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Virus Entry, Assembly, Budding, and Membrane Rafts

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INTRODUCTION

Specific microdomains of the plasma membrane called rafts appear to be involved in many biological events such as biosynthetic traffic, endocytic traffic, and the signal transduction pathway. Among pathogens, viruses, which are obligate intracellular parasites, are confronted with the plasma membrane during their life cycle. They have to enter their host cells by fusion, permeation, or endocytic vesicle discharge and to exit them by budding or membrane disruption.

In this review, we focus on data supporting the involvement of membrane rafts in the virus replication cycle, their role as a viral entry site, a platform for the assembly of viral components, and a scaffold for the budding of virus from infected cells. The elucidation of these interactions requires a detailed understanding of raft structures and dynamics.

DEFINITION OF RAFTS

Composition of Rafts

Although their existence has been debated, the presence of specific microdomains into the biological membranes is now a largely accepted concept (106). According to this concept, the microdomains have been named "rafts" because they can be seen as floating device within the "fluid mosaic" lipid sea of the Singer and Nicholson model (108). In model membranes made of a pure phospholipid-sphingolipid mixture, sphingolipids tend to pack together in microdomains separate from phospholipids because the former have long, largely saturated acyl chains. In the presence of cholesterol, the sphingolipids are organized in microdomains or rafts in the ordered rigid liquid crystal state (L_o), distinct from the disordered fluid liquid phase membranes (L_c) of the surrounding phospholipids (16, 62). The tight packing organization of lipid rafts confers their resistance to some detergents, such as the nonionic detergent Triton X-100 (TX-100) at low temperature, and allows their purification from low-density fractions after flotation in a sucrose gradient. In cell plasma membranes, a similar organization of lipids is likely to occur, and after solubilization in TX-100 at 4°C, membrane microdomains rich in cholesterol and sphingolipids can be similarly isolated by flotation. These microdomains were given various names such as detergent-resistant membrane (17, 38), detergent insoluble glycolipid-enriched complex (48), or Triton-insoluble floating fraction (48) and are now called membrane rafts. The detergent resistance of rafts is critically dependent on the presence of cholesterol.

In addition to biochemical fractionation, several lines of evidence support the in vivo existence of rafts (30, 38, 55). Their in vivo size has been estimated to be between 25 and 700 nm by using fluorescence resonance energy transfer microscopy and single-molecule-tracking microscopy (55, 94, 104, 116). Many, but not all, proteins anchored to the membrane by a lipid moiety associate with membrane rafts. They include the glycosylphosphatidylinositol (GPI)-anchored proteins, which are located on the extracellular leaflet, and palmitoylated or doubly acetylated proteins, which are enriched in the inner cytoplasmic leaflet. However, geranylated proteins are excluded from rafts (see references 48, 105, and 107 for reviews).

Several data point to the existence of different subsets of rafts depending on the combinatorial association of different sphingolipid species with cholesterol and protein contents (72, 104). One particular membrane raft subset is caveolae. Present in many mammalian cells except lymphocytes and neurons, caveolae are 50- to 70-nm plasma membrane invaginations

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which are surrounded by a striated coat made of the 22-kDa caveolins tightly bound to cholesterol (77). Likewise, some bona fide membrane rafts are soluble in 1% TX-100 yet insoluble in a lower concentration of TX-100 or in other nonionic detergents, e.g., Brij or Lubrol (98).

Several techniques to study and characterize membrane rafts have been described in the literature. The simplest one is to collect the pellet from a cell extract solubilized with 1% TX-100 at 4°C and centrifuged at 10,000 × *g*. This technique is not reliable because only the "heaviest" raft structures, which are contaminated with unsoluble material such as protein aggregates, are collected. The classical biochemical experiment involves flotation on a density (sucrose or iodixanol) gradient, with the cell extract being loaded at the bottom of the gradient. The quality of the separation should be checked using bona fide raft and nonraft markers. The alternative approach is to study the colocalization of a protein with a raft marker by microscopic examination (confocal microscopy, fluoresence resonance energy transfer microscopy, electron microscopy, etc.). However, in most cases, rafts are visualized only after clustering of one of the raft components. In any case, disruption of the membrane rafts by treatment of the cells with a cholesterol chelator (methyl-β-cyclodextrin) or a cholesterol-sequestring agent should correlate with the loss of the association of a protein with raft markers.

Functions of Rafts

Membrane rafts are small, mobile, and unstable. They fluctuate in size and composition (55). Their lateral and rotational mobility allows them to act as mobile platforms that carry specific proteins from the *trans*-Golgi network (TGN) to the cell surface, along the plane of the plasma membrane, and from the plasma membrane to the internal membrane (81). Biosynthesis of glycosphingolipid occurs in the Golgi from the glucosylceramide precursor, which is synthesized in the endoplasmic reticulum (ER), and raft microdomains assemble in the TGN. Cholesterol depletion or inhibition of glycosphingolipid synthesis blocks the formation of secretory vesicles from the TGN. and vesicle biogenesis proceeds through the formation of membrane buds which lack necks to undergo fission (53). Rafts, which are abundant at the plasma membrane, act as docking sites for specific proteins involved in many important cellular processes, including (i) polarized sorting along the biosynthetic pathway, (ii) signal transduction, (iii) endocytosis (6, 107), and (iv) receptor for pathogens (see reference 105 for a review). Thus, it comes as no surprise that rafts could potentate steps of the viral life cycle such as virus entry, assembly, and budding (115).

RAFTS AS PLATFORMS FOR VIRUS ENTRY

Virus entry into a host cell involves the specific interaction of virus with receptor molecules which are expressed at the plasma membrane. After attachment to the cellular receptor(s), virus penetration, an energy-dependent process, occurs quickly. Viruses can enter cells by fusion at the plasma membrane or in endocytic vesicles, rupture of the cargo endocytic vesicles, or, more rarely, translocation of viral particles directly into the cytoplasm. Some viruses may enter the host cell in more than one way. Increasing evidence indicates that viruses, including nonenveloped viruses, can use specific membranes microdomains to penetrate the host cell.

Nonenveloped Viruses

After attachment to cell surface receptors, the bound capsids of nonenveloped viruses are internalized mostly if not exclusively by invagination of the plasma membrane and intracytoplasmic vesiculation. The involvement of membrane rafts in mediating this process has been highlighted for several viruses. The most convincing evidence has been provided for simian virus 40 (SV40), a member of the *Papovaviridae,* and echovirus type 1 (EV1), a member of the *Picornaviridae,* which use caveolae for internalization and transport to the perinuclear region.

SV40 initiates infection by binding to the major histocompatibility complex class I molecules (7, 111) which are not targeted to membrane rafts (22). In contrast to viruses that enter cells by typical endocytosis, SV40 is generally considered to penetrate host cells mostly by atypical endocytosis. Biochemical analysis, colocalization studies, and blocking of the virus infection by a dominant negative caveolin showed that this process is mediated by caveolae. Caveolae transport SV40 particles to the ER, where the virus is disassembled (84). It is proposed that SV40 initially binds to major histocompatibility complex class I molecules localized in detergent-soluble membrane and induces a signal which promotes its association with a caveola-containing detergent-insoluble membrane microdomain followed by virus endocytosis (7, 24, 84, 87, 111).

EV1 is internalized into caveolae. It uses $\alpha_2\beta_1$-integrin as cellular receptor. The subsequent entry results in conformational change of the viral capsids, which can be detected after sedimentation through a sucrose gradient. A follow-up study of the internalization process showed that EV1, $\alpha_2\beta_1$-integrin, and caveolin-1 were internalized together in vesicular structures and accumulated in a perinuclear compartment. Purified caveolae contained infectious virus. Depletion of cholesterol by incubating cells with the cholesterol-trapping agent methyl-β-cyclodextrin, or the expression of a dominant negative caveolin markedly inhibit EV1 infection (75).

The use of decay-accelerating factor (DAF or CD55), a GPI-anchored membrane glycoprotein, as receptor by many enteroviruses including enterovirus 70 (57), hemagglutinating echoviruses (11), coxsackievirus B viruses, and coxsackievirus A21 (CAV-21) (82) argues for a possible role of membrane rafts during virus entry. The comparison of DAF-using and non-DAF-using strains of EV11 revealed that DAF-mediated entry is dependent on cholesterol at a postbinding step which precedes the RNA uncoating. The infectivity is blocked by nystatin, a cholesterol-sequestering drug. EV11 copurified with rafts isolated after TX-100 solubilization and sucrose gradient flotation (112).

The receptor of group A rotaviruses (belonging to the *Reoviridae* family) is a complex of several cell components including gangliosides, Hsc70 protein, and $\alpha_2\beta_1$- and $\alpha_V\beta_3$-integrins. All these components might be associated within lipid rafts favoring binding and internalization of rotavirus particles (44, 45).

Enveloped Viruses

The entry of enveloped virus involves virus attachment followed by close apposition of the viral and plasma membranes. Then the two membranes fuse by an energetically unfavorable process involving the destabilization of membrane microenvironment and the formation of a fusion pore to promote the penetration of the viral nucleocapsid. The fusion process requires a major conformational change of the viral fusion protein to expose a hydrophobic fusion peptide. This change can be induced by virus envelope glycoproteins binding to cellular receptors at neutral pH, and it occurs at the plasma membrane. Alternatively, the conformational change of the fusion protein is induced by an acidic pH. In this case, virus particles undergo endocytosis prior to the fusion. Influenza virus is the prototype of viruses which rely on endocytosis and a pH-dependent fusion mechanism to enter the cytoplasm.

Four sets of experimental data argue for a role of membrane rafts in the entry of enveloped virus: (i) anchoring of envelope glycoproteins in rafts, (ii) interaction of the virus envelope glycoprotein with a lipid component of cell membrane rafts, (iii) anchoring of the cellular receptor in rafts, and (iv) inhibition of virus entry after cholesterol depletion and/or sequestration.

The glycoproteins of several viruses, including influenza virus, human immunodeficiency virus (HIV), murine leukemia virus (MLV), measles virus, and Ebola virus, are associated with membrane rafts (10, 74, 91, 103, 119). Maturation of gp120-gp41 palmitoylation sites inhibits viral infectivity, but it is not known whether this is due to a direct effect on virus entry or to reduction in the amount of Env protein incorporated into the viral membrane (99).

The low-pH-dependent penetration of the alphavirus Semliki Forest virus (SFV) and Sindbis virus (SIN), both of the family *Togaviridae*, requires cholesterol in the target membrane (15, 70, 109). Both viruses are strongly dependent on a cholesterol-sphingolipid environment so that they can infect cells via a low-pH-triggered fusion reaction (1, 59, 70, 90, 117, 124). The interaction with cholesterol has been mapped to proline 226 of the SIN E1 spike protein, and a point mutation to alanine results in the loss of cholesterol dependence (70). From studies with liposome, it has been shown that the cholesterol is involved primarily in low-pH-induced virus-liposome binding and the sphingolipid in involved in catalyzing the fusion process. However, SFV and SIN do not seem to require the presence of lipid rafts for fusion with target membranes liposomes because large unilamellar vesicles made of sphingolipids and cholesterol fused to SFV and SIN irrespective of the presence or absence of TX-100-insoluble microdomains (121).

HIV-1 infects permissive cells by binding to CD4, which promotes a conformational change in the surface glycoprotein (gp120), exposing the V3 loop to further interaction with the coreceptors CXCR4 or CCR5. This triggers a conformational change in the transmembrane glycoprotein (gp41), unmasking its fusogenic domain. Glycosphingolipids can act as alternative HIV-1 entry cofactors (35, 47, 51). Physicochemical studies of the interaction of gp120 with different glycosphingolipids, including Gb3 and GM_3, have shown that these compounds mediate HIV-1 entry into $CXCR4^+$ and $CCR5^+$ cells, respectively. Primary and/or secondary interactions between a portion of gp120 and glycosphingolipids are probably required for the gp120 and gp41 conformational changes leading to the fusion process. The interaction of gp120 with galactosylceramide involves the V3 loop, and antibodies against galactosylceramide block HIV entry (34). Depletion of target cells in gangliosides reduced the HIV-induced cell-cell fusion, which was restored by the addition of purified Gb3 (51). Moreover, the HIV-1 gp41 envelope residues 650 to 685 act as a lectin to bind epithelial cell galactosylceramide, and antibodies interacting with this sequence block virus entry (2).

Extensive studies have been performed on the localization of CD4 and coreceptors within rafts, which may be a crucial point for the entry of HIV-1 (21, 28, 64, 73). CD4 is undoubtedly a raft-associated component (56, 79, 86), and its retargeting to nonraft membranes by fusion of its ectodomain to the low-density lipoprotein receptor transmembrane region strongly affects the efficiency of HIV entry at a postbinding step (28). While CCR5 is associated with rafts, CXCR4 is not (61, 93). Accordingly, CCR5 is easily coimmunoprecipitated with CD4 whereas CXCR4 is not unless cells have been preincubated with soluble gp120 (126). After incubation with soluble gp120 at 4 or 12°C, lateral association of CD4 and CXCR4 in GM_1-rich raft microdomains is observed by confocal microscopy (73, 93), but very little CXCR4 is recovered in TX-100 resistant rafts. When incubated at 37°C, gp120 does not induce any redistribution of CXCR4 in GM_1 and CD4 raft microdomains (61). Furthermore, adsorption of HIV particles at 37°C appears to redistribute CD4 outside raft domains. This results suggests that at 37°C, HIV-1 initially binds to CD4 in a raft domain and that the secondary association with CXCR4 requires the shift of proteins and associated lipids away from their preferred lipid environment. This leads to destabilization of the plasma membrane, which may favor the fusion reaction. Rafts would facilitate HIV-1 adsorption onto CD4 and then disperse prior to the ultimate membrane fusion reaction or would stimulate transient CXCR4 motion into rafts as a result of CD4 signaling (61). It should be stressed that the interpretation of the above experiments has to be cautious as far as the relative expression level of CD4 and CXCR4 is concerned: a high expression level will statistically increase the chance of forming loosely interacting complexes of receptor and coreceptor (see below).

Cholesterol depletion of target cells by treatment with methyl-β-cyclodextrin or cholesterol sequestration by nystatin inhibits HIV-1 infection and syncytium formation (64, 66, 73, 93). This type of approach has severe limitations because such treatment has multiple effects, which tend to affect cell viability and can be quickly reversible on serum addition. Nevertheless, it seems that reduction in the cholesterol level reduces the ability of HIV-1 gp120-gp41 form the receptor-coreceptor clusters required to trigger fusion. Accordingly, a high level of CD4 and CXCR4 expression reduces the effect of cholesterol depletion (118).

HIV-1 Nef protein is targeted to membrane rafts (122) and is present in the virus particles. In the presence of normal amount of cholesterol, Nef significantly enhances HIV-1 infectivity, and this effect is abolished when virus is produced from cholesterol-depleted cells (131). Likewise, cholesterol depletion or sequestration from HIV-1 particles inhibits virus internalization, probably by preventing the fusion step. In contrast

to the cholesterol depletion of target cells, incubation of cholesterol-depleted HIV-1 with cholesterol did not result in a recovery of virus internalization (46). Interestingly, in artificial membranes, sphingomyelin and cholesterol promote the surface aggregation of HIV-1 gp41 monomer (100). Replacing the gp160 glycoprotein by the vesicular stomatitis virus (VSV) G protein resulted in pH-dependent virus entry by endocytosis, which is no longer enhanced by the presence of Nef protein (23). Taken together, these data suggest that embedding in a raft can maintain the metastable conformation of fusion-competent gp120-gp41 complex and/or participate in destabilization of the bilayer architecture at the loci of fusion.

Besides these putative direct roles of membrane rafts in HIV-1 entry, the presence of other cellular factors targeted to rafts may promote virus entry. It is important to point out that the penetration of HIV-1 through rafts may direct HIV-1 preintegration complexes into a favorable compartment for a productive infection. It is tempting to speculate that the increased local concentration of receptors, coreceptors, or some other interacting proteins, at a given moment within rafts, can be due in part to the stimulation of signaling pathways within these microdomains. Indeed, the expression of flotillin-1, a protein enriched in lipid rafts and involved in the fusion process, is induced by gp120 binding (25, 29, 64). Likewise, the anchorage of HIV-1 may lead to the accumulation of surface nucleolin within lipid rafts (83). Finally, HIV-1 nonproductively infects brain microvascular endothelial cells via a macropinocytosis mechanism which is dependent on lipid raft integrity and on the mitogen-activated protein kinase signaling pathway (66).

Some enveloped viruses seem to be internalized by endocytosis within caveolae. The cellular receptor of ecotropic MLV is a transmembrane protein named CAT1. It is involved into the transport of cationic amino acids into cells and is physically associated with caveolin in membrane rafts. The disruption of rafts inhibits an early step of ecotropic MLV infection, suggesting that the localization of the receptor within rafts is important for the virus entry step (68). Cholesterol in the target membrane but not in the membrane containing the virus glycoprotein plays a crucial role in enabling membrane fusion (69). The entry of filoviruses such as Ebola virus and Marburg virus is inhibited after cholesterol depletion of the target cells, and after internalization, viral proteins co localized with caveolin as shown by confocal microscopy (33). The putative role of caveolae in mediating MLV and filovirus entry will have to be confirmed by using a dominant negative caveolin.

In summary, further studies should be performed to understand the implication of lipid domains and the molecular organization of cell membranes in the kinetics of events leading to virus entry. The underlying mechanism of the role of membrane rafts in the fusion step of enveloped viruses is unclear. Nevertheless, the available data suggest that rafts might be a platform for virus entry by providing local concentrations of receptors and/or receptor-coreceptor complexes as well as other cell components which can modulate the entry process. However, the composition of the virus envelope also needs to be carefully studies. Some viruses have envelopes made from rafts, and they may or may not require rafts in the target membrane for entry. The way in which some signaling pathways can drive the coalescence of all the components involved in virus entry remains to be determined.

RAFTS AS PLATFORMS FOR VIRUS ASSEMBLY

The late stages of the viral life cycle are the assembly of viral components into virions, maturation into infectious particles, and, in the case of enveloped viruses, release from the cell via a process of budding. Although a great deal of progress has been made in virus assembly (43), it is not yet fully understood. Initially, assembly was studied mostly as a set of events that involved only viral components, but accumulating data have indicated the role of host proteins, the cell membrane, and membrane rafts.

Nonenveloped Viruses

So far, a single report has described the involvement of rafts in the intracellular scaffolding of nonenveloped virus (101). Rotavirus VP4, the most peripheral protein of the triple-layer structure of the virus particle, is first targeted to TX-100-resistant rafts. Later, other viral proteins, including the late nonstructural protein NSP4, accumulate within the rafts (101). The rafts contain infectious virus. VP4 and purified virus interact with artificial lipid rafts and induce a cholesterol-dependent shift of the lipid bilayer into lamellar arrangements. Thus, rafts act as a platform to promote assembly, indicating or suggesting that the final step of rotavirus assembly takes place close to or at the plasma membrane (101).

Enveloped Viruses

Influenza virus hemagglutinin (HA) and VSV G protein, both acting as a receptor binding and fusion protein, were initially characterized as useful markers to study the sorting of transmembrane proteins in polarized cells. Association with detergent-resistant membranes was found to correlate with apical targeting of HA, whereas lack of association correlates with basolateral expression of G protein (106). Accordingly, influenza virus and VSV bud from the apical and basolateral sides, respectively (39). Transport of the influenza virus HA from the TGN to the plasma membrane, is slowed after cholesterol depletion by methyl-β-cyclodextrin (58). Both HA and the other envelope glycoprotein, neuraminidase (NA), preferentially cluster in lipid rafts of polarized and nonpolarized cells (103). A direct association of HA and NA with raft lipids is proposed since they are the unique protein constituent of the virus envelope, which is made of lipid rafts (Fig. 1). Raft-targeting signals mapped to the transmembrane segments and cytoplasmic tails of NA or HA (9, 103, 130) and to the palmitoylation sites of the membrane-proximal cysteine residues in the HA cytoplasmic tail (78). The influenza virus M1 matrix protein plays a critical role in the assembly of virions. When M1 protein is expressed alone, it becomes membrane associated and binds to intracellular membranes, which are TX-100 soluble. In the presence of viral glycoproteins, M1 interacts with HA and NA localized in rafts, and the membrane-M1 interaction becomes TX-100 resistant (3, 130). Thus, the envelope viral glycoproteins, which are targeted to rafts, can drag other viral components so as to promote assembly within rafts. Since influenza virus particles can be formed in the absence of HA or NA, both glycoproteins probably participate in this process (127). Do rafts contribute to the assembly of other viral

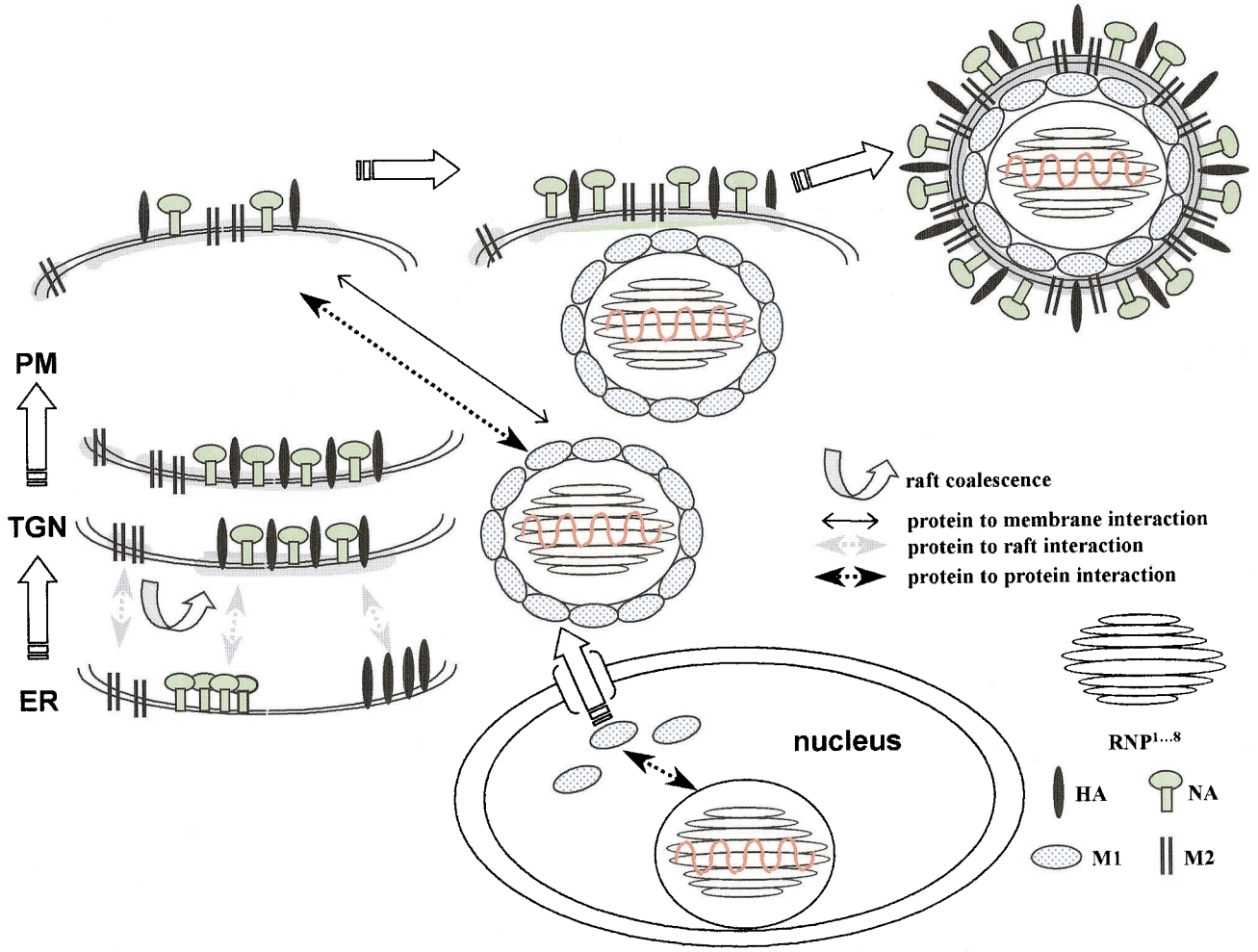

FIG. 1. Model of influenza virus assembly and budding within membrane rafts. The eight RNPs (one for each genomic RNA) are associated with M1 in the nucleus and are exported to the cytoplasm. After synthesis in the ER, the transmembrane HA, NA, and possibly M2 are joined together in the TGN, because of NA-HA interactions and because of their affinity to lipid rafts, before reaching the plasma membrane. There, the M1-RNP1 to M1-RNP8 complexes bind to the HA- and NA-enriched raft membranes because M1 has some affinity to membranes as well as to NA and HA cytoplasmic tails. Then the virus buds away from the membrane rafts. Membrane rafts are represented as shaded grey regions within the lipid bilayer.

components with the virus envelope? Some available data suggest that this can be the case. Influenza virus replication occurs in the nucleus, and the ribonucleoprotein particles (RNPs) are exported to the cytoplasm by their interaction with M1 protein (50, 76), which is itself attracted by the cytoplasmic tails of HA and NA within the rafts. The ion channel M2 protein, which plays a key role in the correct maturation of HA glycoprotein and in pH-dependent virus entry, is associated with the viral envelope (54). M2 protein is palmitoylated (113), targeted to the apical site where virus budding occurs (52), and requires the presence of cholesterol for ion channel activity (26); one can speculate that in the absence of known direct interactions between HA and M2, M2 is targeted to membrane raft, which allows (i) a common membrane transport with HA during exocytosis trafficking and (ii) its incorporation into the virus envelope. However, the lack of protein-protein interaction with other virus components will prevent the accumulation of M2 within the envelope (113) (Fig. 1).

Using a biochemical approach, the assembly of measles virus within membrane rafts has been demonstrated (74, 119) (Fig. 2). In infected cells, all structural proteins including the mature F1–F2 fusion protein and the hemagglutinin (H) are enriched in the raft fraction while the F0 precursor remains excluded from this fraction. Interestingly, the F0 protein is cleaved into the F1–F2 protein in the TGN, where the membrane rafts are formed. Like its functional counterpart, the influenza virus HA, measles F protein, which is palmitoylated (20), is targeted to rafts in the absence of other viral proteins. Because F0 and H proteins associate in the ER (92), the F protein can drag the H protein into rafts, whereas H expressed alone is excluded from these microdomains. When the other structural proteins are expressed alone, only the matrix protein (M) exhibits a low but significant association with membrane rafts. In contrast to what is observed for influenza virus (see above), the coexpression of H and F proteins together with M protein does not result in a significant increase in the amount of M associated

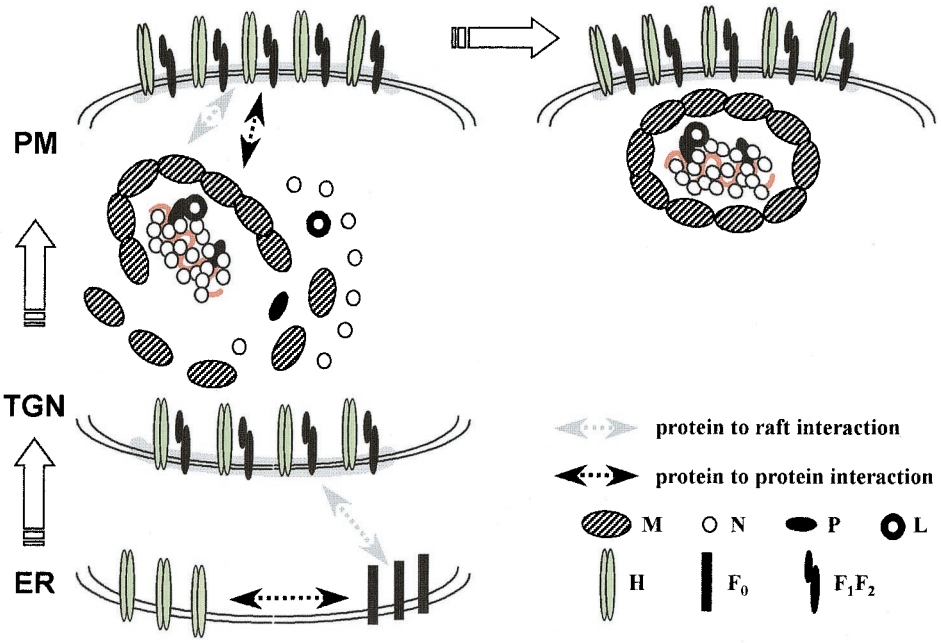

FIG. 2. Model of measles virus assembly within membrane rafts. H and F glycoproteins are synthesized in the ER, where they associate in HF complexes. HF complexes egress to the TGN, where they associate with membrane rafts because F has an affinity for lipid rafts. Then the HF complexes reach the plasma membrane. The RNA genome is encapsidated into N proteins, to which P and L proteins bind. The resulting RNP is wrapped in an M protein shell. The resulting M-RNP complex quickly associates with membrane rafts containing the two glycoproteins, because of the affinity of M for lipid rafts and for the cytoplasmic tails of F and possibly H glycoproteins. The virus is then ready for release, probably from membrane rafts. Membrane rafts are represented as shaded grey regions within the lipid bilayer.

with rafts, although M protein interacts with the cytoplasmic tail of F (110). The inability of the F protein to drag the M protein into rafts is in agreement with the lack of cotransport of the M protein with measles virus glycoproteins for efficient surface targeting (97). Independently of H and F glycoproteins, the genomic RNA, the nucleoprotein (N), the phosphoprotein (P), and the polymerase (L) of measles virus associate into RNPs in the cytosol, allowing the scaffolding of M protein, which probably targets the M-RNP complex to membrane rafts (119) (Fig. 2). Thus, it was proposed that membrane rafts allow H and F glycoproteins, on one hand, and the M-RNP complex, on the other hand, to meet there and assemble. Indeed, detergent-resistant virus-like particles are observed by electron microscopy (14), and assembly in the membrane raft is functional, since rafts isolated from infected cells are infectious (74).

HIV-1 is enclosed in a lipid envelope enriched in cholesterol and sphingolipids, suggesting a specific membrane localization for assembly (5, 21, 96). When expressed alone, HIV-1 gp160 localizes within membrane rafts, and palmitoylation of two cysteines of its cytoplasmic tail serves as targeting signal (99) (Fig. 3). In cells expressing HIV-1 structural proteins, both the mature glycoprotein transmembrane subunit gp41 and the polyprotein precursor $Pr55^{gag}$ are, in part, associated with membrane rafts (63). This localization within rafts is also observed when the precursor is expressed alone. The association is initiated by the myristoylated MA domain and is subsequently stabilized by sequences promoting Gag-Gag interactions (p2 and the N terminus of NC) (85). Replacement of the myristic acid by unsaturated fatty acids results in the loss of association with rafts (65). The loss in the recruitment of non-palmitoylated gp160 by HIV-1 particles could reflect either a misfolding of gp160 due to an unfavorable lipid environment (100) and/or the inability of the glycoprotein to meet with the internal $Pr55^{gag}$ precursor. Indeed, a stable interaction between $Pr55^{gag}$ and the cytoplasmic tail of gp41, stabilizing the gp120-gp41 complex, occurs in immature HIV-1 particles (125). This correlates with basolateral targeting of $Pr55^{gag}$, which is lost in the absence of gp160 (67). In vitro-translated $Pr55^{gag}$ associates with membranes and slowly acquires partial resistance to trypsin cleavage. This property requires the presence a of TX-100-resistant membrane containing intact cellular proteins. This suggests that the association with rafts, probably through interaction with unknown resident cellular factors, concurs with conformational changes, oligomerization, or envelopment with membranes (128). Those changes may switch on the proteolytic processing of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ just prior to the release of HIV-1 particles (S. Alais, D. Hammache, D. Gerlier and N. Chazal, unpublished data). HIV Nef is not a structural protein per se, and it is membrane anchored and targeted to membrane rafts by its myristoylation and positive charge at its N terminus (122, 131). This allows Nef to be present in virus particles and to enrich them in GM_1 (131). A chimeric Nef–N-terminus–green fluorescent protein is efficiently incorporated into HIV particles (123). Interestingly, in vitro, Nef is able to bind to RNA (32) and to reverse transcriptase (36). The targeting of Nef to membrane rafts where HIV-1 assembly occurs can favor these interactions. Taken together, these data indicate that rafts represent a necessary step during HIV-1 assembly (Fig. 3).

Respiratory syncytial virus (RSV) seems to assemble within

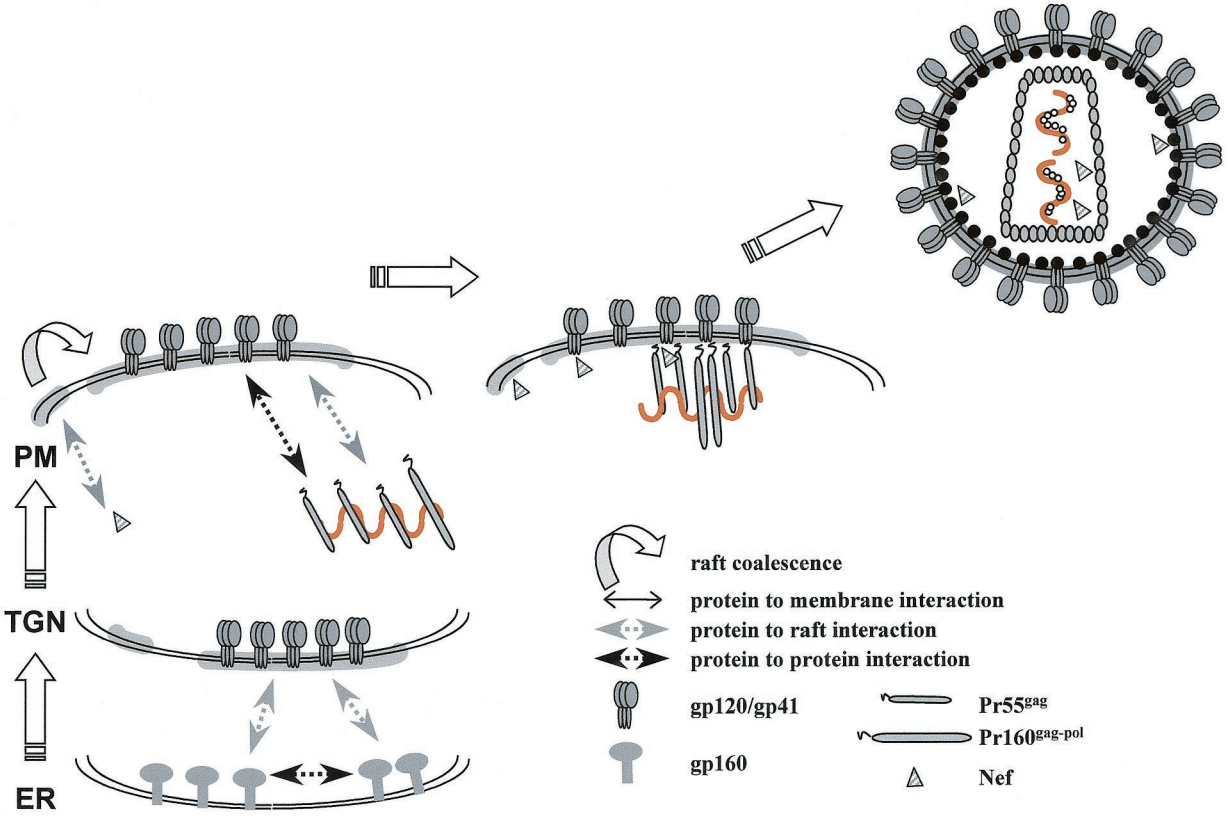

FIG. 3. Model of HIV-1 assembly and budding through membrane rafts. gp160 trimerizes within the ER and, on reaching the TGN, associates with rafts because of its affinity for lipid rafts. It then migrates to the plasma membrane. $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ oligomerize around two genomic RNAs and associate simultaneously with plasma membrane rafts due to the anchoring myristate and intrinsic properties of the MA domain. This allows the binding of MA to the cytoplasmic tail of glycoproteins. The cytoplasmic Nef protein, after palmitoylation, associates with the inner leaflet of the plasma membrane raft. The raft coalescence results in Nef incorporation into HIV-1 particles and in the enrichment of the envelope in lipid rafts. Then HIV-1 matures (cleavage of Gag precursors in MA, CA, NC, p6, and enzymes) and buds from the plasma membrane rafts. Nef protein is initially bound to membrane rafts. When encapsidated into HIV-1 particles, Nef is partly cleaved off by the viral protease into a soluble domain, which is thought to bind to the RNP. Membrane rafts are represented as shaded grey regions within the lipid bilayer.

membrane rafts where viral proteins colocalize with caveolin-1 (18, 19). RSV filaments colocalize with GM_1, and RSV infection induces the redistribution of phosphocaveolin away from focal adhesions. RSV particles contain some caveolin-1 molecules. Accordingly, F and M proteins are found associated with TX-100-resistant membrane rafts (49).

Rafts may be also used by Sendai virus as assembly platforms. When the matrix protein (M), which plays a critical role in Sendai virus assembly, is expressed alone, it is preferentially associated with nonraft membranes, but when M is coexpressed with F or HN glycoproteins, either individually or together, it becomes resistant to an unusually low concentration of TX-100 (4).

When studying the involvement of rafts in the virus assembly process, one has to keep in mind that in contrast to GPI-anchored proteins, only a fraction of viral proteins are found associated with rafts. This could be due to the poor biochemical characterization of rafts subsets or to the transient nature of the association. The elucidation of raft involvement in viral assembly step will have to be more precisely defined by expressing individual viral components independently or together and by using complementary technical approaches. Indeed, available data on raft involvement in the virus life cycle are mostly restricted to the simple descriptions of the distribution of viral proteins within rafts following detergent solubilization and cholesterol depletion.

RAFTS AS PLATFORMS FOR VIRUS BUDDING

While naked viruses are released from the infected cell by disruption of the plasma membrane, enveloped viruses contain a host cell-derived lipid bilayer, which surrounds the nucleocapsid or core and which is acquired during budding (41). Virus detachment from cell is usually described as a pinching-off step. This requires local curvature of the membrane to form a bud, followed by formation a neck (or lipid stalk) and then by fission. The latter consists of the mixing of lipids of the apposing membrane in a manner directly analogous to the fusion event that occurs when the virus enters the cell. The ability of the raft lipid component to regulate budding out of vesicles from the TGN (53) suggests that some virus could use rafts to vesiculate from the plasma membrane, although the topologies of these two vesiculation processes are not equivalent (budding into and away from the cytosol). The mechanisms by which the lipid raft can favor the budding and/or fission process is a matter for speculation (53). Membrane fusion is energetically

unfavorable and, it is almost certain that the stalk contains a (cellular) protein machinery to mediate the fission step (40). Indeed, in the presence of ATP-depleting agents, HIV-1 release is blocked and virus buds anchored by a stalk accumulate (114).

Gateway for Budding Viruses

Although many data suggest that virus budding can occur within membrane rafts, it is difficult to directly demonstrate this process. Indeed, all available tools strongly affect protein targeting to membrane raft and virus assembly, which are prerequisites. Conversely, assembly within membrane rafts is a first indication of the possible involvement of rafts in the budding process of several viruses including influenza virus, measles virus, HIV-1, and Ebola virus.

During the release of enveloped viruses into the extracellular environment, membrane lipids are not randomly incorporated into viral envelope. Virions can have a specific lipid composition different from that of the host cell membrane. Fowl plague virus, which belongs to the influenza virus family, contains large amounts of detergent-insoluble complexes, whereas such complexes are largely absent from the VSV and SFV envelopes (102). The lipid composition of the influenza virus family is due to the intrinsic affinity of the HA and NA glycoproteins for these lipids, as shown by the lower contents of virus envelope in raft lipids when both glycoproteins lacking their cytoplasmic tails are less likely to associate with rafts (130). This suggests that influenza virus buds from raft domains. However, controversial data were obtained for VSV and SFV. The lipid analysis of VSV particles by fluorescence digital microscopy indicates the formation of lipid domains during the budding steps. Both the glycoprotein and the matrix protein induce lateral organization of lipids within the membrane, and the lipid composition of the VSV envelope differs from that of the host cell, suggesting that VSV might bud from "classic" rafts or from a subgroup of rafts (71, 89).

After the assembly of measles virus within membrane rafts, the envelope-RNP complex appears ready for budding. This virus is rather inefficient in budding, and single particles contain several RNPs (95). Viruses released into cell-free supernatant are made partially of nonraft membranes, with recovery in detergent-resistant membrane rafts of H and F glycoproteins but no other viral structural proteins (74). Thus, either the particles assembled in membrane rafts are not precursors of budding mature viruses or, after assembly involving the coalescence of several membrane rafts, virus budding through membrane rafts is associated with the capture of adjacent nonraft membranes and simultaneously initiates a shift of the RNPs from raft to nonraft regions. Two observations argue for the latter hypothesis: (i) whereas RNPs are tightly bound to the plasma membrane of infected cells, they tend to dissociate from the virus envelope after budding (31), and (ii) there is a correlation between a defect in measles virus budding in a murine cell line (120) and the poor localization of the M protein in membrane rafts from infected cells (S. Vincent and D. Gerlier, unpublished data). Demonstration of a raft requirement for measles virus budding awaits further experimental evidence.

Many arguments favor a raft membrane dependence for HIV budding. Treatment of human T lymphocytes cultured in cholesterol-poor medium with lovastatin, an inhibitor of cholesterol synthesis, inhibits HIV-1 production (77). The exclusion of the abundant nonraft CD45 phosphatase from the HIV-1 envelope and the incorporation of raft lipid components (ganglioside GM_1) and resident proteins (the GPI-anchored proteins Thy-1 and CD59) indicates that HIV-1 specifically buds from rafts (80). The blocking of HIV-1 budding after treatment of cells with unsaturated fatty acid directly indicates a critical role of rafts in virus budding. This treatment inhibits $Pr55^{gag}$ targeting to rafts without affecting its association with cell membranes and significantly reduces the number of virus-like particles released into the supernatant (65). The Nef protein, which is recruited into the virus at the raft assembly site, enhances virus release and infectivity (23, 27). Thus, Nef probably participates directly in the formation of the budding scaffold. However, one has to keep in mind that any biochemical data on HIV-1 (and many enveloped virus) relies on the analysis of purified particles, which are hardly devoid of contaminating cell membrane sheets (12, 42).

Recruitment of Cellular Machinery Needed for Virus Budding

Host proteins are implicated in virus budding. Retrovirus gag precursors and Ebola virus, VSV, and rabies virus matrix proteins contains PS/TAP and/or PPXY motifs, which allow interaction with the Tsg101 protein, an E2-like ubiquitin ligase, and the Nedd4 family protein, belonging to an E3 ubiquitin ligase, respectively. The recruitment of these cellular proteins plays a key role in the efficiency of virus budding and release (37, 88). Nedd4, which interacts with RSV and Mazon-Pfizer monkey virus late domains, is specifically localized in the raft domain (60, 129). The release of an HIV-1 mutant lacking the PS/TAP motif is not reduced but is actually increased by cholesterol depletion using methyl-β-cyclodextrin (85). Whether coalescent rafts bring together the virus and cellular proteins or favor the viral protein assembly that promotes local recruitment of the cellular factor remains to be determined.

CONCLUDING REMARKS AND PROSPECTS

Viruses are nanomachines built within the cell factory and designed to invade neighboring cells. Their building relies on timely and dynamically regulated assembly of individual components, including nucleic acids, proteins, and lipids, in specific cell locations. Their invading strategies rely on complex interactions with the cell plasma membrane, involving several viruses and cellular proteins organized in dynamic complexes that are able to mediate viral penetration into the cell interior without rupturing the outside-inside plasma membrane frontier required for cell survival.

The evidence for a great heterogeneity in membrane lipids and their organization in various domains such as rafts has provided new tools to explore the molecular mechanism underlying virus entry, assembly, and budding. It should be stressed that when studying rafts, what matters is the efficiency of the molecule partition within highly exchangeable lipid mi-

crodomains. Therefore, one cannot expect to observe all-or-none phenotypes when attempting to modify raft structure and/or composition. It is very likely that rafts act basically as a planar lipid milieu favoring interactions between molecules which have some intrinsic affinity to each other.

The caveolae, a subset of membrane rafts, are critically involved in the entry of SV40 and EV1. Likewise, embedding of the HIV-1 gp120-gp41 glycoproteins in membrane rafts is required for proper folding and fusion competence. Furthermore, due to their ability to coalesce after cross-linking signals, membrane rafts may control the appropriate clustering of cellular lipid and protein receptors to enable HIV-1 entry.

The opportunity to isolate membrane rafts has allowed us to determine how the H and F envelope glycoproteins and RNPs under scaffolding can reach a common sub membrane location to assemble into a functional measles virus-like particle. A more systematic study of the assembly of all influenza virus components within membrane rafts is likely to be most informative. Likewise, membrane rafts are probably critical for efficient HIV-1 assembly.

Virus budding from membrane rafts does occur, at least for influenza virus and HIV-1, but whether rafts are absolutely required for virus budding is unknown. Solving this question will require the refinement of the raft subset structures, raft lipid boundaries, and raft dynamics. Nevertheless, by carrying accessory proteins, rafts are likely to contribute to the optimization of virus infectivity, as illustrated by the incorporation of Nef into HIV-1 particles.

The following questions need to be addressed in order to decipher any role of membrane rafts in the replication cycle of a virus. Are specific raft subsets involved? Which viral protein (virus receptor) can reach rafts on its own? What are the underlying molecular mechanisms or evidence for specific raft-targeting motif? Which viral protein (virus receptor) is brought about by a partner intrinsically targeted to the raft, and how is this done? Other important questions remain to be answered. What is the role of the cell molecule partners: as cargo for targeting to rafts or as a useful virus cofactor which can thus be efficiently embarked? Are rafts a (compulsory) intermediate oligomerization platform for virus scaffolding? Do raft dynamics control the oligomerization and maturation steps which convert an immature capsid shell into a metastable mature core, ready for uncoating? What is the advantage that viruses gain when using rafts? Do rafts promote local concentrations of useful (cell or virus) molecular partners including specific lipids? Do they promote conformational changes of virus scaffolding components? Do they promote activation (or silencing) of viral or cellular enzymatic activity? Do they promote fusion and/or capsid penetration? Do they promote cell signaling useful for virus replication?

Answering these questions will require the use of several independent approaches such as biochemical purification of rafts after detergent solubilization, colocalization and biosynthesis studies, and reconstitution experiments in artificial bilayers. In any case, one should keep in mind that biochemical characterization of rafts critically relies on the nature of the detergent and the physicochemical conditions of its use. A given detergent exhibits different solubilizing properties toward a class of lipids and protein. These solubilizing properties are modulated by temperature, detergent-to-membrane ratio, pH, and ionic strength (8). Furthermore, rafts are highly dynamic entities: within minutes of receptor stimulation, the raft protein composition drastically changes, with only a small percentage of proteins remaining invariable (13).

Elucidation of the involvement of rafts in the viral life cycle should help to define more precisely the main events of the viral infectious cycle and to provide some clues to fundamental biology. It may also serve to develop new antiviral strategies and to guide the engineering of recombinant viruses useful as experimental tools or therapeutic agents.

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