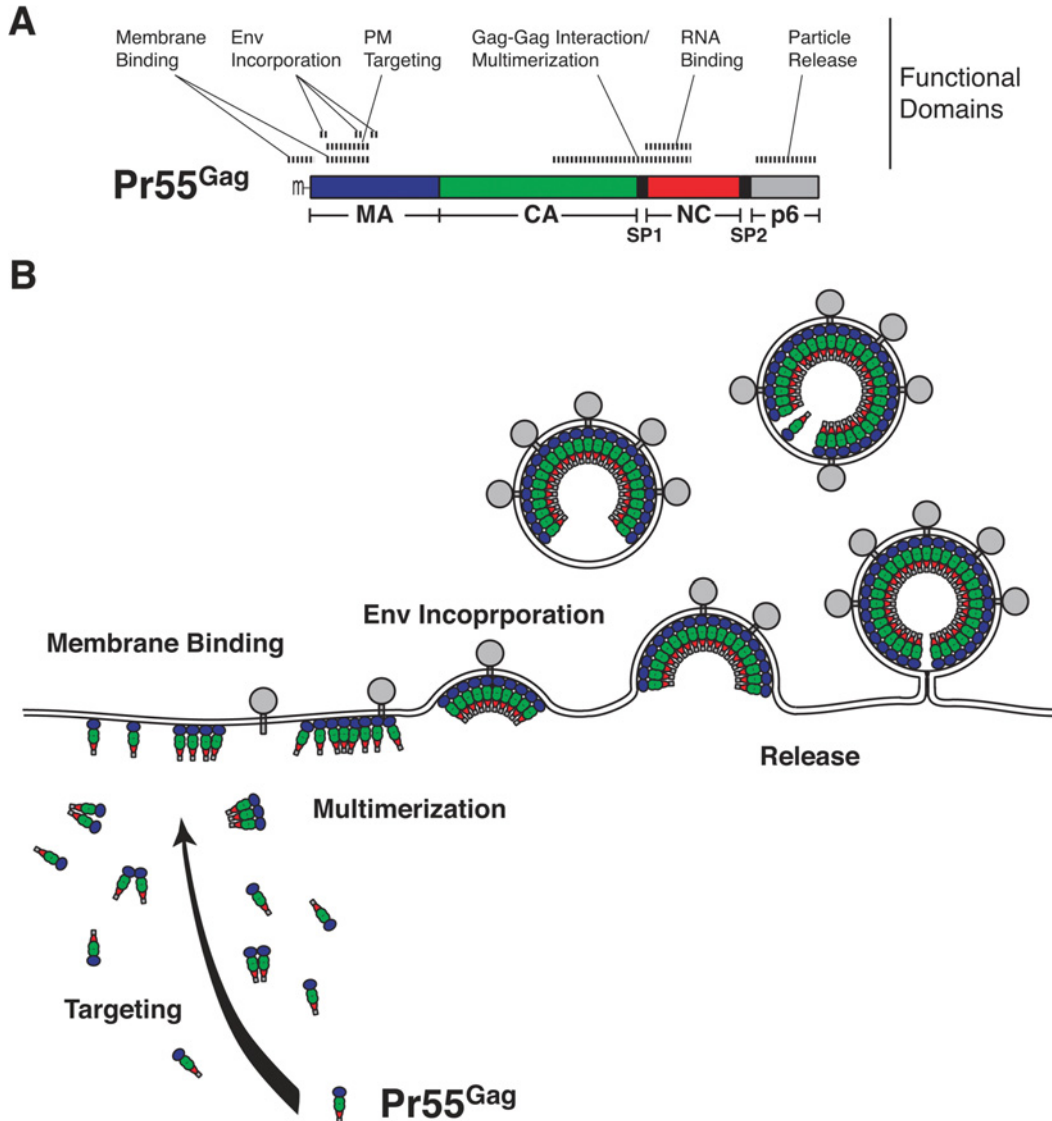
Figure 1 | HIV-1 Gag and virus particle assembly
(**A**) Structural and functional domains are shown. SP, spacer peptide. N-terminal myristylation is shown as (m–). (**B**) A general outline of virus assembly process is shown. For clarity, RNA molecules associated with NC are not depicted.

PM microdomains associated with the late phase of HIV-1 replication cycle
Lipid rafts
Lipid rafts, also known as membrane rafts, are microdomains enriched with cholesterol, glycosphingolipids and other saturated lipids, as well as specific release inhibition and cell-to-cell HIV-1 transmission at VSs (virological synapses).

types of proteins. In the original concept of lipid rafts, formation of these microdomains was thought to rely on the propensity of participating lipids to form a liquid-ordered state through lipid–lipid interactions (Simons and Ikonen, 1997; London and Brown, 2000). According to this model, this cholesterol-dependent liquid-ordered structure co-exists with liquid-disordered domains in cell membranes and membrane-associated proteins partition to either of these domains depending on their membrane binding

modes. Partitioning of molecules with lipid rafts in cells has primarily been assessed based on their association with DRM (detergent-resistant membrane) fractions (Brown and Rose, 1992). However, it is of note that DRM association reflects only a preference for rafts by proteins or lipids of interest and does not prove that raft association precedes experimental manipulation (Lichtenberg et al., 2005). Using this and other methods, proteins anchored to the extracellular leaflet of the PM by a GPI (glycosylphosphatidylinositol) moiety are identified as raft-associated proteins (Brown and London, 2000; Simons and Toomre, 2000). Cytoplasmic proteins modified with saturated acyl chains such as palmitoyl moieties constitute another representative class of raft proteins. Unsaturated lipid modifications such as prenylation are less favoured in rafts (Melkonian et al., 1999; Zacharias et al., 2002). Some transmembrane proteins such as TfR (transferrin receptor) and CD45 are also known to be excluded from rafts.

Lipid rafts are thought to serve as delivery or concentration platforms in various cellular functions, including signalling, protein sorting and cell polarity. However, the involvement of rafts in these cell functions, and even the very existence of rafts in intact cells, have been matters of debate because of their submicroscopic size and because of concerns over methods for studying rafts, in particular, the use of detergent-resistance-based isolation methods (Edidin, 2003; Munro, 2003; Hancock, 2006). Nonetheless, advanced biophysical and cell biological techniques have provided strong support to the presence of cholesterol-dependent microdomains in the PM (Kusumi et al., 2004; Mayor and Rao, 2004; Hancock, 2006; Jacobson et al., 2007; Day and Kenworthy, 2009). The definition of rafts put forth in the 2006 Keystone Symposium of Lipid Rafts and Cell Function summarizes the current consensus as following: "Lipid rafts are small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" (Pike, 2006).

As noted in this consensus definition of lipid rafts, in addition to lipid–lipid interactions, protein–protein and protein–lipid interactions have been gaining recognition as important factors in the dynamics of these microdomains (Douglass and Vale,

2005; Gaus et al., 2005; Larson et al., 2005; Hancock, 2006). In particular, oligomeric proteins that bind rafts (e.g. caveolins and flotillins) modulate stability, size and/or structure of membrane domains, creating specific subsets of microdomains (Langhorst et al., 2005; Morrow and Parton, 2005; Parton and Simons, 2007).

TEMs

TEMs are another type of membrane microdomains primarily organized by tetraspanins, a family of proteins with four transmembrane domains (Hemler, 2005; Levy and Shoham, 2005; Berditchevski and Odintsova, 2007; Charrin et al., 2009; Yanez-Mo et al., 2009). The importance of protein–protein interactions has been well recognized for formation of TEMs. These interactions include homo- and hetero-oligomerization of tetraspanins and interactions of tetraspanins with other proteins (e.g. integrins) (Hemler, 2005; Levy and Shoham, 2005; Berditchevski and Odintsova, 2007; Charrin et al., 2009; Yanez-Mo et al., 2009). Because of these interactions with other proteins, TEMs are involved in a wide variety of cellular functions, including intra- and intercellular signalling and cell–cell adhesion (Hemler, 2005; Levy and Shoham, 2005; Berditchevski and Odintsova, 2007; Charrin et al., 2009; Yanez-Mo et al., 2009).

TEMs are detected as microscopically visible patches using antibodies recognizing specific tetraspanins (Claas et al., 2001; Nydegger et al., 2006; Espenel et al., 2008). Live cell microscopy showed that these patches are stable in shape and localization (Espenel et al., 2008). In addition to these patches, single-molecule tracking of the tetraspanin CD9 demonstrated that there is another population of tetraspanins not associated with TEMs and that this population of molecules is in a dynamic exchange with those associated with TEMs (Espenel et al., 2008). Somewhat analogous to lipid rafts, at least a portion of the non-TEM-associated CD9 appears to form small, dynamic clusters (Espenel et al., 2008). Notably, formation of these clusters is dependent on PM cholesterol and CD9 palmitoylation (Espenel et al., 2008). Furthermore, under some experimental conditions, tetraspanins were observed to associate with DRM fractions (Claas et al., 2001; Charrin et al., 2002, 2003). However, lipid rafts and TEMs (or related clusters) are generally regarded

as distinct types of microdomains because detergent resistance and sensitivity to cholesterol depletion displayed by tetraspanins are qualitatively different from those of raft proteins (Claas et al., 2001; Charrin et al., 2002, 2009; Hemler, 2005; Le Naour et al., 2006; Yanez-Mo et al., 2009). As described later, this view is also supported by recent microscopy-based experiments that demonstrated clear segregation of tetraspanins from raft-associated proteins (Nydegger et al., 2006; Barreiro et al., 2008; Espenel et al., 2008).

Relationships between microdomain organization and HIV-1 assembly

Roles played by lipid rafts during HIV-1 assembly

During virus particle assembly, Gag and Env associate with DRM that presumably originates from rafts (Nguyen and Hildreth, 2000; Lindwasser and Resh, 2001, 2002; Ono and Freed, 2001; Ding et al., 2003; Halwani et al., 2003; Holm et al., 2003; Ono et al., 2005; Bhattacharya et al., 2006; Dou et al., 2009). Immunofluorescence microscopy studies showed that Gag co-localizes with lipid raft markers in cells (Nguyen and Hildreth, 2000; Holm et al., 2003; Ono and Freed, 2005). The lipid bilayer of the viral envelope is enriched in raft-associated lipids and proteins (Aloia et al., 1993; Saifuddin et al., 1995; Graham et al., 2003; Brugger et al., 2006; Chertova et al., 2006; Chan et al., 2008; Ott, 2008) and adopts cholesterol-dependent liquid-ordered structure (Lorizate et al., 2009). Cellular cholesterol depletion, which disrupts rafts, inhibits virus particle production (Ono and Freed, 2001; Pickl et al., 2001) by impairing Gag membrane binding, multimerization and/or mobility at the PM (Gomez and Hope, 2006; Ono et al., 2007). Substitution of myristate at the Gag N-terminus with an unsaturated analogue blocks Gag-DRM association and impairs virus particle production (Lindwasser and Resh, 2002). Owing to technical limitations inherent to the methodologies applied in these studies, some of these data can be explained without implicating rafts in HIV-1 assembly. Collectively, however, these reports are consistent with the notion that lipid rafts or cholesterol-dependent microdomains play an important role in HIV-1 particle assembly.

Not only other retroviruses [e.g. HTLV-1 (human T-cell lymphotropic virus 1) (Pickl et al., 2001; Feng et al., 2003) and murine leukaemia virus (Pickl et al., 2001; Nitta et al., 2010)], but also other families of enveloped and non-enveloped viruses, are thought to utilize lipid rafts during the late phase of their life cycles (Suomalainen, 2002; Briggs et al., 2003; Ono and Freed, 2005; Metzner et al., 2008). These viruses include paramyxoviruses (e.g. measles virus, Sendai virus and respiratory syncytial virus) (Sanderson et al., 1995; Ali and Nayak, 2000; Manie et al., 2000; Vincent et al., 2000; Henderson et al., 2002; McCurdy and Graham, 2003), orthomyxoviruses (e.g. influenza A virus) (Scheiffele et al., 1999; Ali et al., 2000; Barman and Nayak, 2000; Zhang et al., 2000; Leser and Lamb, 2005), filoviruses (e.g. Ebola virus) (Bavari et al., 2002; Panchal et al., 2003), herpes viruses (e.g. herpes simplex virus and pseudorabies virus) (Lee et al., 2003; Favoreel et al., 2004; Lyman et al., 2008) and reoviruses (e.g., Bluetongue virus) (Bhattacharya and Roy, 2008). In most cases, lipid rafts are postulated to serve as delivery vehicles or concentration platforms for viral structural elements, thereby facilitating assembly and release of infectious virions. However, the exact nature of association between lipid rafts and structural proteins of these viruses (including HIV-1), in particular, the impact of viral proteins on lipid raft organization, is less well understood.

MA as the interface between Gag and lipid rafts

In the case of HIV-1 Gag, both the N-terminal myristate and a highly basic region of MA, which are essential for binding of MA to the PM bilayer (Gottlinger et al., 1989; Bryant and Ratner, 1990; Zhou et al., 1994; Hill et al., 1996), are implicated in lipid raft association. The MA highly basic domain has been shown to interact with a PM-specific phospholipids, PtdIns(4,5)P_2 (Saad et al., 2006; Shkriabai et al., 2006; Chan et al., 2008; Chukkapalli et al., 2008, 2010; Alfadhli et al., 2009a, 2009b; reviewed in Ono, 2009). This interaction promotes Gag-membrane binding and Gag localization to the PM, thereby facilitating HIV-1 particle assembly and release (Ono et al., 2004; Chan et al., 2008; Chukkapalli et al., 2008). Gag proteins of other retroviruses, including murine leukaemia virus, Mason–Pfizer monkey virus and HIV-2, also require PtdIns(4,5)P_2 for efficient virus release (Stansell et al.,

Figure 2 | Association of Gag with lipid raft microdomains
NMR studies suggest that binding of PtdIns(4,5)P_2 to the MA highly basic region induces exposure of the N-terminal myristate moiety as well as sequestration of the highly unsaturated 2′-acyl chain of PtdIns(4,5)P_2 (**A**). Two exposed saturated acyl chains, i.e., the N-terminal myristate and the 1′ acyl chain of PtdIns(4,5)P_2, are postulated to promote partitioning of the Gag molecule to a raft domain, which may become stabilized or coalesce with other rafts upon Gag multimerization (**B**). Multiple saturated acyl chains associated with a Gag cluster may also induce formation of a stable raft-like domain (**C**). Possibilities shown in (**B**) and (**C**) are not mutually exclusive.
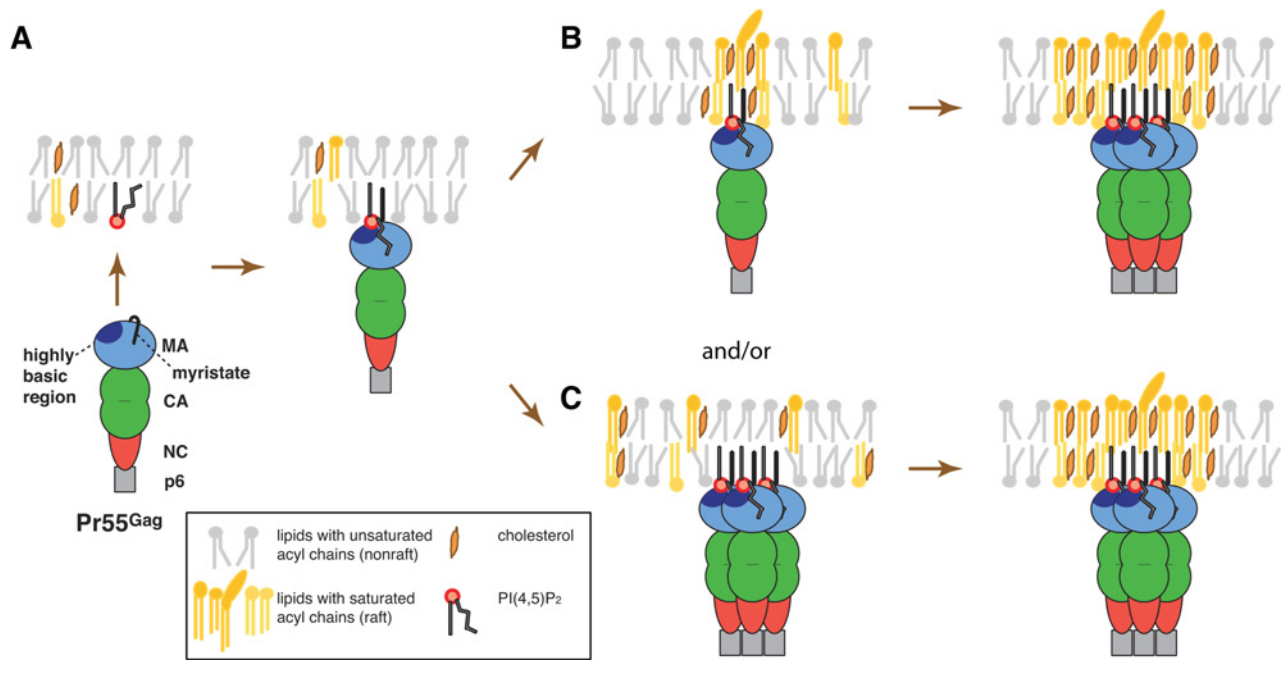
2007; Chan et al., 2008; Saad et al., 2008; Hamard-Peron et al., 2010). Notably, NMR studies showed that exposure of the myristate moiety, which is otherwise sequestered inside MA, is induced by binding of PtdIns(4,5)P_2 to the MA highly basic domain (Saad et al., 2006). The NMR studies further suggest that, when HIV-1 MA binds PtdIns(4,5)P_2, it sequesters the highly unsaturated 2′ acyl chain of PtdIns(4,5)P_2, leaving the saturated 1′ chain available for association with membrane (Saad et al., 2006) (Figure 2). This mode of MA-PtdIns(4,5)P_2 binding is predicted to promote Gag interaction with lipid rafts (Saad et al., 2006). In addition, as described earlier, substitution of myristate with unsaturated analogues blocks Gag association with DRM, suggesting that the N-terminal myristate moiety plays a key role in Gag association with lipid rafts (Lindwasser and Resh, 2002). Therefore, it is likely that MA does not simply function as a membrane-binding domain, but also serves as a specific interface between Gag and membrane microdomains.

Potential effects of HIV-1 assembly on the organization of lipid rafts

Besides MA, NC may affect Gag interaction with lipid rafts. Although NC-mediated higher-order multimerization is not essential for initial DRM association of Gag (Ono et al., 2005), NC increases density of the DRM with which Gag associates (Lindwasser and Resh, 2001). Therefore, as postulated previously (Lindwasser and Resh, 2001; Ono and Freed, 2001; Dalton et al., 2007), Gag membrane binding and multimerization may alter the size and/or structure of lipid rafts (Figure 2). In this regard, it is important to note that, except for caveolae, which constitute a unique subset of rafts organized by caveolin, lipid rafts are generally considered to be small, with an estimated diameter of 5–50 nm (Pralle

et al., 2000; Prior et al., 2003; Sharma et al., 2004; Eggeling et al., 2009). In contrast, the diameter of an HIV-1 particle is approx. 100–150 nm. Therefore, it is unlikely that a virus particle assembles within and buds from a single lipid raft. Rather, it is more likely that virus particle assembly involves recruitment and coalescence of small rafts into large stable rafts at assembly sites. When Gag forms multimers at the PM, saturated acyl chains associated with MA likely create a lipid environment suitable for recruiting raft-associated molecules in the PM cytoplasmic leaflet (Figure 2). Therefore, during virus assembly, Gag may induce formation of lipid-raft-like microdomains *de novo*. As mentioned earlier, oligomeric membrane-bound proteins such as flotillin can stabilize lipid rafts to form microscopically visible structures (Langhorst et al., 2005; Morrow and Parton, 2005; Parton and Simons, 2007). It is tempting to speculate that membrane-bound Gag multimers play an analogous role during association with lipid rafts.

Roles played by TEMs during HIV-1 assembly

TEMs have also been shown to co-localize with assembling Gag in T cells as well as other cell types (Mazurov et al., 2006; Nydegger et al., 2006; Jolly and Sattentau, 2007; Grigorov et al., 2009; Hogue et al., 2009). Biochemical studies showed that HIV-1 Gag can be co-immunoprecipitated with some tetraspanins from cell lysates (Grigorov et al., 2009). In some cases, somewhat larger membrane domains enriched with tetraspanins, termed ELD (endosome-like domain), are observed to be sites of virus assembly (Booth et al., 2006). Because of their similarity, these two domains will be hereafter referred to as TEM/ELDs in this review. In macrophages, HIV-1 Gag has been observed to assemble virus particles within apparently intracellular, tetraspanin-enriched compartments (Raposo et al., 2002; Pelchen-Matthews et al., 2003). At least subpopulations of these compartments were later shown to be convoluted invaginations of the PM (Deneka et al., 2007; Jouve et al., 2007; Welsch et al., 2007; Bennett et al., 2009). Therefore, these compartments may be derived from TEM/ELDs.

Like raft components, tetraspanins are incorporated into retrovirus particles (Nguyen et al., 2003; Pelchen-Matthews et al., 2003; Chertova et al., 2006; Jolly and Sattentau, 2007; Khurana et al., 2007; Medina et al., 2008; Sato et al., 2008).

Notably, these virion-associated tetraspanins impair virus infectivity by inhibiting Env-mediated fusion (Sato et al., 2008; Grigorov et al., 2009; Krementsov et al., 2009; Weng et al., 2009). Consistent with the effect of tetraspanins on virus–cell fusion, tetraspanins also inhibit cell–cell fusion mediated by cell-associated Env (Sato et al., 2008; Grigorov et al., 2009; Krementsov et al., 2009; Weng et al., 2009). It is of note that this inhibitory effect seems to require Gag-dependent clustering of Env and resulting localization of Env to TEMs (Weng et al., 2009). Therefore, HIV-1 assembly in TEM/ELDs likely prevents virus-expressing cells in contact with uninfected cells from fusing prematurely and forming syncytia (Krementsov et al., 2009; Weng et al., 2009). In contrast to the inhibitory effect on Env-mediated fusion, a consensus has not yet emerged on whether TEM/ELDs or particular tetraspanins actively regulate the HIV-1 particle assembly process. RNAi (RNA interference)-mediated knockdown of tetraspanins have produced contradictory data thus far (Chen et al., 2008; Ruiz-Mateos et al., 2008; Grigorov et al., 2009; Krementsov et al., 2009). Careful comparison of cell types, expression levels of each of the major tetraspanins in these cells, and timing after expression of Gag and siRNA (small interfering RNA) molecules are likely needed for determining the effect of TEM/ELDs on virus assembly and release.

Interestingly, it has been shown that association of proteins with TEM/ELDs requires membrane binding and higher-order multimerization of the proteins (Fang et al., 2007). Therefore, considering the dynamic nature of tetraspanin partitioning to membrane domains described above (Barreiro et al., 2008; Espenel et al., 2008), it is possible that, like lipid rafts, TEM/ELDs are also recruited to or stabilized at virus assembly sites due to Gag multimerization at the PM.

Relationships between lipid rafts and TEM/ELDs at HIV-1 assembly sites

As described earlier, biochemical data suggest that lipid rafts and TEM/ELDs are distinct microdomains. Microscopy studies that examined co-patching of raft proteins and tetraspanins also support this view (Nydegger et al., 2006; Espenel et al., 2008). Co-patching has been frequently used for studies of membrane microdomains that are submicroscopic in size

(Harder et al., 1998; Janes et al., 1999; Shvartsman et al., 2003; Gri et al., 2004; Meder et al., 2006; Lingwood et al., 2008). In a co-patching assay, membrane proteins are homotypically cross-linked with specific bivalent antibodies before fixation. This induces formation of microscopically visible patches. If two proteins share affinity to the same microdomain, and cross-linked proteins retain association with the microdomain stably enough, they co-localize in the same patch or co-patch. For example, a transmembrane raft marker, influenza HA, and another raft marker, PLAP (placental alkaline phosphatase; a GPI-anchored protein), co-localize at discrete patches when each are cross-linked by specific antibodies before fixation. In contrast, when stained after fixation, both show diffuse localization over the cell surface (Harder et al., 1998). Such co-patching is not observed between PLAP and a non-raft marker, TfR (Harder et al., 1998). Using this method, tetraspanins were observed to segregate from HA and CD55, a GPI-anchored raft protein (Nydegger et al., 2006; Espenel et al., 2008; I.B. Hogue and A. Ono, unpublished data). Consistent with co-patching experiments, live cell microscopy coupled with advanced techniques, including fluorescence recovery after photobleaching and single-molecule tracking, further showed that dynamic behaviours of tetraspanins and GPI-anchored proteins are clearly distinct (Barreiro et al., 2008; Espenel et al., 2008).

The findings described above indicate that, in normal cells, TEM/ELDs and lipid rafts are distinct microdomains. As described earlier, however, HIV-1 particle assembly associates with both lipid rafts and TEM/ELDs. This raises a question whether HIV-1 induces coalescence of these two types of microdomains at virus assembly sites. One study compared distributions of HA and tetraspanins in the presence of other influenza proteins and HIV-1 Gag (Khurana et al., 2007). In this study, Gag co-patched well with TEM/ELD markers but HA remained segregated (Khurana et al., 2007). However, because other influenza proteins such as M1 likely restricted free movement of HA or HA-containing lipid rafts, it remains unknown whether lipid rafts and TEM/ELDs can be co-recruited to the HIV-1 assembly sites or whether they form separate assembly sites. In this regard, it is notable that a recent report suggests that two viral glycoproteins (HIV-1 Env and Ebola GP) are incorporated into distinct populations of progeny virions even when both glycoproteins are expressed in the same cells (Leung et al., 2008). Incorporation of viral glycoproteins into retrovirus particles has been suggested to involve lipid rafts or other membrane microdomains (Pickl et al., 2001; Briggs et al., 2003; Bhattacharya et al., 2004, 2006; Metzner et al., 2008; Jorgenson et al., 2009; but see Yang et al., 2010). Therefore, the observation of bimodal incorporation of HIV-1 Env and Ebola GP was interpreted as indicating that a single Gag particle quantally associates with a microdomain containing single species of viral glycoprotein (Leung et al., 2008). Thus, it is possible that qualitatively different microdomains undergo homotypic but not heterotypic coalescence and independently constitute or become recruited to different assembly sites. Clearly, further investigation is needed for a better understanding of dynamic reorganization of PM microdomains during HIV-1 assembly.

Potential roles played by microdomains in steps following virus particle assembly
Involvement of PM microdomains in a cellular defence mechanism

PM microdomains may be involved not only in particle assembly and viral glycoprotein incorporation but also in a recently discovered cellular defence mechanism that inhibits a post-assembly process. The HIV-1 protein Vpu has been known to enhance virus release in a cell-type-specific manner (Klimkait et al., 1990; Varthakavi et al., 2003; Neil et al., 2006). In the absence of Vpu, virus particles are tethered to the surface of non-permissive virus-producing cells and are eventually endocytosed (Klimkait et al., 1990; Neil et al., 2006; Harila et al., 2007). These tethered virions can be released after treatment with a protease, subtilisin (Neil et al., 2006). Recent studies demonstrated that a cellular protein known as HM1.24, CD317, BST-2 or tetherin (hereafter referred to as BST-2/tetherin) is a subtilisin-sensitive, Vpu-responsive inhibitor of virion release (Neil et al., 2008; Van Damme et al., 2008). Expression of this protein, which can be augmented by IFN (interferon) (Neil et al., 2007), renders permissive cells non-permissive to HIV-1 lacking Vpu (Neil et al., 2008; Van Damme et al., 2008). Depletion of BST-2/tetherin rescues virion release from non-permissive cells in the absence of Vpu (Neil et al., 2008; Van Damme et al., 2008). Vpu antagonizes this protein

Figure 3 | Structure and function of BST-2/tetherin
(**A**) Key structural features and a possible mode of microdomain partitioning are shown. Cysteine residues that form disulfide bonds in BST-2/tetherin dimers are depicted. Glycosylation sites are not shown. (**B**) Proposed modes for BST-2/tetherin-mediated physical linkage between virions and the PM are illustrated.
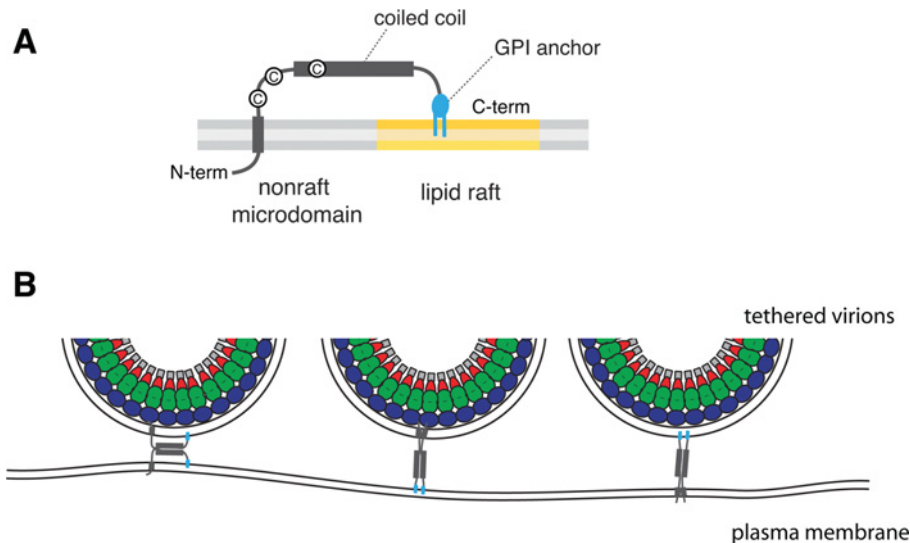
at least partly through down-regulation from the cell surface by ubiquitin-dependent mechanisms and/or through retention in the *trans*-Golgi network (Bartee et al., 2006; Van Damme et al., 2008; Douglas et al., 2009; Dube et al., 2009; Goffinet et al., 2009; Gupta et al., 2009; McNatt et al., 2009; Mitchell et al., 2009; Miyagi et al., 2009; Rong et al., 2009; Sato et al., 2009).

BST-2/tetherin is a type II transmembrane protein that consists of a short N-terminal cytoplasmic domain, a transmembrane domain, a dimerizing extracellular region containing a coiled-coil structure and a C-terminal GPI anchor (Kupzig et al., 2003) (Figure 3). Based on detergent resistance, this protein was identified as a raft-associated protein (Kupzig et al., 2003; Rollason et al., 2007). The GPI anchor, but not the transmembrane domain, mediates this raft association (Kupzig et al., 2003). Based on these findings, this protein was speculated to reside at the boundary between raft and non-raft regions (Kupzig et al., 2003). Because of its topology and dimerization ability, BST-2/tetherin is proposed to tether virus particles to the surface of producer cells by physically linking viral membrane and PM (Neil et al., 2008) (Figure 3), although an alternative or additional mechanisms may be possible

(Goffinet et al., 2009; Miyagi et al., 2009). Supporting the physical tethering model, BST-2/tetherin has been observed to co-localize with Gag puncta on the cell surface (Neil et al., 2008; Van Damme et al., 2008; Goffinet et al., 2009; Jouvenet et al., 2009a; Mitchell et al., 2009) and associate with budding virus particles (Perez-Caballero et al., 2009). Interestingly, BST-2/tetherin can inhibit the release of a wide variety of enveloped viruses including retroviruses, filoviruses and arenaviruses (Jouvenet et al., 2009a; Kaletsky et al., 2009; Sakuma et al., 2009) and inhibit HIV-1 release in cells derived from various species (Sato et al., 2009). Notably, a completely artificial protein designed to contain key structural features of BST-2/tetherin inhibits virus release like native BST-2/tetherin, despite the lack of sequence homology (Perez-Caballero et al., 2009). Therefore, if physical association between BST-2/tetherin and assembling virus particles is needed for its antiviral activity, such association is likely dependent on a common cellular structure rather than specific viral or cellular co-factor proteins. Together with the notion that many enveloped viruses associate with lipid rafts or other microdomains (Suomalainen, 2002; Briggs et al., 2003; Ono and Freed, 2005; Metzner et al., 2008), it was suggested that microdomain association of BST-2/

tetherin may promote incorporation of this protein into assembling particles (Jouvenet et al., 2009b). Consistent with a potential role for microdomains in BST-2/tetherin function, a cholesterol-binding compound amphotericin B methyl ester blocks the antagonistic activity of Vpu against BST-2/tetherin (Waheed et al., 2008). Furthermore, a BST-2/tetherin derivative lacking the GPI anchor, which is unable to associate with DRMs (Kupzig et al., 2003), fails to inhibit virus release despite proper transport to the PM (Neil et al., 2008; Perez-Caballero et al., 2009). However, further investigation is needed to determine whether association of BST-2/tetherin with microdomains is essential for inhibition of virus release by this protein.

Involvement of PM microdomains in cell-to-cell virus transmission

Another post-assembly process that may involve PM microdomains is cell-to-cell virus transmission. Virus transmission from infected cells to adjacent uninfected cells at cell–cell contacts has been shown to allow more efficient virus spread than infection by cell-free virions (Sato et al., 1992; Chen et al., 2007; Sourisseau et al., 2007; Sattentau, 2008; Sherer and Mothes, 2008). Recent microscopy-based studies revealed that retrovirus-producing cells form several different types of contact structures with target cells through which nascent virions can be transferred (Haller and Fackler, 2008; Sattentau, 2008; Sherer and Mothes, 2008). These structures include filopodial bridges, tunnelling or membrane nanotubes and VSs (Haller and Fackler, 2008; Sattentau, 2008; Sherer and Mothes, 2008). Attachment of tips of filopodia extended from one cell to another initiates filopodial bridges (Sherer et al., 2007), whereas nanotubes are likely formed after two conjugated cells detach from each other, at least in the case of T-cell nanotubes (Sowinski et al., 2008). Live cell imaging demonstrated that nascent virus particles move along the surface of these thin membranous structures from infected cells to uninfected cells (Sherer et al., 2007; Sowinski et al., 2008; Jin et al., 2009; Rudnicka et al., 2009). In contrast to filopodial bridges and nanotubes, the VS facilitates massive virus transfer at a short distance. The VS is a zone of contact formed between HIV- or HTLV-infected T cells and target T cells. This contact zone is enriched in viral Env and Gag proteins, cell adhesion and signalling mo-

Figure 4 | VS and potential involvement of uropods in its formation

(**A**) Key features of the VS formed between a virus-producing T cell and a target T cell are shown. Major components of the VS are listed. MTOC, microtubule-organizing centre. (**B**) Accumulation of assembling and assembled viruses to uropods enriched in cell adhesion molecules may facilitate formation of VSs upon contact of uropods with a new target cell. Alternatively, virus-laden platforms may be formed at uropods and laterally move over the PM to cell–cell contacts at other areas of virus-producing cells.
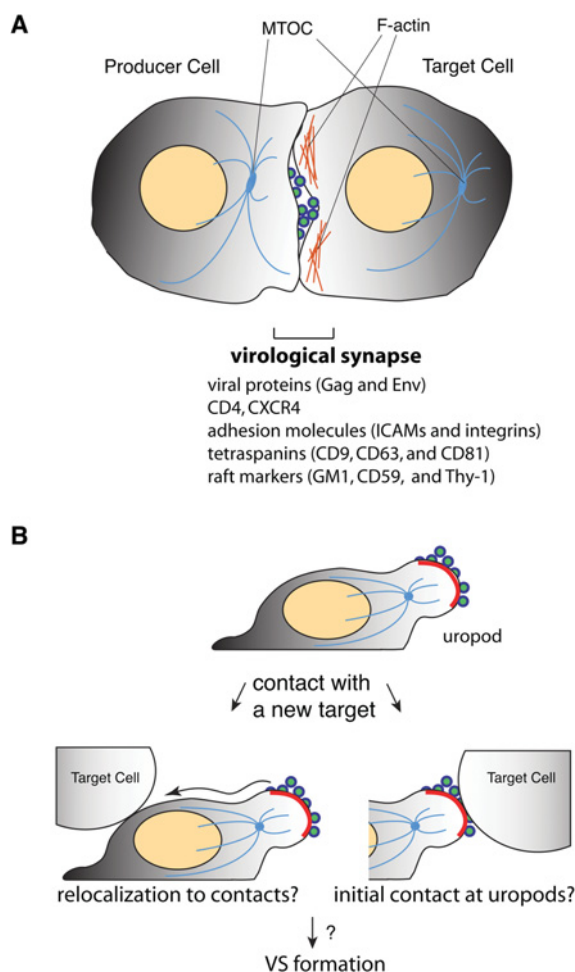
lecules and cytoskeletal proteins (Igakura et al., 2003; Jolly et al., 2004, 2007a, 2007b; Sol-Foulon et al., 2007; Arthos et al., 2008; Vasiliver-Shamis et al., 2009). A large number of assembled or assembling virus particles are observed in this junction (Figure 4) (Jolly et al., 2004; Hubner et al., 2009; Rudnicka et al., 2009). Notably, markers for lipid rafts and

TEM/ELDs are also enriched at the VS (Jolly and Sattentau, 2005; Jolly and Sattentau, 2007; Rudnicka et al., 2009). Furthermore, cholesterol depletion and treatment with anti-tetraspanin antibodies reduce formation of the VS (Jolly and Sattentau, 2005, 2007). However, it is currently unknown what roles lipid rafts and/or TEM/ELDs play in VS formation.

Although intact cytoskeletons (microtubules and actin filaments) (Jolly et al., 2007b; Sol-Foulon et al., 2007) and cell adhesion molecules (Jolly et al., 2007a; Arthos et al., 2008; but see also Puigdomenech et al., 2008) have been shown to facilitate VS formation, little is understood about cellular events leading to establishment of the VS. In particular, whether precursors of the VS exist or whether the VS is formed *de novo* upon cell–cell contact remain to be determined. In recent live cell microscopy experiments, pre-existing Gag-containing patches were observed to laterally move towards contact sites (Hubner et al., 2009; Rudnicka et al., 2009). Therefore, it is possible that Gag multimers and assembling particles first accumulate at a specific membrane region and form Gag-laden patches, which eventually constitute the VS upon cell–cell contact. In this regard, it is of note that, in T cells, viral proteins often accumulate at one cellular pole (Fais et al., 1995; Deschambeault et al., 1999; Nguyen and Hildreth, 2000; Chen et al., 2007; Fang et al., 2007; Grigorov et al., 2009). In some cases, Gag was observed to localize to a protrusion resembling a uropod (Nguyen and Hildreth, 2000; Chen et al., 2007), a rear-end structure formed in migrating T cells (Sanchez-Madrid and Serrador, 2009). Indeed, we have observed that Gag highly co-localizes with several uropod markers including PSGL-1 (P-selectin glycoprotein ligand 1) and CD43 in polarized primary CD4$^+$ T cells (G.N. Llewellyn and A. Ono, unpublished data). Such specific localization of Gag to uropods may be particularly relevant for cell-to-cell spread, because T cells are known to adopt a polarized morphology while migrating within lymphoid organs where cell-to-cell HIV-1 transmission likely occurs frequently (Germain et al., 2006; Krummel and Macara, 2006; Cahalan and Parker, 2008; Sanchez-Madrid and Serrador, 2009). The uropod is enriched in adhesion molecules and observed to mediate T cell–T cell contacts (Sanchez-Madrid and Serrador, 2009). Thus, polarized localization of viral components to a uropod may form a putative precursor for the VS. Notably, markers for lipid rafts and TEM/ELDs are known to accumulate to uropods in polarized T cells (Gomez-Mouton et al., 2001; Sala-Valdes et al., 2006). Lipid rafts containing flotillin are implicated in trafficking of PSGL-1 to uropods (Rossy et al., 2009). We observed that the tetraspanin CD81 co-patches substantially with Gag at uropods (G.N. Llewellyn and A. Ono, unpublished data). Notably, siRNA-mediated depletion of CD81, but not other tetraspanins, was observed to disperse polarized localization of Gag in T cells (Grigorov et al., 2009). Therefore, it is conceivable that PM microdomains associated with Gag multimers mediate polarized localization and assembly of Gag at a uropod, which eventually serves as a preformed platform for the VS. Filopodial contacts were found to induce polarized virus assembly in infected cells during cell-to-cell virus transmission between adherent cells (Jin et al., 2009). In the case of VS formation between migrating T cells, however, such polarity may already be established before cells initiate contact. In future studies on the cell-to-cell HIV-1 transmission, elucidating mechanisms promoting polarized localization of Gag and associated microdomains will likely be an important part of efforts towards a better understanding of this mode of virus spread.

Concluding remarks

Biochemical and microscopy-based studies showed that two types of PM microdomains, lipid rafts and TEMs, are associated with the process of HIV-1 particle assembly. This association may depend on intrinsic affinity of HIV-1 Gag for pre-existing microdomains, but it is also likely that Gag multimerization on the cytoplasmic leaflet creates a membrane environment suitable for recruiting microdomain components. Interactions of Gag with these microdomains likely facilitate particle assembly, Env incorporation into nascent virions and/or efficient virus spread through cell–cell contacts. On the other hand, these interactions may also set the stage for host cells to impose restrictions on virus particle release by BST-2/tetherin or restrictions on Env-mediated virus–cell fusion by tetraspanins. With advances in our understanding of dynamic aspects of PM organization, future studies will no doubt elucidate molecular mechanisms underlying relationships between microdomains and assembling particles.

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