Do lipid rafts mediate virus assembly and pseudotyping?

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Co-infection of a host cell by two unrelated enveloped viruses can lead to the production of pseudotypes: virions containing the genome of one virus but the envelope proteins of both viruses. The selection of components during virus assembly must therefore be flexible enough to allow the incorporation of unrelated viral membrane proteins, yet specific enough to exclude the bulk of host proteins. This apparent contradiction has been termed the pseudotypic paradox. There is mounting evidence that lipid rafts play a role in the assembly pathway of non-icosahedral, enveloped viruses. Viral components are concentrated initially in localized regions of the plasma membrane via their interaction with lipid raft domains. Lateral interactions of viral structural proteins amplify the changes in local lipid composition which in turn enhance the concentration of viral proteins in the rafts. An affinity for lipid rafts may be the common feature of enveloped virus proteins that leads to the formation of pseudotypes.

INTRODUCTION


Physicists and structural biologists have long been attracted by the precision and order of virus assembly. Some believe that the process is similar to the crystallization of isolated proteins. The cell, however, is a more complex and structured environment than a crystallization tray. Viruses, the most exploitative and resourceful of cellular parasites, take advantage of this organization to optimize their life cycles. The process is therefore not so appealingly simple and consideration of the cellular environment is essential to an understanding of virus assembly.

Pseudotype formation is a feature of virus assembly that is only comprehensible in the context of a structured cell. Membrane viruses normally assemble their own spikes and internal proteins. However, during a mixed infection of a host cell with two different enveloped viruses, particles may be produced that contain the genome of one virus packaged within a membrane that contains the envelope proteins of the other, or both viruses. These are pseudotypes. This phenomenon was described first with RNA viruses but examples were soon found in DNA viruses (Huang et al., 1974; Lee et al., 2001; Pastorekova et al., 1992; Witte & Baltimore, 1977). The consequences of sharing envelope proteins include an increased breadth in the virus host range. A virus infection that would normally be restricted to certain cells can be mediated in other cells through the envelope proteins of the co-infecting virus. This behaviour is essential to the infectivity of replication-defective acute transforming retroviruses, which lack envelope glycoproteins and therefore require the presence of a co-infecting helper virus (Granger & Fan, 2001). It may also be desirable for the production of vectors for gene therapy (Kang et al., 2002; VandenDriessche et al., 2002). The largest numbers of examples of pseudotyping were reported for vesicular stomatitis virus (VSV) (Závada, 1972a, b; Závada et al., 1972, 1978, 1979, 1983; Závada & Rosenbergova, 1972; Zavadova & Závada, 1980) and other matrix (M) protein-containing viruses (Závada, 1976); this may simply reflect the fact that tools for assaying for pseudotypes were well developed in these systems (Závada, 1977). In contrast to the plasticity of surface component incorporation, attempts to demonstrate mixing of internal components between unrelated envelope viruses failed (McSharry et al., 1971). An insightful review by Závada (1982) summarized the breadth of examples and emphasized the paradoxical nature of the phenomenon. As described by Závada (1982), this ‘pseudotypic paradox’ arose from the combination of three observations: (1) enveloped viruses non-selectively incorporate both their own glycoprotein (gp) as well as the gp of viruses belonging to other families; (2) cell membranes assemble both cellular and virus surface gp; and (3) enveloped viruses seem to exclude cellular gp from mature virions.

The ability of membrane viruses to accommodate the proteins of an unrelated virus, while excluding most cellular proteins, suggested a sophisticated mechanism for recognizing viral proteins in preference to cellular ones. ‘The only
explanation that occurs to us is that the enveloped viruses must share a common, highly specific mechanism of assembly of virus surface structures’ (Závada, 1982). The identification of these common features, including perhaps the protein motifs responsible, represented a formidable challenge. One of the models put forward by the review appears prescient in predicting that ‘some alteration in the ultrastructure of the lipid membrane is induced by attachment of virus M protein or its equivalent and that this alteration must be the same in all enveloped viruses. It is our guess that this might function by phase partitioning’ (Závada, 1982).

This review will summarize research implicating lipid domains in the assembly pathways of a number of enveloped viruses and discuss how they provide a ready explanation for the phenomenon of pseudotyping (Pickl et al., 2001).

A suggestive image

How might membrane domains feature in the assembly of enveloped viruses? Cryo-electron micrographs (Fig. 1) of a preparation of feline foamy virus (FFV) grown in Crandel feline kidney (CrFK) cells show that it contains two morphologically distinct viruses: FFV (Fischer et al., 1998; Wilk et al., 2000, 2001b; Winkler et al., 1997; Yu et al., 1996; Zemba et al., 1998) and feline leukaemia virus (FLV) (Bolognesi, 1974; Essex et al., 1973; Gallo & Wong-Staal, 1982). These are seen as individual particles in Fig. 1(A). The FFV particle (black annotation) displays the double-layered angular core structure (hexagon, small arrowheads), clear membrane leaflets (arrows) and prominent rod-shaped spikes (~130 Å long, large arrowhead) that characterize FFV in cryo-electron microscopy (Wilk et al., 2000, 2001a). The FLV particle is annotated in white. The morphology of this particle is quite distinct from that of FFV. The FLV core is round rather than angular and displays a radial arrangement of rod-like features (small arrows) characteristic of Gag in other retroviruses (Fuller et al., 1997; Wilk et al., 1999, 2001b). The membrane lacks prominent surface spikes and appears broader (large arrow) due to the presence of a submembrane protein layer.

The morphological differences between these two viruses make it possible to interpret the hybrid particle seen in Fig. 1(B). The simplest explanation is that separate assemblies of FFV and FLV proteins have budded into the same envelope. Two distinct cores are seen within the single particle. Each core is associated with a portion of the envelope, which has a distinct local appearance. The rod-like FFV spikes are localized near the angular FFV core and away from the round core and broad membrane layer of FLV.

Microscopists are notorious for their tendency to elaborate a complex biological framework from a single image (Baker et al., 1999; Wilk & Fuller, 1999). We need to remain cautious in distinguishing the illustrative from the probative. Nevertheless, this image of a hybrid particle suggests a particular view of retrovirus assembly in which interactions with membrane domains select both envelope and core proteins. Initially, lipid domains select viral membrane proteins by their affinity for the domains. Viral proteins

Fig. 1. Cryo-electron micrographs of particles found in a preparation of FFV grown in CrFK cells. The preparation contains two morphologically distinct viruses: FFV and FLV. (A) Individual virions. An FFV particle is marked in black. The particle displays an angular core structure (hexagon) surrounded by a double protein layer (small arrowheads), clear membrane leaflets (arrows) and prominent rod-shaped spikes (large arrowhead) that are characteristic features of foamy virus in cryo-electron microscopy. An FLV particle is marked in white. It has a round core (circle) with three protein layers (arrowheads) exhibiting a radial arrangement (small arrows). The membrane appears broad due to the presence of a submembrane protein layer. (B) A hybrid virion, for description see text. Bar, 50 nm.
then select other viral components by cooperative interaction and other membrane components by their affinity for the gathered viral ones. This gathering of components is self-reinforcing so that the domains become stable entities as further components are recruited. In the FFV/FLV example, interactions with lipids localize the two viruses to the same domain within the membrane, while protein–protein interactions maintain the separate nature of the two virus assemblies. The hybrid particle could result from the budding of the adjacent assemblies into one particle. The co-localization and resulting incorporation of envelope proteins from co-infecting membrane viruses provides an explanation for the creation of pseudotypes. This hybrid particle would have the broadened host range of a pseudotype, although the classical pseudotypic particle would contain the internal proteins of only one of the partners. Lipid rafts are the chief candidates for the domains that recruit the viral components.

Enveloped virus assembly and sorting

Virus assembly shares features with other macromolecular assembly processes in cells. Both processes incorporate mechanisms for regulation and control. Both require the bringing together of many components so that an ordered series of specific interactions leads to recognition and assembly. Often the components to be assembled are concentrated and segregated from the other cellular components so that the appropriate interactions become more likely and can occur without interference. A further common feature is cooperation. Oligomerization is used to generate the template that is recognized in later steps of assembly. This enhances the effect of the individual recognition steps so that a weak preference for interaction between two components becomes amplified by cooperation among many.

Enveloped virus formation exhibits the general features of this assembly process (Simons & Fuller, 1985) as well as the distinctive characteristic of membrane assemblies: that the selection and incorporation of components occurs in a two-dimensional milieu. In this environment, an inappropriate component can be included more easily through being trapped in a growing two-dimensional array, even in the absence of a specific interaction. Consequently, assembly in two dimensions must use a hierarchy of sorting steps that allow selection of the correct components and exclusion of incorrect ones.

Incorporating an inappropriate surface component could have significant consequences for the biology of the virus. Enveloped viruses use their surface components for entry. Typically, the surface proteins function in receptor recognition and membrane fusion. We have already seen that incorporation of inappropriate components could allow interactions with a broader range of cell surface proteins and hence alter the specificity of entry. The effect on fusion could be equally dramatic. In the case of influenza virus, oligomerization of haemagglutinin (HA) is required to form the fusion pore (Ellens et al., 1990; Gutman et al., 1993; White et al., 1996). A reduction in HA concentration, equivalent to an increase in the concentration of foreign proteins, leads to a large decrease in the efficiency of fusion (Ellens et al., 1990).

How effective is the sorting during virus formation and budding? Budding from the plasma membrane occurs in an environment of ~30,000 plasma membrane proteins per square micron (Griffiths et al., 1984; Quinn et al., 1984; Simons & Fuller, 1985). The surface density of viral proteins in a budded virion is easiest to calculate for an icosahedral enveloped virus such as Semliki Forest virus (SFV), which has ~25,000 spike complexes per square micron (Fuller et al., 1995; Mancini et al., 2000; Simons & Fuller, 1985). Hence, virus budding does not lead to a significant change in the concentration of surface proteins within the budding particle but rather selection of the proteins from the mixed population at the cell surface. The selectivity is extreme. Even in a relatively late stage of infection, the viral proteins contribute only ~1% of the surface proteins of the infected cell and yet the budded SFV virion does not contain detectible cellular proteins (Fuller et al., 1995; Mancini et al., 2000; Simons & Fuller, 1985).

These considerations also apply to non-icosahedral viruses, such as retroviruses, where an even smaller fraction of the protein on the cell surface is contributed by the virus (Vogt, 1997). In such viruses, a subset of cellular plasma membrane proteins is also incorporated into virions, while others are excluded. Human immunodeficiency virus (HIV), for example, incorporates more than 20 cellular proteins (Esser et al., 2001; Ott, 2002). Some cellular proteins, such as cyclophilin A and HLA-II in HIV, may be included through specific interaction with viral proteins. As discussed below, others may simply be co-localized with viral proteins through raft association.

Lipid rafts

Cellular membranes contain hundreds of different lipid components, including glycerophospholipids, sphingolipids and cholesterol, each of which have different chemical, physical and biological properties. Accumulating evidence suggests that sphingolipids and cholesterol can become segregated from other membrane lipids to form ordered lipid micro-domains, called rafts, floating in a glycerophospholipid-rich environment (Cheong et al., 1999; Harder et al., 1998; Harder & Simons, 1997; Lafont & Simons, 2001; Lusa et al., 2001; Rietveld et al., 1999; Scheiffele et al., 1999; Simons et al., 2000; Verkade et al., 2000).

Raft lipids are probably held together weakly, establishing a dynamic equilibrium of raft and non-raft regions within the plasma membrane (Harder & Simons, 1997). The sphingolipids interact laterally through van der Waals interactions and extensive hydrogen bonding between the sugar head groups and between the sphingosine backbones. Moreover, the majority of sphingolipids have saturated, and therefore uninkinked, acyl chains that allow tighter packing of laterally
associating lipids and a higher gel-to-liquid phase transition temperature (Boggs & Wang, 2001). These interactions lead to segregation of sphingolipid-rich domains from their glycerophospholipid-rich surroundings. The degree of lateral interaction is increased further by the presence of cholesterol. The 3-β-hydroxyl group of cholesterol hydrogen bonds with the ceramide group of sphingolipids, while its planar sterol ring interacts with the saturated acyl chain. In this way cholesterol fills the voids between neighbouring sphingolipids (Harder & Simons, 1997) and increases the local rigidity of the bilayer.

The most common biochemical tool used for studying membrane rafts has been extraction with cold non-ionic detergents such as Triton X-100 or NP-40 (Fig. 2). This treatment results in the formation of detergent-resistant membranes (DRMs), which are also referred to as detergent-insoluble glycolipid-enriched complexes or DIGs. DRMs exhibit a buoyant density which allows them to be separated from membranes and soluble proteins by flotation in density gradients. Many cellular proteins are found to be associated with DRMs.

**The raft passengers**

The nature of the association between ‘raftophilic’ proteins and rafts varies. Only a few proteins are permanent residents. These are important for maintaining a specific raft organization and include the caveolins (Galbiati et al., 2001; Harder & Simons, 1997; Monier et al., 1996). Many other proteins, however, are transient passengers or guests, taking advantage of the remoteness of the lipid island for intracellular targeting (Lusa et al., 2001) or specific signal transduction processes (Cheng et al., 2001; Drake & Braciale, 2001; Drevot et al., 2002; Dykstra et al., 2001; Langlet et al., 2000; Sharkey et al., 1990; van der Goot & Harder, 2001). The abundance of signalling molecules and immunoreceptors suggests a central role for rafts in modulating T-cell receptor function (Langlet et al., 2000).

Integral and peripheral membrane proteins use different strategies for raft association. Integral membrane proteins like CD36, SNAP-25 and caveolin-1 contain several palmitoylation (Buser et al., 1994; Rodgers et al., 1994; Sigal et al., 1994; Webb et al., 2000) sites adjacent to, or just within, the palmitoylated leaflet of the membrane (Jochen & Hays, 1993; Monier et al., 1996; Veit et al., 1996a, b). Insertion of the palmitoyl chain into the lipid bilayer is energetically favourable and may provide the driving force for partitioning the modified protein into the raft environment (Schroeder et al., 1994).

Peripheral membrane proteins contact only one side of the raft membrane. Association with the inner leaflet of the bilayer is frequently mediated by multiple N-terminal acylations with saturated fatty acids such as myristate or palmitate. The binding energy they provide is approximately $10^{-4}$ M $K_d$, which is not sufficient to anchor a protein to a membrane. Therefore, myristoylated proteins can only maintain an efficient membrane interaction when a second membrane-binding site is present. This second membrane-binding site can be created by a nearby stretch of basic residues or palmitoyl chains, as in the Src family of protein kinases or the $\alpha$ subunits of heterotrimeric G proteins (Buser et al., 1994; Robbins et al., 1995; Rodgers et al., 1994; Sigal et al., 1994).

Glycosylphosphatidylinositol (GPI)-linked proteins such as CD55, PLAP and Thy-1 (Calafat et al., 1983; Harder et al., 1998; Marschang et al., 1995) are the most abundant...
proteins associated with the outer leaflet of the raft bilayer. They are synthesized initially as transmembrane proteins. The transmembrane region is then proteolytically cleaved and replaced by a pre-assembled glycolipid. Consequently, the modified polypeptide chains are anchored to the membrane only through the glycolipid modification. Most GPI-anchored proteins are raft associated through the long, saturated acyl and alkyl chains of their lipid anchors (Benting et al., 1999; Brown, 1994, 1998; Brown & London, 1998; Brown & Rose, 1992; Melkonian et al., 1999; Rodgers et al., 1994; Schroeder et al., 1994).

The presence of GPI-anchored proteins in the outer leaflet of the raft bilayer can be regulated by phospholipase C activity, resulting in the release of GPI-anchored molecules from the plasma membrane (Brown et al., 1994). Likewise, integral membrane proteins and proteins attached to the cytosolic face of the raft membrane can be controlled by reversible palmitoylation (Robinson et al., 1995). These mechanisms allow flexible control and suggest that raft microdomains are dynamic membrane assemblies.

In summary, rafts are dynamic structures that restrict their lipid and protein composition quantitatively but not absolutely. Once formed, rafts recruit other components by their affinity with the initial raft components. Exclusion appears passive. Components that lack affinity for the initial ones will not be concentrated in the raft region. This effect is cooperative; as the composition of the raft changes due to the incorporation of components, the strength of the attraction increases (Benting et al., 1999; Brown & London, 1998; van der Goot & Harder, 2001).

Evidence for rafts in virus assembly

Similar modifications to those seen on cellular raft proteins are found on several viral proteins. Retroviral Gag proteins are myristoylated and the glycoproteins of retroviruses, filoviruses and influenza viruses may be palmitoylated (Ito et al., 2001; Schmidt, 1982, 1984; Yang et al., 1995). There is increasing evidence that a number of virus proteins, with or without modifications, are raft associated and that assembly and budding of virions may take place through rafts. The acid test of whether or not a virus assembles and buds through lipid rafts is whether or not the lipid composition of its membrane reflects the lipid composition of a raft and differs from the bulk cellular lipid composition. In practice, however, viruses are usually classified as raft viruses based on an assortment of more indirect methods that assay instead for raft-associated phenomena. They can be divided loosely into five general approaches: (1) co-floation with DRMs and associated marker proteins in density gradients after cold detergent treatment (see above). This is the most widely used raft assay for both viruses and cellular proteins; (2) observing punctate co-localization with raft markers in the plasma membrane by immunofluorescence; (3) measuring the incorporation of raftophilic molecules into virions; (4) biophysical experiments to define the fluidity of the lipid environment in the viral membrane; and (5) looking for blocks to virus assembly and budding after raft disruption, normally induced by cholesterol depletion – it is important to remember the limitations of these methods when interpreting resulting observations.

The Triton X-100-extractability assay, while widespread, is not used with sufficient uniformity that comparison of results from different laboratories is straightforward. In the case of HIV there is evidence that the coarseness of commonly used density gradients disguises the fact that Gag–membrane complexes float at a slightly higher density than conventional rafts (Lindwasser & Resh, 2001). Other combinations of detergent and temperature can be used to isolate raft-like membrane domains, which may have different compositions to those preserved in Triton X-100 at 4 °C (Roper et al., 2000). It was reported recently that T-cell receptor-containing rafts could be isolated using polyoxyethylene ether detergents (Brij) at 37 °C (Drevot et al., 2002).

In the absence of an analysis of lipid composition, it is difficult to conclusively link flotation at a particular density to presence in membrane microdomains. Furthermore, when viral proteins are observed in association with detergent-insoluble fractions in infected cells, the possibility that this occurs during transport of the proteins prior to assembly should be considered.

The incorporation of raftophilic proteins into the viral envelope provides evidence of raft-mediated assembly only if relative concentrations in the plasma membrane are considered properly.

Biophysical results indicating reduced flexibility of lipid tails in the viral membrane may be difficult to interpret in the presence of highly ordered viral proteins, which may themselves induce ordering of lipids.

Interpreting the effects of raft disruption on virus assembly is also potentially dangerous. The depletion of cholesterol is likely to have widespread effects on cellular processes and it is difficult to attribute exclusively any deleterious effects on virus assembly to raft disruption. Alternatively, cholesterol may have a structural role in the budded virus beyond any contribution to raft formation, as is the case in SFV (Ahn et al., 2002; Lu & Kielian, 2000).

Despite these considerations, there is compelling evidence for the involvement of rafts in the assembly of a number of different non-icosahedral enveloped viruses.

Retroviruses

The majority of retrovirus studies are on HIV-1. Flotation assays have been carried out on the Gag protein and its cleavage product, MA, the Env protein and its cleavage products, TM and SU, and the Nef protein. Triton X-100 treatment ranged from 3 min at 0.5% to 20 min at 0.25% to 1 h at 1%. Three-step gradients have been commonly used and varied from 73→10% gradients to 40→5%
gradients. This means that DRMs were isolated at interfaces ranging between 65 and 10% and 30 and 5%. HIV proteins were found throughout the gradients, but typically a number of fractions at the bottom of the gradient and around the interface were pooled to represent the soluble and raft components. Any attempt to quantify must therefore be critically dependent on the gradient used. Given that no two groups have used identical conditions, it is impossible to accurately compare their results. Nevertheless, some consistent patterns do emerge. Nguyen & Hildreth (2000) found 90% of myristoylated Gag associated with DRMs and approximately 35 and 25% of MA and TM, respectively. Ono & Freed (2001) found 25% of Gag in the raft fractions, which was half of the membrane-associated Gag. Given that the DRMs represent only a small fraction of the plasma membrane, this represents a significant concentrating effect. Zheng et al. (2001) found that the concentration of Gag in the DRMs was 14 times higher than that in the soluble fraction and almost three times that in the plasma membrane. This concentrating effect was Nef dependent (Zheng et al., 2001). Nef itself is also enriched in DRMs (Wang et al., 2000; Zheng et al., 2001). Gp160 was found to be raft associated using both a flotation assay (Pickl et al., 2001) and a simple test for insolubility in cold Triton X-100 (Rousso et al., 2000). Rousso and colleagues observed that insolubility in cold Triton X-100 was dependent on the palmitoylation of at least one of the two cytoplasmic palmitoylation sites.

Lindwasser & Resh (2001) examined the flotation of Gag on multiple-step Optiprep gradients and found that while 6–8% of Gag floats in the raft fraction (marked by GM1 and caveolin-1), about 30% of Gag was found at a slightly higher density. A reduction in Gag multimerization relocates some of this Gag to the raft fraction. These authors (Lindwasser & Resh, 2001) termed these protein-containing domains ‘barges’. Such observations highlight the limitations of three-step gradients. A recent article (Ding et al., 2003) explores this issue and shows that HIV-1 Gag rafts are more dense than classical lipid rafts.

Gag and Env co-localize in a punctate pattern in transfected Cos cells (Hermida-Matsumoto & Resh, 2000). A similar pattern of co-localization is seen between Env and GM1 (Pickl et al., 2001). Nguyen & Hildreth (2000) observed co-localization of an anti-HIV polyclonal antibody with a number of raft markers in infected Jurkat cells, but in broad patches rather than the small points observed by the other groups. Non-raft proteins were excluded from these areas. The same authors demonstrated that raftophiles proteins and GM1 were all incorporated into virions, despite being present in low concentrations on the cell surface. In contrast, the highly expressed CD45 was not incorporated into virions.

Cholesterol depletion leads to a decrease in virus infectivity (Ono & Freed, 2001; Zheng et al., 2001), which is Nef dependent (Zheng et al., 2001), as expected for a system in which assembly is mediated by rafts. Crucially, the viral membrane is known to be enriched in sphingolipids and cholesterol relative to the plasma membrane (Aloia et al., 1988, 1993). There is therefore a significant weight of evidence supporting a role for lipid rafts in the assembly of HIV.

Moloney murine leukaemia virus was also studied by Pickl et al. (2001). They observed flotation of Gag and Env, co-localization of Env and GM1, incorporation of raftophiles into virions and a reduction in virus titre after cholesterol depletion, implying that this retrovirus also assembles at raft domains. They also observed pseudotyping of virus particles with Env proteins from HIV, VSV and influenza virus.

Influenza virus

Neuraminidase (Barman & Nayak, 2000; Zhang et al., 2000) and HA (Pickl et al., 2001; Zhang et al., 2000) both float in the raft fraction of cell lysates. HA is also found floating in density gradients of Triton X-100-treated virus preparations. This behaviour is dependent on their cytoplasmic tails (Zhang et al., 2000). Co-localization of HA and raft markers in patches on the cell surface can be observed after cross-linking and GM1 is found incorporated into virions (Pickl et al., 2001). Electron paramagnetic resonance suggests the presence of two different domains with fast and slow oxygen collision rates (Kawasaki et al., 2001). The authors attributed the less mobile domain to a protein-rich raft domain. Diphenylhexatriene fluorescence polarization, used as a measure of acyl chain order, also suggests the presence of highly ordered domains within the viral envelope (Scheiffele et al., 1999).

Zhang et al. (2000) demonstrated that reducing the raft association of HA and neuraminidase, by deleting regions in their cytoplasmic tails, lowered the amount of cholesterol and sphingomyelin in the virion. Scheiffele et al. (1997) showed that HA expressed in a different lipid environment, namely the membrane of VSV, is not DRM associated (Scheiffele et al., 1997). They also demonstrated that the detergent resistance of the sphingomyelin within the influenza virus membrane is dependent on the presence of cholesterol. These results demonstrate the inter-dependence of both lipid and protein components in moulding the properties of the influenza virus membrane.

Vesicular stomatitis virus

Flotation assays with VSV give contradictory results. Pickl et al. (2001) found the VSV glycoprotein (VSV-G) associated with DRMs, whereas Scheiffele et al. (1999) found that it did not. The first group were working with extracts from cells expressing HA-tagged VSV-G, whereas the second group were working with purified virus. Although essentially the same gradient was used, the second group carried out a significantly shorter centrifugation step (2 h as opposed to 16 h), confounding comparisons between the findings. In the same work, Pickl et al. (2001) observed co-localization of HA-tagged VSV-G with GM1 in patches on the plasma membrane.
There is a significant body of work on the lipid composition of the VSV envelope. It is clear that the envelope is enriched in cholesterol and sphingomyelin when compared to the membrane from which it buds (Patzer et al., 1978; Pesin & Glaser, 1980). The high cholesterol content leads to an increase in membrane rigidity (Lisi et al., 1993). Glycoprotein (G) and M protein can induce the formation of domains with a similar composition to the viral envelope when incorporated into large vesicles in vitro (Luan et al., 1995).

There is a significant body of work on the lipid composition of VSV. Pseudotyping of VSV has been observed with togaviruses, retroviruses, bunyaviruses, arenaviruses, paramyxoviruses, orthomyxoviruses, coronavirus, herpesviruses and poxviruses (Altstein et al., 1976; Calafat et al., 1983; Dragunova & Závada, 1979; Lukashevich & Závada, 1982; Pastorekova et al., 1992; Schnitzer et al., 1977; Zajac et al., 1980; Závada, 1972b; Závada et al., 1972, 1978, 1983; Zavadova & Závada, 1980). CD4 is incorporated efficiently (Schnell et al., 1996). Confusingly, although murine leukaemia virus envelope proteins can pseudotype with VSV (Witte & Baltimore, 1977), HIV-1 gp160 is excluded (Johnson et al., 1998; Owens & Rose, 1993). Mutagenesis revealed that if all of the gp160 cytoplasmic domain is removed, the protein is incorporated efficiently into VSV virions, but truncations that leave 10 or 29 amino acids are still excluded. Strangely, replacement of the cytoplasmic domain with that of CD4 (which is incorporated efficiently into VSV) does not permit incorporation (Johnson et al., 1998). Failure to incorporate correlates with a different cell surface localization to that of VSV-G. Johnson et al. (1998) propose that the cytoplasmic domain of gp160 contains a signal inhibiting co-localization with VSV-G and suggest that a similar signal is generated in the CD4 tail by a change in folding when it is in combination with the gp160 transmembrane domain. If VSV-G and gp160 are expressed in the absence of other VSV proteins, the differences in localization become less dramatic but nevertheless persist. It appears difficult to interpret these observations in the context of raft association. All three of the truncated tail constructs lack both palmitoylation sites, at least one of which appears necessary for raft association of gp160 (Rouso et al., 2000).

These results may perhaps be reconciled by suggesting that VSV proteins associate with raft-like membrane domains with a composition that differs to some extent from that of classical rafts. The properties of these domains would be influenced by the proteins within them, leading to the observed changes in localization of VSV-G and gp160 in the absence of VSV-M. The differences between these raft-like domains might also be blurred when raft-associated proteins were expressed at extremely high levels, such as during virus co-infections. Certain proteins, such as CD4, would be enriched in both types of domains, whereas others would be more specific to one or the other. This might provide a partial explanation for contradictory flotation results and also help to explain the exclusion of HIV gp160 from VSV virions.

The results of double infections in polar cells are informative. Rodriguez-Boulan et al. (1983) showed that influenza viruses, such as WSN, bud from the apical surface of polarized Madin–Darby canine kidney cells, while VSV buds from the basolateral surface. This has been demonstrated amply in singly infected cells (Fuller et al., 1984; Fuller, 1987; Pfeiffer et al., 1985; Roth et al., 1979; Simons & Fuller, 1985). During early infection in doubly infected cells, both viruses maintain their polar budding (Roth & Companys, 1981). At later times in infection, pseudotypic particles are formed in which WSN glycoproteins are incorporated into VSV virions (Roth & Companys, 1981). Up to three-quarters of VSV infectivity can be neutralized by anti-VSV antibody. The start of pseudotype formation correlates with the loss of tight junction integrity, and therefore polarity, as a result of the cytopathic effect of infection. Doubly infected non-polarized baby hamster kidney cells produce pseudotypes without any lag time. Hence, pseudotype formation requires that both glycoproteins be present in the same epithelial cell domain.

Other potential raft viruses

A number of measles virus proteins float in sucrose density gradient of Triton X-100-treated cells in a cholesterol-dependent fashion (Manie et al., 2000). Similar observations have been made for Ebola and Marburg viruses (Bavari et al., 2002). These filoviruses were also shown to incorporate GM1, but not the transferrin receptor. The viral glycoprotein and GM1 also co-localize on the cell surface. Respiratory syncytial virus has been shown to assemble within GM1-rich microdomains (Brown et al., 2002) and will form pseudotypes with VSV (Kahn et al., 1999, 2001).

The role of rafts in virus assembly

It is becoming clear that rafts play a role in the assembly of a number of different non-icosahedral viruses. One way of addressing this function of rafts is to contrast the mechanism of assembly of membrane viruses that appear to employ rafts with those that do not.

SFV is a typical example of an icosahedral virus that does not appear to utilize lipid rafts in its formation. SFV and other alphaviruses such as Sindbis virus and Ross River virus (Cheng et al., 1995; Kielian & Helenius, 1986; Schlesinger & Schlesinger, 1986; Zhang et al., 2002) assemble in a well-defined manner (Kielian, 1995; Kielian & Helenius, 1986; Kielian et al., 1990; Schlesinger & Schlesinger, 1986; Simons & Garoff, 1980; Simons & Warren, 1984). A capsid is formed in the cytoplasm during incorporation of the single strand of genomic RNA (Simons & Warren, 1984). The capsid has a T = 4 hexamer–pentamer arrangement involving 240 copies of the capsid protein surrounding the RNA (Fuller et al., 1995). The envelope proteins of the virus are transported through the trans-Golgi network, where they are processed
to generate the fusion active mature spike, to the cell surface. There is reasonable evidence that the spikes form a hexagonal array at the cell surface (von Bonsdorff & Harrison, 1978), which presents an array of spike tails on the cytoplasmic side of the bilayer. This acts as an interaction site for the capsid. The icosahedral nature of the final structure allows the visualization of the geometry. The assembled virus shows complementarity between the array of spikes and the capsid that allows precise interaction over the icosahedral surface (Fuller et al., 1995; Mancini et al., 2000). The hexagonal arrangement of the spikes is spaced so that it can interact with the hexamer–pentamer arrangement of the capsid proteins. This results in the incorporation of precisely 80 copies of the trimeric spike complex to match the 240 copies of capsid protein. The arrangement is complementary, since individual trimeric spikes interact with two hexamers and one pentamer of capsid (Fuller et al., 1995; Mancini et al., 2000). This complementarity yields a very precise control of composition. Loss of a single spike would result in lack of complementarity over the array and loss of interactions over several spikes (Lescar et al., 2001; Mancini et al., 2000). There are no convincing reports of the incorporation of unrelated virus envelope proteins into SFV.

This model for SFV assembly differs from the one suggested by the hybrid retrovirus particles in Fig. 1. An important distinction is that the curvature of the membrane in a non-icosahedral raft virus is accomplished both by protein–protein interactions of the internal proteins of the virus, and their interactions with the inner leaflet of the lipid bilayer. Compare this with an icosahedral virus such as SFV, where the curvature of the membrane is dictated exclusively by the curvature of the two organized protein layers that sandwich it. This gives a raft virus greater flexibility in both the protein composition of the viral membrane and the geometry of the virion. In addition, assembly is dependent on the composition of the lipid bilayer. Retroviruses are an extreme example of this. Virus-like particles can form by expression of Gag alone without the presence of the Env proteins. The geometry of the particles formed does not show the consistent shape or size expected for a non-raft virus.

The steps leading to assembly of a raft virus take advantage of the dynamic properties of lipid rafts and the fact that the viral proteins are raftophilic. The cellular membrane never has the smooth, random distribution of lipids and proteins described in the early fluid membrane model (Singer & Nicolson, 1972). This model is too simple a description, since cellular proteins and lipids will tend to form micro-domains of selected lipid composition. As shown in Fig. 3, pre-existing rafts are dynamic structures but they turn the smooth homogeneous fluid membrane of the early model into a landscape of locally varying lipid composition (1). Virus infection leads to the production of viral proteins and their expression at the cell surface (2). The affinity of the viral proteins for particular lipid populations will lead to recruitment of more of those lipids and their enrichment in the neighbourhood of the proteins (3). This will result in the formation of rafts enriched in lipids that have affinity for the viral protein and the recruitment of further lipids and proteins with similar affinity. This process would continue until the collection of viral proteins and their interaction with the inner leaflet of the membrane results in curvature and budding (4).

There are several consequences of such a model. Assembly
would not be dependent on interactions between viral capsid and transmembrane proteins, nor on a well-defined geometry or shape. Budding could result from the coalescence of separate domains so long as the result provided sufficient curvature to close the membrane. Cooperativity would be key to the budding process, but there is inherent flexibility in the protein composition of the resulting membrane. The pseudotypic paradox can be resolved by this limited flexibility. Gag and other M-like proteins would be raftophiles. Their recruitment by rafts and ability to recruit and concentrate other raftophiles is shared across many virus families. The protein layer forming under the membrane is selective, but not tightly so, and can incorporate proteins non-specifically or be found adjacent to a protein lattice of differing composition within the same raft. Pseudotyping is not a paradox if one gives up the search for a common protein structural feature that is shared between a broad range of viruses. Raft viruses are not precise builders like their non-raft counterparts and when they are sharing a crowded building site it is inevitable that they occasionally pick up each others’ bricks.

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