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Interactions Between Virus Proteins and Host Cell Membranes During the Viral Life Cycle

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The structure and function of cells are critically dependent on membranes, which not only separate the interior of the cell from its environment but also define the internal compartments. It is therefore not surprising that the major steps of the life cycle of viruses of animals and plants also depend on cellular membranes. Indeed, interactions of viral proteins with host cell membranes are important for viruses to enter into host cells, replicate their genome, and produce progeny particles. To replicate its genome, a virus first needs to cross the plasma membrane. Some viruses can also modify intracellular membranes of host cells to create a compartment in which genome replication will take place. Finally, some viruses acquire an envelope, which is derived either from the plasma membrane or an internal membrane of the host cell. This paper reviews recent findings on the interactions of viral proteins with host cell membranes during the viral life cycle.

KEY WORDS: Cell membranes, Viral entry, Viral replication, Virus budding, Protein–membrane interactions.

I. Introduction

Viruses are unable to replicate on their own, and they need the intracellular environment and energy supplies to replicate. Indeed, they use the host translation machinery to synthesize their proteins, and the cell provides structures and/or host factors to achieve the synthesis of viral genomes. They also use cellular proteins and/or structures for intracellular transport and posttranslational modifications. Due to the structural and functional

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roles of cell membranes, major steps of the life cycle of viruses also depend on cellular membranes. A classic viral life cycle can be divided into three stages: (1) early events (attachment to host cells, penetration, and uncoating) (2) viral biosynthetic events (replication of the viral genome, transcription, and translation), and (3) virion assembly and release (Fig. 1). Studying how viral proteins interact with host cell membranes is therefore important to understand how viruses enter host cells, replicate their genome, and produce progeny particles.

Because they are obligate intracellular parasites, viruses need to cross the plasma membrane in order to initiate their replication. To enter the cell, a

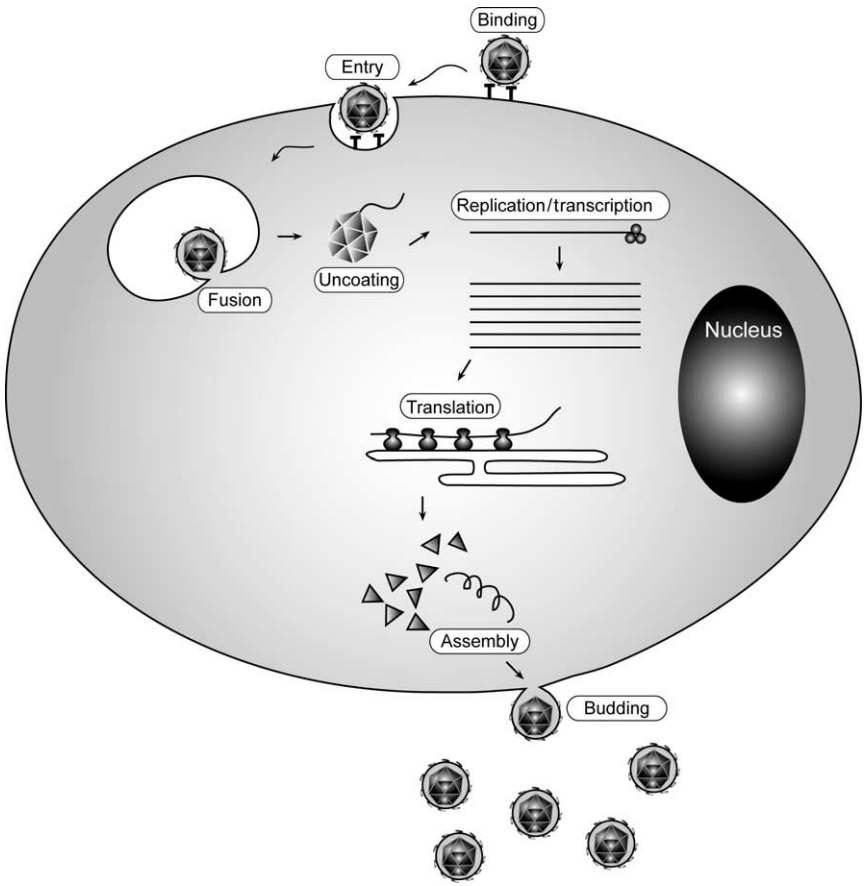

FIG. 1 Viral life cycle in eukaryotic cells. Viruses are obligate intracellular parasites. They enter host cells, they use host cell machineries to replicate their genome and synthesize their proteins, and they produce progeny virions that are released in the extracellular environment.

virus needs to bind a receptor and sometimes a coreceptor before delivering its genome into the cytosol by crossing either the plasma membrane or the membrane of an endocytic vesicle. For enveloped viruses, specialized envelope glycoproteins (fusion proteins) will trigger fusion of the viral membrane with the cell membrane. Recent structural data on some fusion proteins allow a better understanding of how these proteins interact with cell membranes to induce the fusion process.

Some viruses require membrane surfaces on which to assemble their replication complex and they lead to alterations that include proliferation and reorganization of some internal organelles. Depending on the virus family, these membranes can be derived from diverse subcellular compartments. Interestingly, many viruses also induce the formation of membrane-invaginating spherules, which play a major role in some steps of genomic replication and provide a microenvironment isolated from the cytosol. There is a growing interest in understanding how viral proteins interact with these membranes to modify their architecture and support genome replication.

Some viruses contain an envelope consisting of viral glycoproteins associated with a host cell-derived lipid bilayer. The viral envelope surrounds the nucleocapsid or core and is acquired at a late step of the viral life cycle, called budding. Depending on the virus species, budding can occur at the plasma membrane or an intracellular membrane of the secretory pathway. Virus budding is a complex phenomenon requiring concerted actions of many viral and host components. Various types of interactions are engaged in this process. They include interactions of viral proteins with the lipid bilayer as well as protein–protein interactions of viral components.

II. Interactions Between Virus Proteins and Host Cell Membranes During Entry

The viral genome has to go through a membrane barrier to gain access to the cytosol. For most viruses, entry into the cytosol is a multistep process, during which the host cell assists the incoming virus. Viruses first attach themselves to various components of the plasma membrane, which they use as nonspecific attachment factors or as specific cell surface receptors. Receptor binding activates cellular endocytic pathways through which viruses are internalized in endosomes. Viruses have evolved diverse mechanisms of entry, which are adapted to the variety of cellular endocytic routes. When they reach the appropriate intracellular location, they are activated for penetration by cellular signals and make their way through the membrane of the endosome, or through the plasma membrane for those that do not enter by endocytosis. Enveloped viruses fuse their lipid envelope with the plasma membrane or the

membrane of an endosome, resulting in the release of the capsid into the cytosol. For nonenveloped viruses, the penetration step occurs by disruption of the endosome, or through a pore formed in the plasma or endosomal membrane. In most cases, the penetration is under the control of structural components of the virion that are in a metastable state, and is induced by the relaxation of their conformation, when the cell provides the proper trigger. This activation step promotes the exposure of hydrophobic domains on the viral particle, which initiate membrane association and a cascade of conformational changes that leads to the transfer of the viral genome into the cytosol. Viral entry can be seen as an interplay between viruses and cellular membranes, with viruses being able to induce endocytosis and signaling in the target cell, and cells providing triggers to the viral penetration program.

A. Viral Attachment

The first step in virus entry is the attachment of the viral particle to the surface of a target cell. Viruses can bind to a variety of molecules on the cell surface, including membrane proteins, polysaccharides, and lipids. The interactions range from nonspecific attachment to abundant low-affinity sites, such as cell surface heparan sulfate proteoglycans, for example, to specific binding to high-affinity receptors, which are responsible for initiating endocytosis, for transducing specific signaling events into the cytoplasm of the cell, or for inducing conformational changes in the structure of the envelope glycoproteins of the virus that lead to virus entry. The tropism of a virus depends to a large extent on the nature and the tissue distribution of these high-affinity specific receptors.

1. Attachment Factors and Entry Receptors

The first contact of a virus with the membrane of a target cell may not occur by direct interaction with a specific receptor, but rather with a nonspecific attachment factor. Cell surface heparan sulfate proteoglycans play this role for many viruses. Interactions with heparan sulfate proteoglycans have been documented for herpesviruses (Spear and Longnecker, 2003), papillomaviruses (Giroglou *et al.*, 2001), paramyxovirus 3 (Bose and Banerjee, 2002), pestiviruses (Hulst *et al.*, 2000), flaviviruses (Chen *et al.*, 1997; Mandl *et al.*, 2001), Sindbis virus (Byrnes and Griffin, 1998; Klimstra *et al.*, 1998), adenoviruses (Dechecchi *et al.*, 2000), and adeno-associated viruses (Opie *et al.*, 2003; Summerford and Samulski, 1998). In most cases, these interactions are thought to be nonspecific and probably based on electrostatic attractions between the highly charged sulfated proteoglycans and arginine residues in viral particles (Chen *et al.*, 1997; Hulst *et al.*, 2000;

Klimstra *et al*., 1998; Mandl *et al*., 2001; Opie *et al*., 2003). They provide initial docking sites, which concentrate viruses in the vicinity of the cell surface and facilitate interactions with specific receptors. For some viruses, the ability to interact with heparan sulfate is an acquired adaptation to *in vitro* culture (Hulst *et al*., 2000; Klimstra *et al*., 1998; Mandl *et al*., 2001).

In addition to heparan sulfate proteoglycans, viruses may also interact with other types of carbohydrates. Gangliosides are receptors for murine polyomavirus and simian virus 40 (SV40) (Gilbert and Benjamin, 2004; Tsai *et al*., 2003). The polyomavirus major capsid protein VP1 binds to oligosaccharides terminating in α-2,3-linked sialic acid present on gangliosides (Stehle *et al*., 1994). Sialogangliosides may also function as initial attachment receptors for rotavirus (Dormitzer *et al*., 2002; Rolsma *et al*., 1998). Orthomyxoviruses and paramyxoviruses hemagglutinin (HA) glycoproteins bind sialic acid groups present on glycolipids or glycoproteins (Crennell *et al*., 2000; Skehel and Wiley, 2000). The affinity of a single interaction between an HA molecule of influenza virus or a VP1 monomer of the polyomavirus capsid and a sialic acid unit is rather low (Skehel and Wiley, 2000; Stehle *et al*., 1994). With respect to this low affinity, sialylated proteins and lipids are therefore similar to nonspecific attachment factors. However, a strong binding is achieved through multiple contacts between the virus, which is multimeric in nature, and sialic acids, which are abundant on the surface of the cell. The clustering of gangliosides triggers intracellular signaling that promotes the uptake of the viral particle. Therefore gangliosides also play the role of specific entry receptors. Influenza particles contain a second envelope glycoprotein with neuraminidase activity, which is called NA. The NA glycoprotein hydrolyzes sialic acid from oligosaccharides. Its function is to release the virus from the cell surface if the particle is not internalized after binding, and to facilitate the release of newly formed virions from the cell surface by removing receptors for the virus. Recent data indicate that although gangliosides do function as attachment factors for influenza virus, they are not sufficient to promote entry. Internalization requires additional unidentified N-linked glycoprotein(s) at a postbinding step, which may function as entry receptors (Chu and Whittaker, 2004).

2. Multiple Receptor Usage

Multiple receptor-binding events are often required for efficient entry. Adenovirus is an example of this process (Nemerow, 2000). Adenoviruses have a receptor responsible for the specific attachment to target cells and another one that induces endocytosis. A number of adenoviruses (group C) bind to the coxsackievirus and adenovirus receptor (CAR), a member of the immunoglobulin superfamily (Bergelson *et al*., 1997). CAR interacts with a conserved loop of the fiber protein of the virus and promotes its attachment

to the cell surface (Bewley *et al.*, 1999). However this binding is not sufficient to induce the uptake of the virus. Instead, integrin $\alpha_V\beta_3$ or $\alpha_V\beta_5$ association with a conserved RGD motif present on the penton base protein promotes virus internalization (Wickham *et al.*, 1993). Both receptors are required for efficient adenovirus entry.

Interestingly, fiber–CAR interactions have another function during adenovirus infection of airway epithelia (Walters *et al.*, 2002). CAR is a tight junction protein located at the basolateral plasma membrane in epithelia, and is engaged in homodimeric interactions between proteins located on adjacent cells. Following the infection of an individual cell of the epithelium, virions are initially released only to the basolateral surface. Together with the virus, a large excess of fiber protein is also released, which saturates CAR binding sites and dissociates CAR homodimers. This results in a local disruption of cell junctions in the epithelium, which facilitates adenovirus escape to the apical surface, and the spread of the virus.

As mentioned above, Herpes simplex virus 1 interacts first with heparan sulfate (Spear and Longnecker, 2003). This interaction is mediated by the glycoprotein gC. Then, more specific interactions occur between gD, another viral glycoprotein, and alternative cellular receptors. Cellular receptors for Herpes simplex virus 1 include nectin-1 and nectin-2, two intercellular adhesion molecules belonging to the immunoglobulin superfamily, and herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor family. Heparan sulfate could also serve as specific receptors initiating the entry of Herpes simplex virus 1 through the interaction of 3-*O*-sulfated monosaccharide units of the proteoglycans with gD (Shukla *et al.*, 1999). It is thought that the binding to specific receptors induces conformational changes in gD. These changes in turn activate the fusion machinery of the virion, which includes glycoproteins gH and gB, and eventually leads to the translocation of the nucleocapsid into the cytoplasm of the cell.

Another well-documented example of a virus that uses multiple receptors is the human immunodeficiency virus (HIV). HIV infects cells of the immune system via binding to cell surface CD4. When the envelope protein subunit gp120 binds to CD4, it undergoes a conformational change that allows the interaction with the coreceptor CXCR4 or CCR5 (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Feng *et al.*, 1996). The binding to these coreceptors is strain specific, but similarly triggers the conversion of the envelope subunit gp41 from its native inactive state to a fusion-competent conformation.

In addition, HIV can also bind to the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (Bashirova *et al.*, 2001; Geijtenbeek *et al.*, 2000). In contrast to CD4 binding, this interaction does not lead to conformational changes in the envelope glycoprotein and does not lead to infection of dendritic cells. It retains the virus in an infectious form and facilitates its delivery to permissive T cells.

Many other enveloped viruses interact with DC-SIGN and the related liver and lymph node-specific L-SIGN. DC-SIGN and L-SIGN are mannose-binding C-type lectins that bind high mannose N-linked glycans on the envelope glycoproteins of HIV-1 (Feinberg *et al*., 2001), Sindbis virus (Klimstra *et al*., 2003), Dengue virus (Navarro-Sanchez *et al*., 2003; Tassaneetrithep *et al*., 2003), human cytomegalovirus (Halary *et al*., 2002), hepatitis C virus (HCV) (Gardner *et al*., 2003; Lozach *et al*., 2003; Pohlmann *et al*., 2003), Ebola virus (Lin *et al*., 2003; Simmons *et al*., 2003a), and severe acute respiratory syndrome (SARS) coronavirus (Jeffers *et al*., 2004). Dengue virus, SARS coronavirus, Ebola virus, and Sindbis virus apparently use L-SIGN and/or DC-SIGN as an entry receptor, whereas HIV (and also probably HCV) can bind to the same lectins but do not use them to mediate virus entry. Interestingly, DC-SIGN is expressed in skin-epidermal Langerhans cells, which are the primary target cells of Dengue virus after an initial bite by an infected mosquito, and DC-SIGN interacts with insect cell-derived high-mannose N-linked glycans of Dengue virus envelope protein E (Navarro-Sanchez *et al*., 2003). Therefore, it has been suggested that DC-SIGN could be the receptor for Dengue virus transmitted by a mosquito bite.

B. Endocytosis

Endocytosis is the main entry route into host cells for a large number of animal viruses. One advantage of this mode of entry is that endocytic vesicles, in which viruses are packaged during their internalization, are designed to traverse the cortical actin cytoskeleton and travel inside the cell. In this way, the entire virus can be efficiently transported through the cytoplasm up to an intracellular location that is close to its site of replication, leaving no sign of its entry at the cell surface to be detected by the immune defenses of the host. Endocytic pathways may deliver viruses to different endosomal compartments from which they can penetrate into the cytosol, or be further transported to other intracellular compartments, such as the endoplasmic reticulum (ER) (Kartenbeck *et al*., 1989) or the Golgi complex (Bantel-Schaal *et al*., 2002). The advantage of being internalized in an endosome may also be important for nonenveloped viruses that use a lytic mechanism of penetration, like adenovirus (Greber *et al*., 1993), because rupture of an endosomal membrane may be less damaging to the host cell than rupture of the plasma membrane. For many viruses, the acidic environment of endosomes triggers the mechanism of penetration. Because acidic pH is usually not met outside the cell, endocytosis provides pH-dependent viruses a simple way to prevent inappropriate activation of the fusion. In contrast, viruses that enter directly through the plasma membrane must have pH-independent modes of entry. The reverse is not necessarily true. Viruses

that are pH independent may use endocytosis for entry (Breiner and Schaller, 2000; Gianni *et al.*, 2004).

Recent studies have revealed a surprising variety of cellular endocytic routes (Conner and Schmid, 2003; Pelkmans and Helenius, 2003; Sieczkarski and Whittaker, 2002a). It appears that viruses have evolved strategies adapted to this variety of entry routes. Endocytic pathways fall into two main types: those targeting "classic" acidic endosomes and those targeting newly discovered neutral compartments such as caveosomes and related structures (Fig. 2). The best-documented endocytic pathway begins with the formation of clathrin-coated vesicles at the plasma membrane. It delivers cargoes to early endosomes, from which they are sorted to various intracellular destinations including late endosomes and lysosomes. A second entry route is mediated by the internalization of caveolae from the cell surface. Extracellular ligands internalized in caveola-derived vesicles are not delivered to acidic endosomes but to caveosomes, which represent another class of intracellular endocytic compartments. Like early endosomes, caveosomes are sorting compartments. However, there is no evidence that the caveola/caveosome pathway leads to any lysosome-like degradative organelle. It is not yet clear how these two pathways are connected to each other. Other uncharacterized mechanisms of internalization from the plasma membrane may also deliver cargoes to early endosomes in a clathrin-independent manner, or to caveosome-like structures in a caveola-independent manner.

1. Clathrin-Mediated and Other Endocytic Pathways Targeting Acidic Endosomes

The role of clathrin-coated vesicles in virus internalization has been known for many years. Several viruses, including Semliki forest virus (Marsh and Helenius, 1980), influenza virus (Matlin *et al.*, 1981), vesicular stomatitis virus (VSV) (Matlin *et al.*, 1982), West Nile virus (Gollins and Porterfield, 1985), and many more were visualized in electron-dense coated vesicles at early times of infection by transmission electron microscopy. However viral particles were also detected in uncoated vesicles during these experiments. These uncoated structures could originate from the release of the clathrin coat from clathrin-coated vesicles or from a non-clathrin-mediated internalization pathway.

The functional importance of clathrin in virus entry has been assessed by microinjection of anticlathrin antibodies (Doxsey *et al.*, 1987) or by expression of dominant-negative forms of dynamin (DeTulleo and Kirchhausen, 1998) or Eps15 (Sieczkarski and Whittaker, 2002a). Dynamin is a large GTPase required for the pinching off of vesicular carriers of several endocytic pathways from the plasma membrane (Hinshaw, 2000). Dominant-negative forms of dynamin may thus be used to probe the requirement for endocytosis

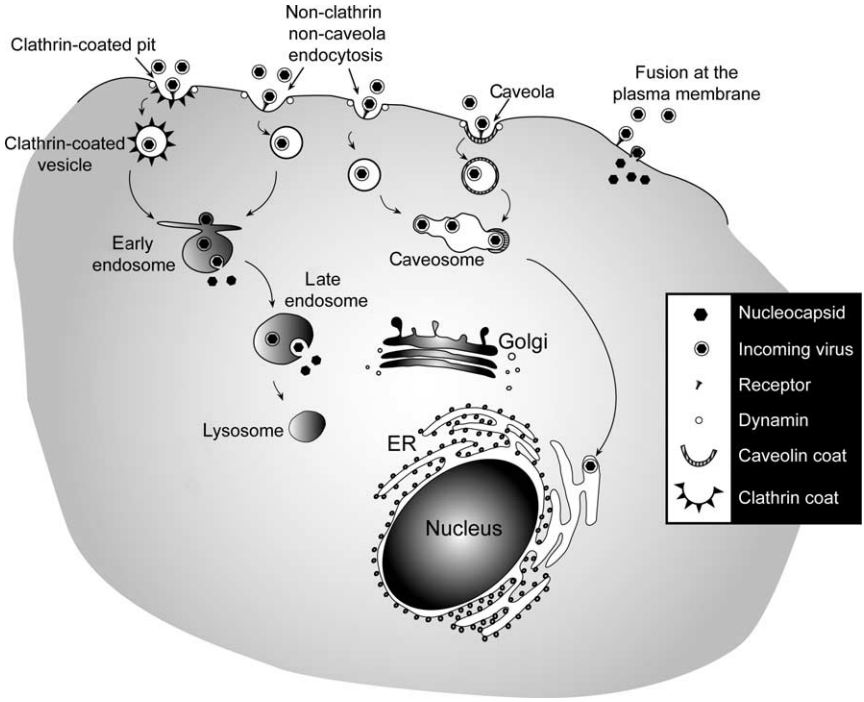

FIG. 2 Entry routes of viruses into host cells. Incoming viruses bind to attachment factors and entry receptors at the plasma membrane. Receptor binding may trigger endocytosis or direct entry at the plasma membrane. Four endocytic routes have been described for viruses. Many enveloped and nonenveloped viruses are internalized from the plasma membrane in clathrin-coated vesicles. They are transported to early endosomes, and for some of them to late endosomes. At low endosomal pH, viral proteins undergo conformational changes that initiate the penetration of the nucleocapsid into the cytoplasm by fusion of the viral envelope with the endosomal membrane for enveloped viruses, or by endosomal lysis or formation of a pore-like structure for nonenveloped viruses. Other viruses are internalized from the plasma membrane via caveolar endocytosis. They are delivered to caveosomes from which they are sorted to other intracellular destinations, such as the smooth endoplasmic reticulum (ER) for SV40, for penetration into the cytoplasm. Unlike early and late endosomes, caveosomes have a neutral content. Nonclathrin, noncaveola endocytic routes also occur, which appear to traffic viruses toward early and late endosomes or toward caveosome-like structures. Most of these endocytic routes are dependent on dynamin function.

(although there are a few examples of dynamin-independent endocytosis), but are not necessarily indicative of a clathrin-mediated uptake. On the other hand Eps15 dominant-negative forms can be used to probe functionally clathrin-mediated endocytosis. Eps15 is a protein that associates with AP-2, the major adaptor complex of clathrin-coated pits at the plasma

membrane. The overexpression of truncated forms of Eps15 imposes a strong blockade of clathrin-mediated endocytosis without affecting other internalization pathways and other intracellular transport steps controlled by clathrin (Benmerah *et al.*, 1998; Carbone *et al.*, 1997). Clathrin-mediated endocytosis requirement for entry has been demonstrated with the use of dominant-negative forms of Eps15 for several viruses including Sindbis virus (Carbone *et al.*, 1997), Semliki forest virus (Sieczkarski and Whittaker, 2002b), adenovirus-2 (Meier *et al.*, 2002), Hantaan virus (Jin *et al.*, 2002), JC virus (Querbes *et al.*, 2004), West Nile virus (Chu and Ng, 2004), and bovine viral diarrhea virus (Lecot *et al.*, 2005). Dominant-negative forms of other proteins of the clathrin coat at the plasma membrane were used to demonstrate clathrin-mediated entry of rhinovirus type 2 (Snyers *et al.*, 2003).

With the use of a dominant negative form of Eps15 as a functional probe for clathrin-mediated endocytosis, the entry of influenza virus was recently revisited. In electron microscopy studies, incoming influenza virions were detected in clathrin-coated pits and vesicles and in another type of uncoated membrane invaginations and smooth-walled vesicles, which were negative for incoming VSV (Matlin *et al.*, 1981). The suppression of clathrin function with Eps15 mutants did not inhibit influenza virus infection (Sieczkarski and Whittaker, 2002b). This led to the hypothesis that influenza virus may use either clathrin or nonclathrin endocytic pathways for productive infection. More recently, this hypothesis was confirmed by the direct observation of the uptake of fluorescently labeled influenza virions in cells expressing YFP-tagged clathrin light chain, using dual color video microscopy (Rust *et al.*, 2004). Incoming virions were shown to enter both through clathrin-coated vesicles and through a clathrin-independent pathway of internalization in the same cell. The incoming virions that were internalized by a clathrin-independent mechanism were not associated with caveolae. The alternative endocytic pathway of influenza virus is still poorly characterized and it is not known how it is related to the uncoated vesicles that were originally detected in electron microscopy (Matlin *et al.*, 1981). Influenza can enter even in the presence of both Eps15 dominant-negative mutant and cholesterol-sequestering drugs (Sieczkarski and Whittaker, 2002b). This indicates that influenza virus can enter by a clathrin- and lipid raft-independent pathway. Both influenza virus entry pathways appear to deliver viruses to acidic endosomes.

The arenavirus lymphocytic choriomeningitis virus is another example of a virus that can enter by a clathrin-independent pathway. Lymphocytic choriomeningitis virus entry is still poorly characterized. Inhibition studies and immunoelectron microscopy indicated that the entry process involves a pH-dependent fusion step and that virions are internalized in uncoated vesicles (Borrow and Oldstone, 1994). The uncoated vesicles containing

incoming lymphocytic choriomeningitis virus particles are reminiscent of the uncoated vesicular carriers observed during influenza virus entry (Matlin *et al.*, 1981). It is not known at the present time if the entry route of lymphocytic choriomeningitis virus is related to the alternative endocytic pathway of influenza virus. Future studies with this and other viruses may provide new insights on novel endocytic pathways.

2. Endocytic Transport After Internalization

Following entry by clathrin-mediated or alternative pathways, viral particles are delivered to early endosomes. Viruses may penetrate from this compartment, or be transported to late endosomes. The risk for a virus that is entering by this route is to be transported to lysosomes, the degradative compartment normally targeted by the endocytic pathway. To avoid lysosomal delivery, viruses that enter by this endocytic route have evolved mechanisms of penetration adapted to the environment of endosomes. Many viruses are activated by acidic endosomal pH, and the threshold of pH for activation usually matches the pH values of early or late endosomes. The endosomal compartment where the fusion occurs may be experimentally characterized with the GTPases Rab5 and Rab7. Rab family members are small Ras-like GTPases, which regulate membrane traffic events in the endocytic and secretory pathways. Overexpression of a dominant-negative form of Rab5, which controls entry into early endosomes, decreases the uptake of adenovirus (Rauma *et al.*, 1999), rhinovirus (Snyers *et al.*, 2003), influenza virus, Semliki forest virus, and VSV (Sieczkarski and Whittaker, 2003). Influenza virus is also sensitive to a dominant-negative form of Rab7, which controls the transport to late endosomes (Sieczkarski and Whittaker, 2003). In contrast, Semliki forest virus and VSV are not affected. These observations indicate that influenza virus must reach late endosomes before being activated for fusion, and that it transits in early endosomes on route to late endosomes, whereas Semliki forest virus and VSV can fusion from within early endosomes. This is consistent with the threshold pH for the activation of influenza virus fusion protein, which is about 5.0–5.5 (White *et al.*, 1981), and is in the range of pH that is found in late but not in early endosomes. For Semliki forest virus and VSV, the fusion is induced at slightly higher pH, 6.0 and 6.1, respectively (White *et al.*, 1981), which fit with the pH of early endosomes.

An acidic pH may not be the only factor important for penetration that the endosomal environment provides to the incoming virus. A short exposure of West Nile virus docked at the plasma membrane in conditions where endocytosis is inhibited induces the fusion of the envelope with the plasma membrane but does not lead to a productive infection (Gollins and Porterfield, 1986), whereas a similar low pH-induced fusion at the plasma

membrane leads to a productive infection for Semliki forest virus (White *et al.*, 1980). This suggests that the endocytic entry provides additional factors that are essential for a productive West Nile virus infection. Even for Semliki forest virus, it has been suggested that the ionic content of endosomes may be essential for envelope fusion, in addition to acidic pH (Helenius *et al.*, 1985).

3. Caveolae and Lipid Raft-Mediated Pathways

Another cellular entry route that is provided by cells to incoming viruses is the internalization of caveolae. Caveolae are small invaginations of the plasma membrane. They represent a subdomain of cholesterol- and sphingolipid-rich lipid rafts that are specifically associated with the cholesterol-binding protein caveolin. Virus entry through caveolae was described with the study of SV40 (Anderson *et al.*, 1996; Pelkmans *et al.*, 2001). After binding to the cell surface SV40 is quickly transferred to preexisting caveolae. Caveolae usually are immobile at the surface of the cell, but may be induced to detach themselves from the plasma membrane by SV40-triggered, tyrosine kinase-mediated signaling and to move inside the cell. Caveolae internalization requires dynamin function, involves actin remodeling, and is independent of clathrin-mediated endocytosis. Caveolar endocytosis is characterized by a lag period at the cell surface before internalization and by slow kinetics. Caveolar endocytosis is also sensitive to agents that disrupt lipid rafts by altering the cholesterol content of membranes. However, this criterion alone cannot be used to define caveolar entry, because other endocytic pathways are also sensitive to cholesterol depletion. Caveolae are also used for entry by BK virus (Eash *et al.*, 2004) and human echovirus type-1 (Pietiainen *et al.*, 2004). In some cell types, polyomavirus could also enter through caveolae endocytosis (Gilbert and Benjamin, 2004). Based on studies with Ebola virus and Marburg virus GP-pseudotyped virions, caveolae-mediated entry of filoviruses was proposed (Empig and Goldsmith, 2002). However, the entry pathway of Ebola virus still remains controversial (Simmons *et al.*, 2003b).

SV40 is quite unusual, in that incoming virions accumulate in the smooth ER (Kartenbeck *et al.*, 1989). Video microscopy studies revealed that virions internalized in caveola-derived vesicles are initially delivered to caveosomes and bypass "classic" early endosomes. Unlike early endosomes, caveosomes have a neutral pH and do not receive cargoes internalized by clathrin-coated vesicles. Caveosomes are sorting compartments from which SV40 virions are transported toward the ER by caveolin-negative vesicular carriers (Pelkmans *et al.*, 2001). The penetration into the cytosol is thought to occur through the ER membrane. The cellular signal that triggers the penetration of SV40 in the ER and the mechanism by which SV40 actually

traverses the ER membrane are unknown. SV40 particles are then transported to the nucleus through the nuclear pore.

In addition to caveolae, cells are endowed with other lipid raft-dependent endocytic pathways that deliver endocytosed material in caveosomes or caveosome-like structures (Nichols, 2002; Pelkmans and Helenius, 2003). These pathways, which are still very poorly characterized, may be used by polyomavirus (Gilbert *et al*., 2003) and by SV40 in cells that lack caveolae (Damm *et al*., 2005).

4. Signaling During Entry

The endocytosis of most membrane proteins is inducible and regulated. It is very likely that viruses and their receptors are no exception to this rule. Upon binding to their receptors, viruses can induce various cellular signaling pathways. Because of the multimeric nature of viruses, receptor clustering probably is a common strategy for viruses to induce intracellular signaling.

Studies from adenovirus, SV40, and several other viruses have provided evidence that viruses stimulate host cell signaling pathways that will in turn regulate their endocytosis and intracellular transport. SV40 activates tyrosine kinase-induced signaling events from within caveolae that lead to local rearrangements of the actin cytoskeleton (Pelkmans *et al*., 2002) and the enclosure and internalization of caveolae (Chen and Norkin, 1999; Pelkmans *et al*., 2001). During its intracellular transport, SV40 also activates signaling events from within caveosomes. This second set of signaling appears necessary for the sorting of the virus away from caveolin-containing membrane domains and its further transport toward ER (Pelkmans and Helenius, 2003). Tyrosine kinase-regulated endocytosis has also been documented for other viruses, like JC virus, which is internalized by clathrin-coated vesicles (Querbes *et al*., 2004), and human cytomegalovirus, which activates epidermal growth factor receptor- and integrin-mediated signaling pathways (Evers *et al*., 2004).

Adenovirus entry requires the function of phosphoinositol-3-OH kinase (PI3K) and protein kinase C (PKC). The binding to cell surface integrins activates PI3K, the inhibition of which reduces adenovirus endocytosis (Li *et al*., 1998b). Downstream targets of PI3K activation, like Rho family GTPases Rac and Cdc42, are also activated by adenovirus. These small GTPases regulate the dynamics of cortical actin cytoskeleton and participate in adenovirus entry (Li *et al*., 1998a; Meier *et al*., 2002).

Adenovirus binding to integrin is involved in adenovirus internalization (Wickham *et al*., 1993) and membrane permeabilization (Wickham *et al*., 1994). These actions are consistent with the role of PKC in integrin endocytosis (Panetti *et al*., 1995) and in endosomal acidification (Zen *et al*., 1992). The activation of PKC by adenovirus binding to integrins also

stimulates macropinocytosis (Meier *et al.*, 2002). However, the role of macropinocytosis in adenovirus entry is not clear at the present time, and may be a side effect of the activation of PKC signaling pathways by adenovirus. PKC inhibitors impair the entry of several enveloped viruses (Constantinescu *et al.*, 1991). Influenza virus entry is inhibited by PKC inhibitors at a postinternalization step. More specifically, cells overexpressing a dominant-negative form of PKC-βII accumulate incoming influenza virions in late endosomes, with no apparent defect in endosome acidification (Sieczkarski *et al.*, 2003).

C. Viral Penetration

Following receptor binding and endocytosis, virus entry is completed with the transfer of the viral genome across a cellular membrane. This penetration step is often coupled with the uncoating of the genome for RNA viruses that replicate in the cytosol. The penetration of enveloped viruses occurs by fusion of the viral envelope with a cellular membrane, either the plasma membrane or the membrane of an endosome. The fusion creates a physical continuity between the interior of the virion and the cytoplasm of the cell. When the fusion is completed, the viral core is delivered to the cytosol. The process is similar in principle to cellular membrane fusion events, the protein machinery involved in virus entry being much simpler. In this manner, the viral genome does not have to be physically transferred across the membrane. For nonenveloped viruses, the process is completely different. They must breach the membrane barrier to transfer their genome into the cytoplasm. The mechanisms of penetration of nonenveloped viruses are less understood than those of enveloped viruses. They probably involve the lysis of the membrane or the formation of a pore-like structure. As for enveloped viruses, the penetration step of nonenveloped viruses is triggered by cellular signals, such as receptor binding or the endosomal environment.

1. Mechanisms of Membrane Fusion

The fusion of the viral envelope with a cellular membrane is catalyzed by fusion proteins. Most fusion proteins contain a large ectodomain, a single transmembrane domain, and a short cytoplasmic tail. Fusion proteins are present on the surface of the virion in a metastable state. The ectodomain contains a fusion peptide, which is a short hydrophobic sequence frequently rich in glycine and alanine residues. In the metastable state, the fusion peptide is buried inside the structure of the fusion protein ectodomain. Following activation, the fusion peptide is exposed and inserts into the target membrane. The free energy released from the metastable state during the

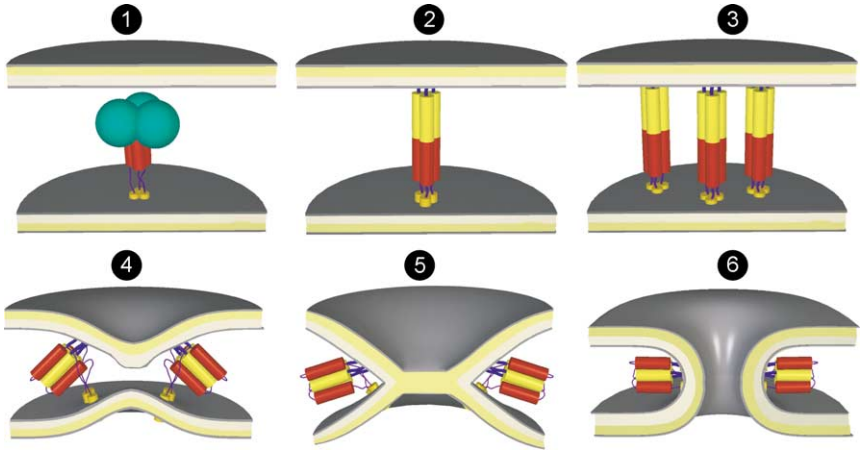

FIG. 3 Mechanism of membrane fusion induced by viral envelope proteins. Class I fusion proteins form trimers on the surface of the viral envelope, with fusion peptides buried within the trimer interface (1). In the case of retroviral fusion proteins, upon exposure to receptor, the envelope proteins undergo conformational changes leading to exposure of fusion peptides, which then insert into the target membrane (2). Multiple fusion proteins may cluster to form a fusion site (3). Additional conformational changes lead to the formation of a six-helix bundle (4), resulting in hemifusion, and mixing of the outer leaflets of the viral and cellular membranes (5). A fusion pore then forms (6), and enlarges.

activation is then converted into a conformational change of the fusion protein that forces the membranes in a close proximity.

Models predict that membrane fusion proceeds in two steps (Chernomordik and Kozlov, 2003). During a first step, both lipid bilayers are bent toward one another (Fig. 3). Lipid rearrangements in the external leaflet of each membrane (the external leaflet being the one in contact with the other membrane) facilitate the formation of protrusions, which culminates with the merging of both external leaflets. This results in the formation of a stalk between both membranes. This stalk generates a local hemifusion, in which a physical continuity between the external leaflets occurs without any merging of internal leaflets. A local hemifusion diaphragm may be formed by the apposed internal leaflets. In a second step of the fusion process, small pores are transiently formed in the bilayer of the hemifusion diaphragm. When one of these small pores expands or if several of them merge, the continuity of both bilayers is restored and the fusion is completed. The content mixing (which allows for the transfer of the viral genome into the cytoplasm) is only possible after the completion of this second step.

Biological membranes do not spontaneously bend or undergo pore formation. Thus the function of viral fusion proteins probably involves the generation of local rearrangements in lipid bilayers that foster the formation of the

stalk during step one and of the pore during step two, in addition to pulling the membranes toward one another. Experimental evidence has been documented in favor of these functions with HIV fusion protein Env (Markosyan *et al.*, 2003; Melikyan *et al.*, 2000). Mutations in viral fusion proteins have been reported, which block various steps of the fusion process. When the transmembrane domain of influenza virus fusion protein HA is replaced with a glycosylphosphatidylinositol anchor, the fusion reaction is blocked at the hemifusion state (Kemble *et al.*, 1994; Melikyan *et al.*, 1997). The length of the transmembrane domain is also critical for the transition from hemifusion to fusion (Armstrong *et al.*, 2000).

In addition, the lipid content of fusing membranes is critical for some viruses. Alphaviruses require cholesterol and sphingolipids in the target membrane for fusion (Kielian and Helenius, 1984; Nieva *et al.*, 1994). Cholesterol promotes membrane binding and trimerization of the fusion protein of alphaviruses and flaviviruses (Stiasny *et al.*, 2003). The fusion proteins of these viruses directly interact with cholesterol, and the interactions involve the 3β-hydroxyl group at position C-3 of the sterol. For influenza virus and HIV, the cholesterol content of the viral envelope, rather than that of the target membrane, is essential for fusion (Guyader *et al.*, 2002; Sun and Whittaker, 2003). All these observations support the notion that the fusion is a complex multistep process of lipid–protein interactions.

Fusion peptides are critical to the function of fusion proteins. They have the unusual property of being able to insert themselves into target membranes only after the activation of the fusion protein. Once inserted, they function as stable membrane anchors but they probably also function at different steps of the fusion by producing local lipid rearrangements that facilitate the formation of the stalk and of the hemifusion pore. Fusion peptides may be located at the N-terminus or in an internal loop of the fusion protein. Their sequences are quite different among different virus families, but within a single family, sequences are highly conserved among different virus strains. The structure of a synthetic influenza HA fusion peptide (an N-terminal fusion peptide) docked to lipid bilayers has been determined by nuclear magnetic resonance (NMR) and by site-directed spin-labeled electron paramagnetic resonance (EPR) spectroscopy (Han *et al.*, 2001). At pH 5, which corresponds to HA fusogenic conformation, the fusion peptide has a V-shaped form, made of two amphipathic helices separated by a turn. Each helix inserts obliquely into the outer leaflet of the membrane and does not penetrate the inner leaflet. When the first residue of its fusion peptide is changed from a glycine to a serine, HA only mediates lipid exchange but no content mixing in *in vitro* fusion assays, indicating that the fusion mediated by this mutant is blocked at the hemifusion step (Qiao *et al.*, 1999). Interestingly, the membrane insertion of this mutant fusion peptide appears different from that of the wild type (Li *et al.*, 2003). These

observations indicate that fusion peptides do not associate with membranes as transmembrane domains do. It is likely that whether they are located at the N-terminus of the fusion protein or in an internal loop, fusion peptides do not span the lipid bilayer. It is proposed that such a mode of insertion could perturb lipid packing and facilitate lipid mixing between juxtaposed membranes (Han *et al*., 2001).

2. Type I Fusion Proteins

Structural data have revealed two different types of fusion proteins. Type I fusion proteins are present on the surface of the virion as spike glycoproteins, which project perpendicularly to the viral membrane. The influenza virus HA glycoprotein is the best documented type I fusion protein (Skehel and Wiley, 2000). HA is a homotrimer that is held together largely by coil–coil interactions between long α-helices. HA is posttranslationally processed in a late step during the assembly of the viral particle. The proteolytic cleavage leaves the fusion peptide at the N-terminus of the membrane-anchored fusion subunit HA2, which remains disulfide bound to the receptor-binding subunit HA1. During entry, the acidic pH of late endosomes induces a dramatic conformational change, which mediates the fusion. The final postfusion state of HA is a highly stable conformation, referred to as trimer of hairpins, with the fusion peptide and the transmembrane domain located at the same end of the molecule. This suggests that the protein folds back on itself following its low pH-induced activation. Type I fusion proteins are also found in retroviruses, paramyxoviruses, filoviruses, and coronaviruses. Like HA, they are all cleaved into two subunits by posttranslational proteolytic processing and the cleavage is thought to generate the metastable state required for their fusion-promoting function. The postfusion conformations of HIV gp41 (Eckert and Kim, 2001) and of paramyxovirus SV5 fusion protein F (Baker *et al*., 1999) are also trimers of hairpins, suggesting that pH-dependent and pH-independent type I fusion proteins mediate membrane fusion by very similar mechanisms.

3. Type II Fusion Proteins

Alphaviruses and flaviviruses are endowed with type II fusion proteins. In contrast to type I proteins, type II envelope proteins are synthesized as heterodimers with another membrane protein, which usually plays a role of chaperone during the folding. In flaviviruses, the fusion protein E is later released by proteolytic cleavage of the associated protein prM in the trans-Golgi network, before secretion of the virus. In alphaviruses, the fusion protein E1 remains associated with the glycoprotein E2 in the envelope of the mature virion.

Under native conditions, type II fusion proteins do not form homotrimeric spikes on the surface of the virion. They are associated as homodimers, which are positioned parallel to the viral membrane. Their ectodomains are associated in an antiparallel manner in the homodimer (Kuhn *et al.*, 2002; Lescar *et al.*, 2001). The conformation dramatically changes when they are exposed to an acidic environment such as the inside of endosomal compartments (Allison *et al.*, 1995; Wahlberg and Garoff, 1992). The homodimers dissociate to acquire their fusogenic conformation. Following this dissociation, type II fusion proteins reorient themselves, rise up away from the bilayer of the viral envelope, and form homotrimers. An internal fusion peptide is exposed, which can insert into the target membrane (Gibbons *et al.*, 2003). The insertion of the fusion peptide into a target membrane facilitates the formation of homotrimers (Heinz and Allison, 2000). Following membrane insertion and homotrimerization, type II fusion proteins fold back and adopt a postfusion conformation relatively similar to the trimer of hairpin conformation of type I fusion proteins, in which the transmembrane domain and the fusion peptide are in close proximity (Bressanelli *et al.*, 2004; Gibbons *et al.*, 2004; Modis *et al.*, 2004).

4. Mechanisms of Activation

In addition to structural criteria, fusion proteins can also be categorized according to mechanisms of activation. For some viruses, the interaction with specific receptors and coreceptors is essential for activation, whereas for other viruses it does not play any role. As previously mentioned, a well-documented mode of activation is the exposure to low pH. A third mechanism was recently proposed, which combines the consecutive priming of the fusion protein through receptor interactions at the cell surface and further activation at low pH in endosomes (Mothes *et al.*, 2000).

Enveloped viruses that are activated at low pH include alphaviruses, flaviviruses, orthomyxoviruses, rhabdoviruses, bunyaviruses, and filoviruses. The requirement for low pH can be experimentally assessed with drugs that interfere with endosome acidification, such as bafilomycin or lysosomotropic agents (Mothes *et al.*, 2000), or by briefly warming up in acidic medium viruses that were prebound to cells (White *et al.*, 1980). Most viruses with a pH-dependent mechanism of fusion are inactivated by pretreatment at low pH in the absence of cells. It is thought that under acidic conditions, pH-dependent fusion proteins undergo irreversible conformational changes, which prevent any further activation. This property can be used to assess whether a virus requires low pH to fuse. However, some pH-dependent fusion proteins, such as VSV glycoprotein G, are not inactivated by low pH pretreatment (Puri *et al.*, 1988).

Viruses that do not require low pH to fuse include herpesviruses, paramyxoviruses, poxviruses, and most retroviruses. These viruses are often

referred to as pH-independent viruses. The fusion proteins of these viruses are activated by direct or indirect interactions with specific receptors and coreceptors. As mentioned before, the activation of HIV gp41 occurs through sequential binding of the receptor-binding subunit gp120 with CD4 and CCR4 or CXCR5 (Eckert and Kim, 2001). For other pH-independent viruses, the receptor-binding domain and the fusion protein are not subunits of a single envelope protein. Paramyxoviruses express an attachment protein and a fusion protein at the surface of the virion. The binding of the attachment protein to a cell receptor probably induces conformational changes in the attachment protein, which in turn cause the activation of the fusion protein (Lamb and Kolakofsky, 2001). A similar mode of activation probably also occurs in herpesviruses (Cocchi *et al.*, 2004).

A third type of activation mechanism was recently proposed, based on the study of a retrovirus, the avian leukosis virus (Mothes *et al.*, 2000). Like pH-dependent fusion proteins, avian leukosis virus fusion protein Env requires a low pH step for activation. However, in contrast to influenza HA and other pH-dependent fusion proteins, the low pH activation step is only possible after interaction with a receptor. This mode of activation combines a step of "priming" by receptor binding followed by a step of activation by low pH exposure. The receptor-binding step at neutral pH promotes the insertion of the fusion peptide into the target membrane (Hernandez *et al.*, 1997). Membrane fusion is triggered by the low pH exposure step (Melikyan *et al.*, 2004). The two consecutive steps induce different conformational changes in Env (Matsuyama *et al.*, 2004).

5. Mechanisms of Penetration of Nonenveloped Viruses

As noted above, the mechanisms of penetration of nonenveloped viruses are very different from those of enveloped viruses. However, the molecular mechanisms that lead to the activation of viral proteins present some similarities. Like enveloped viruses, nonenveloped viruses are activated for penetration by receptor binding and/or low pH in endosomes. These triggering signals induce conformational changes in structural proteins of the particle. It has been proposed that viral proteins of nonenveloped viruses are present in the infectious particle in a metastable state, and that the release from the metastable state leads to the exposure of membrane-interacting hydrophobic domains initially located in an inner part of the viral protein (Hogle, 2002). According to this model, the release from a metastable state leading to more hydrophobic viral particles that are able to interact with cellular membranes is a common paradigm in enveloped and nonenveloped virus entry.

In the case of poliovirus, the penetration is triggered by interaction with the receptor. Changes in the conformation of the viral particle have been identified (Hogle, 2002). It is probable that these conformational changes

occur directly at the plasma membrane, because poliovirus infection does not require endocytosis (DeTulleo and Kirchhausen, 1998). They lead to the formation of the so-called A particle, which is more hydrophobic and protease sensitive than the native particle. On the A particle, the hydrophobic N-terminus of the capsid protein VP1 and a myristate group of VP4 are exposed and allow interaction with the membrane. Following membrane insertion, other conformational changes lead to the formation of a pore through which the genomic RNA translocates.

The penetration of reovirus is similar to that of poliovirus in that it also involves structural changes in a protein of the virion, which result in the formation of a more hydrophobic viral particle prone to interact with membranes (Chandran and Nibert, 2003). Reovirus penetration is under the control of the μ1 viral protein. During penetration, conformational changes occur in μ1, which lead to the exposure of the myristylated N-terminal hydrophobic peptide. Activated μ1 then inserts in the membrane of the endosome. This results in the transfer of the core into the cytosol of the target cell by an unknown mechanism. A major difference between poliovirus and reovirus penetration lies in the mode of activation. Whereas poliovirus is activated by receptor interactions, reovirus penetration is initially triggered by the endosomal environment. However, in the case of reovirus the acidic pH of endosomes is not responsible for the activation. Reovirus penetration is triggered by the proteolytic degradation of σ3, a protein that interacts with μ1 in the viral particle. This proteolytic degradation of σ3, which releases μ1 in its activated form, is mediated by endosomal cathepsins L and B (Ebert *et al*., 2002).

In the case of rotavirus entry, conformational transformations associated with membrane penetration have been defined by structural data. Two consecutive transformations occur in VP4, the major spike protein of the virion (Dormitzer *et al*., 2004). VP4 is first primed by trypsin cleavage in the intestinal lumen before cell entry. This converts VP4 from a flexible undefined state into spikes made of VP4 dimers, which project away from the surface of the virion and present receptor-binding sites at the top of the spike. Then, unknown cellular events trigger dramatic structural rearrangements in primed VP4 during entry. VP4 associates in trimers through coil–coil interactions of α-helices, a potential membrane-interacting loop rich in glycine and hydrophobic residues is exposed at the top of the spike by shedding of the receptor-binding subunit, and the trimer of cleaved VP4 adopts a final conformation in which each subunit folds back in a conformation reminiscent of trimers of hairpins formed by membrane fusion proteins of enveloped viruses. The mechanism of action of rotavirus VP4 is thus very similar in principle to that of fusion proteins, with a proteolytic cleavage step that activates VP4 and a cellular signal that triggers structural transformation of the protein and the formation of a final, folded-back conformation, in

which the membrane-interacting loop is located close to the C-terminal domain that anchors the protein in the virion. How this structural transformation actually leads to the transfer of the virion across the membrane is not yet understood.

The effector mechanisms of nonenveloped virus penetration are still poorly characterized. It is generally assumed that it involves the formation of a pore-like structure, as in poliovirus entry (Hogle, 2002), or the lysis of the endosome, as in adenovirus entry (FitzGerald *et al.*, 1983). The lytic activity of adenovirus capsid is borne by the penton protein. Rhinoviruses appear to use either pore formation or membrane rupture in endosomes for endosomal escape, depending on the serotype (Schober *et al.*, 1998). However, the molecular mechanisms leading to endosomal membrane rupture are not understood. For parvoviruses, a phospholipase A_2 activity has been identified in the N-terminal extension of the capsid protein VP1 (Zadori *et al.*, 2001). This phospholipase is required for endosomal escape. It is proposed that this domain, which is positioned inside the capsid in the native virion, is exposed following exposure to low pH in late endosomes, and acts on phospholipids of the endosomal membrane to facilitate the egress of the particle.

III. Interactions Between Virus Proteins and Host Cell Membranes During Genomic Replication

Some viruses require membrane surfaces on which to assemble their replication complex. Such interactions have been well documented for positive-strand RNA viruses. These viruses share fundamental similarities in genome replication despite apparent differences in genomic organization, particle morphology, and host range. Members of this group encapsidate positive-stranded RNA genomes, and replicate in the cytoplasm through negative-stranded RNA intermediates, with no DNA phase. Upon entering the cell, the messenger-sense genomes are translated by cellular machinery to yield viral structural and nonstructural proteins. Whereas structural proteins and genomic RNA are incorporated in the next viral progeny, nonstructural proteins are required for proteolytic processing of viral precursors, and for the replication of the viral RNA. To carry out these functions, all or some of the nonstructural proteins will direct the assembly of a multisubunit ribonucleoprotein structure called RNA replication complex, which is usually associated with host-derived membranes. The assembly of these complexes involves specific interactions between virus and host factors, and often results in the proliferation and rearrangement of cellular membranes within the infected cell. Thus, by associating with cellular membranes, replication

complexes not only provide a microenvironment required for the multiple reactions but also may generate the recruitment of cellular factors to regulate viral genome replication.

Within the replication complex, the viral RNA directs genome replication by one of the nonstructural proteins: the RNA-dependent RNA polymerase, in addition to other nonstructural accessory proteins and cellular factors involved in RNA synthesis. First, the genomic RNA serves as a template to produce a negative-stranded RNA. This negative-stranded RNA synthesis is thought to result in a double-stranded RNA intermediate called a replicative form. Second, negative-stranded RNA is used to generate an excess of positive-stranded RNA genomes that can be packaged into virions or can act as templates for the synthesis of viral proteins through subsequent rounds of translation (Fig. 4).

The role of cellular membranes in viral RNA synthesis is not well understood, but some roles have been proposed: (1) the physical support for assembly and organization of the RNA replication complex, (2) the compartmentalization and local concentration of substrates, (3) the proliferation of membranes may increase the total surface available for replication, and for storage of viral precursors and products, (4) provision of lipid compositions crucial for genome synthesis, (5) physical protection of the viral RNA and proteins from host defense mechanisms and degradation. The replication of many positive-strand RNA viruses is associated with ER membranes, for example, picorna-, flavi-, hepaci-, bromo- and tomaboviruses. However, endosomes and lysosomes, chloroplasts, and mitochondria have also been identified as target sites for viral RNA replication of other positive-strand RNA viruses.

A. Viral Replication Associated with Membranes Derived from the ER-Golgi Apparatus

Interactions between cellular membranes and poliovirus replication have been extensively described. After virion entry, the incoming poliovirus genome migrates to specific perinuclear sites, where the genome is directly translated by the cellular machinery. The synthesis of the viral polyprotein takes place in association with cytoplasmic membranes. Soon after translation, newly synthesized nonstructural viral proteins become associated with the viral RNA into an RNA replication complex, which is assembled on intracellular membranes (Bienz *et al.*, 1987, 1990). Poliovirus replication complexes consist of groups of vesicles of 70–400 nm in diameter, and after isolation become associated as large rosette-like shells of many vesicles interconnected with tubular extensions (Bienz *et al.*, 1992). *In vitro*, the rosettes can dissociate reversibly into tubular vesicles, and are able to resume

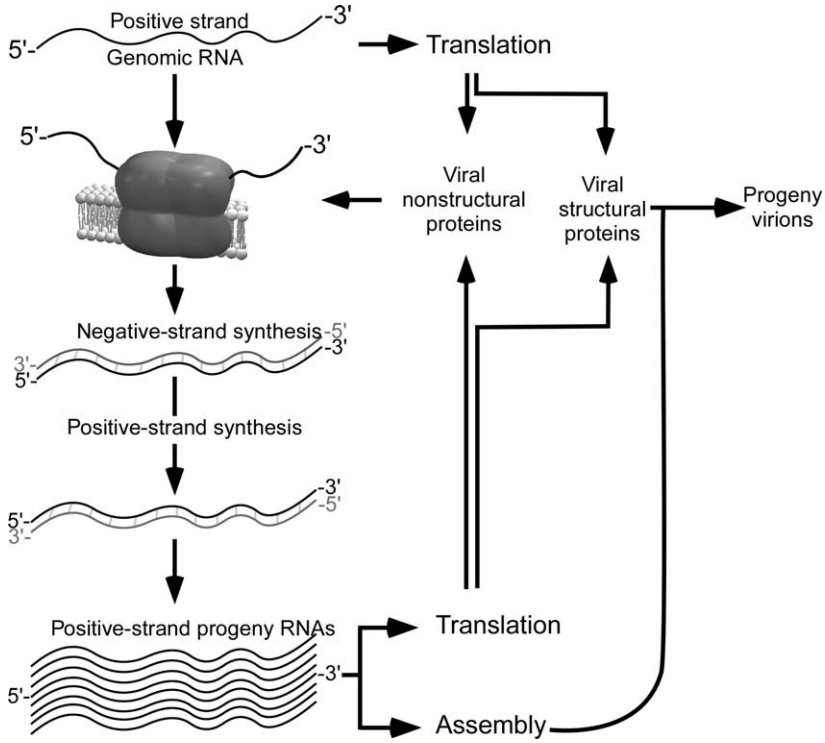

FIG. 4 Replication of positive-strand RNA viruses. After entry, the incoming viral RNA directs translation to generate viral proteins. Viral nonstructural proteins along with viral RNA will assemble on cellular membranes into a multisubunit RNA replication complex. The genomic RNA serves as a template to produce a negative-strand RNA, which results in double-stranded RNA intermediates called replicative forms. Then, the negative-stranded RNA is used to generate excess of positive-stranded RNA genomes that can be packaged into virions or can act as a template for the synthesis of viral proteins through subsequent rounds of translation.

RNA synthesis (Bienz *et al.*, 1992). Immunoisolated poliovirus-specific vesicles contain viral nonstructural proteins, and cellular markers of the ER, lysosomes, and trans-Golgi network, suggesting a complex biogenesis of the RNA replication complexes (Schlegel *et al.*, 1996). Electron microscopy analyses indicated that intracellular membrane rearrangements in poliovirus-infected cells lead to the formation of double-membrane vesicles similar to immature autophagic vacuoles carrying markers from the early and late secretory pathways (Suhy *et al.*, 2000).

The use of drugs targeting the secretory pathway of the host cell contributed to elucidating the biogenesis of the poliovirus replication complex. Brefeldin A (BFA) is a fungal macrocyclic lactone that has multiple targets

in vesicular transport, and blocks membrane traffic between the ER and the cis- and trans-Golgi compartments, leading to the disruption of the trans-Golgi apparatus (Nebenfuhr *et al.*, 2002). BFA completely inhibits viral RNA synthesis in poliovirus-infected cells (Irurzun *et al.*, 1992; Maynell *et al.*, 1992) as well as in an *in vitro* system (Cuconati *et al.*, 1998). The role of membrane trafficking and subcellular localization of viral RNA replication was further defined by confocal laser scanning microscopy. Poliovirus vesicles are generated at the ER by the cellular COPII budding mechanism, and are homologous to the vesicles of the anterograde membrane transport pathway (Rust *et al.*, 2001). ER resident proteins are excluded from the released vesicles, which are not destined to the Golgi apparatus, but that accumulate in the cytoplasm (Rust *et al.*, 2001). These results are consistent with other observations, showing that poliovirus infection inhibits the transport of both plasma membrane and secretory proteins. This transport inhibition does not require viral RNA replication or the inhibition of host cell translation by poliovirus (Doedens and Kirkegaard, 1995). During poliovirus infection, cells undergo extensive proliferation and rearrangement of intracellular smooth membranes and loss of ER membranes takes place (Cho *et al.*, 1994). Furthermore, the addition of cerulenin, an inhibitor of phospholipid synthesis, to poliovirus-infected cells results in inhibition of (1) the synthesis of the progeny genome, (2) the synthesis of phospholipids, and (3) the proliferation of membranes, indicating that continuous phospholipid synthesis is required for efficient genomic replication (Guinea and Carrasco, 1990). Egger and co-workers (2000) proposed that a functional poliovirus replication complex is assembled in a coupled and complex process involving viral translation, modification of membranes and budding, and viral RNA synthesis.

Poliovirus encodes a single polyprotein, which is cotranslationally cleaved into three viral precursor proteins, designated as P1, P2, and P3, according to their role during infection (Racaniello, 2001) (Fig. 5). P1 is the polypeptide precursor of the structural proteins (proteins associated with the viral particle), whereas P2 and P3 correspond to nonstructural proteins required for the replication of genomic RNA. The primary cleavage event of several picornaviruses occurs at the junction between the P1 and P2 precursor proteins and is mediated by the viral proteinase $2A^{pro}$ (Toyoda *et al.*, 1986). The nonstructural precursor P2 yields precursor 2BC, which in turn is cleaved to 2B and 2C (viral NTPase). P3 yields 3AB and 3CD, which are processed to 3A and 3B (VPg), and to $3C^{pro}$ (viral proteinase) and $3D^{pol}$ (viral RNA-dependent RNA polymerase), respectively.

The ectopic expression of poliovirus nonstructural proteins in cell culture has contributed to defining their biochemical roles during infection as well as their function in membrane association during the biogenesis of the poliovirus replication complex. Expression of both P2 and P3 poliovirus precursors

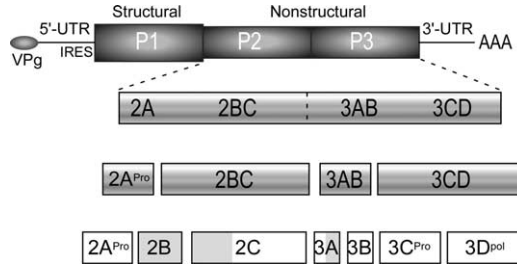

FIG. 5 Processing of poliovirus polyprotein. Poliovirus polyprotein is cleaved into three precursor proteins. P1 is the polypeptide precursor of the structural proteins, and P2 and P3 correspond to nonstructural proteins. The primary cleavage event occurs at the junction between the P1 and P2 precursor proteins and is mediated by the viral proteinase $2A^{pro}$. The nonstructural precursor P2 yields the 2BC precursor, which in turn is cleaved to 2B and 2C. P3 yields 3AB and 3CD, which are processed to 3A and 3B, and to $3C^{pro}$ and $3D^{pol}$, respectively. Shadowed regions in mature proteins illustrate their membrane-binding properties.

without structural proteins results in membrane alterations similar to those observed in infected cells (Teterina *et al.*, 2001). Individual expression of viral 2B protein results in its targeting to ER membranes and the Golgi complex (de Jong *et al.*, 2003), and it has been found that 2B expression interferes with the secretory pathway in mammalian (Doedens and Kirkegaard, 1995) and yeast cells (Barco and Carrasco, 1995). Viral 2B protein has also been reported to disassemble the Golgi complex (Sandoval and Carrasco, 1997). The 2B protein has a predicted cationic amphipathic α-helix within its N-terminal region, and a potential transmembrane domain, which can form dimers or tetramers in membranes (Aguirre *et al.*, 2002). Poliovirus 2B protein has been classified as a viroporin, a group of small viral proteins whose insertion into membranes is followed by their oligomerization that creates a typical hydrophilic pore, leading to membrane destabilization, and enhancing membrane permeability (Gonzalez and Carrasco, 2003).

The individual expression of poliovirus 2C protein results into its localization to the ER membranes, causing expansion of the organelle into tubular structures. As opposed to poliovirus 2B, the overexpression of protein 2C does not disrupt glycoprotein trafficking of VSV G protein to the plasma membrane (Suhy *et al.*, 2000). The sequence responsible for membrane binding of 2C has been mapped to its N-terminal region, which has been predicted to fold into an amphipathic α-helix (Echeverri and Dasgupta, 1995; Paul *et al.*, 1994; Teterina *et al.*, 1997). Poliovirus 2C protein displays ATPase (Pfister *et al.*, 2000) and GTPase activities (Rodriguez and Carrasco, 1993) and is involved in genomic replication (Banerjee *et al.*, 1997). Poliovirus 2BC, like 2B, is also a membrane protein, which interferes with the vesicular transport in both animal and yeast cells (Aldabe *et al.*, 1996; Doedens and

Kirkegaard, 1995). Thus, the 2B moiety in the 2BC protein accounts for transport inhibition (Doedens and Kirkegaard, 1995). The 2BC protein induces vesicles similar to those observed in poliovirus-infected cells, and causes an increase in permeability of the plasma membrane, like 2B (Teterina *et al.*, 1997).

Poliovirus 3A protein ectopically expressed in cell culture can inhibit the vesicular trafficking of secretory proteins from the ER to the Golgi complex (Doedens *et al.*, 1997; Doedens and Kirkegaard, 1995). The 3A protein remains associated with ER membranes but can be delivered into vesicles, similar to those found in infected cells and by expression of 2BC (Dodd *et al.*, 2001). In poliovirus-infected cells, the 3AB precursor contains a 22-amino acid–long sequence corresponding to 3B and called VPg. Only the membrane-associated 3AB protein can be cleaved by the viral proteases ($3C^{pro}$ and $3CD^{pro}$), and thus serves as the source of VPg (Lama *et al.*, 1994). The 3AB precursor associates tightly with cellular membranes, resembling the binding of integral membrane proteins (Datta and Dasgupta, 1994). The binding domain has been mapped to the C-terminal region, within a hydrophobic sequence (Towner *et al.*, 1996). However, the exact binding mechanism is not known. The 3B (VPg) portion of 3AB has affinity to the catalytic subunit $3D^{pol}$ and its precursor 3CD, which in turn recruits the template RNA into the membrane-associated replication complex by interaction with 3C and 3D (Egger *et al.*, 2000).

As for the picornaviruses, the genome of viruses of the Flaviviridae family is directly translated into a large polyprotein. For hepatitis C virus (HCV), the viral polyprotein is synthesized on ER-associated ribosomes, and is cleaved co- and posttranslationally by cellular and viral proteases into the mature structural and nonstructural proteins. The nonstructural proteins involved in replication (NS3, NS4A, NS4B, NS5A, and NS5B) have been shown to interact with ER membranes (Dubuisson *et al.*, 2002) (Fig. 6). However, the soluble protease/helicase NS3 protein associates with the membrane by interaction with NS4A, a cofactor of the protease domain of NS3. NS4A is a 54-amino acid cofactor for both serine protease and helicase activities of NS3, and its cofactor activity requires stable complex formation between NS3 and NS4A, an interaction that also stabilizes NS3 (Bartenschlager *et al.*, 1995; Pang *et al.*, 2002; Tanji *et al.*, 1995). NS3 is found in association with ER or ER-like membranes when coexpressed with NS4A, but it is distributed diffusely throughout the cytoplasm and nucleus when expressed in the absence of NS4A (Wolk *et al.*, 2000). Deletion analyses have shown that the hydrophobic N-terminal domain of NS4A is required to target NS3 to ER membranes.

NS4B is an integral ER membrane protein (Hugle *et al.*, 2001; Lundin *et al.*, 2003). NS4B is predicted to be a polytopic protein with both N- and C- terminal regions facing the cytoplasm. Interestingly, an intact N-terminal

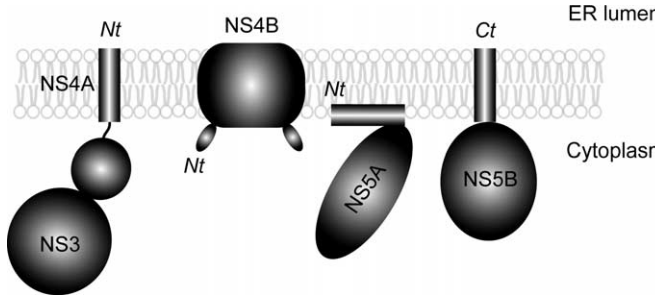

FIG. 6 Model of the insertion of hepatitis C virus nonstructural proteins into the endoplasmic reticulum membranes. NS3, NS4A, NS4B, NS5A, and NS5B are shown. The N- and C-terminus are indicated as Nt and Ct, respectively.

amphipathic helix can mediate correct membrane association of HCV nonstructural proteins and RNA replication, indicating a role for NS4B in the formation of membrane structures required for RNA replication (Elazar *et al.*, 2004). Expression of NS4B induces the formation of a seemingly ER-derived membranous web that is able to harbor all HCV structural and nonstructural proteins (Egger *et al.*, 2002). When NS4B is expressed alone, its association with ER membranes occurs cotranslationally, presumably via engagement of the signal recognition particle by an internal signal peptide (Hugle *et al.*, 2001), but no canonical signal peptide has been identified. Apparent activities in nucleotide binding and hydrolysis (Einav *et al.*, 2004), translation inhibition (Kato *et al.*, 2002), modulation of NS5B enzymatic function (Piccininni *et al.*, 2002), and transformation (Park *et al.*, 2000) have been reported for NS4B.

NS5A is a large, hydrophilic phosphoprotein of unknown function, which is a component of the HCV replication complex. Membrane association of NS5A is independent of the expression of other HCV nonstructural proteins, and it has been shown to be posttranslationally associated with ER-derived membranes via an N-terminal amphipathic α-helix (Brass *et al.*, 2002). This amphipathic helix has been proposed to be partially buried in one leaflet of the cellular membrane to give a monotopic topology (Penin *et al.*, 2004). Disruption of this helix leads to a diffuse cytoplasmic localization of NS5A and is lethal for HCV RNA replication (Elazar *et al.*, 2003).

HCV RNA-dependent RNA polymerase NS5B associates with membranes independently of other viral proteins. The conserved C-terminal 21-amino acid residues of NS5B are necessary and sufficient to target NS5B to the cytosolic side of ER membranes (Schmidt-Mende *et al.*, 2001). Membrane association of NS5B occurs by a posttranslational mechanism and results in integral membrane association and cytosolic orientation of the

functional protein domain (Schmidt-Mende *et al.*, 2001). Within membrane proteins, NS5B has been classified as a tail-anchored protein (Kutay *et al.*, 1993; Wattenberg and Lithgow, 2001). Recent results have demonstrated that the NS5B insertion sequence crosses the membrane phospholipid bilayer as a transmembrane segment (Ivashkina *et al.*, 2002). NS5B has been shown to be retained in the ER or an ER-derived modified compartment following transient transfection or in the context of a subgenomic replicon (Schmidt-Mende *et al.*, 2001). Interestingly, in addition to being a membrane anchor, the conserved C-terminal region of NS5B, *in vitro*, can regulate the polymerase activity modulating template binding and NTP substrate selection (Adachi *et al.*, 2002; Leveque *et al.*, 2003; Vo *et al.*, 2004), and *in vivo* is essential for viral RNA replication (Lee *et al.*, 2004; Moradpour *et al.*, 2004).

HCV nonstructural proteins form a membrane-associated replication complex together with viral RNA, altered cellular membranes, and additional as yet unidentified host cell components. In this context, physical interactions among nonstructural proteins have been described (Dimitrova *et al.*, 2003). However, the protein–protein interactions and dynamics within a functional replication complex are poorly defined. Expression of the entire HCV polyprotein has been shown to induce a prominent alteration, designated the membranous web, which contains all the viral proteins (Egger *et al.*, 2002). These observations have been confirmed in the context of a subgenomic HCV replicon (Gossert *et al.*, 2003). The membranous web can be induced by NS4B alone and is very similar to the ''sponge-like inclusions'' previously observed by EM in the liver of HCV-infected chimpanzees (Moradpour *et al.*, 2003).

Flavivirus RNA replication takes place within the cytoplasm of infected cells, in association with virus-induced membrane structures, which are separable by sedimentation from cellular membranes and retain RNA-dependent RNA polymerase activity (Chu and Westaway, 1992; Grun and Brinton, 1988). Membrane fractionation by sedimentation has been used to purify Kunjin virus replication complexes. Ultrastructural analyses have shown that the characteristics of flavivirus-induced membranes are associated with purified RNA-dependent RNA polymerase activity. RNA polymerase-active fractions were shown to contain nonstructural proteins as well as genomic replicative forms (Chu and Westaway, 1992). Systematic electron microscopy analyses of Kunjin virus-infected cells have revealed dramatic morphological changes and proliferation of the ER. Three different membrane structures have been identified: convoluted membranes (CM), paracrystalline structures (PC), and vesicle packets (VP) of smooth membranes (Westaway *et al.*, 1999). Most viral nonstructural proteins and the replicating RNA have been localized to VPs, which are derived from trans-Golgi membranes late during infection (Mackenzie *et al.*, 1999). VPs are sacs of vesicles surrounded by a membrane with a diameter of about 50–100 nm. Importantly, unlike CMs and PCs, these VPs are not observed following

expression of Kunjin virus subgenomic replicons (Khromykh and Westaway, 1997). In these cells, the replicating RNA localized throughout the cytoplasm in small isolated foci, suggesting that not all the structures induced by the nonstructural proteins are sites of RNA replication (Mackenzie *et al.*, 2001; Westaway *et al.*, 1999). Comparison between replicon cell lines producing RNA and nonstructural proteins with different efficiencies suggests that induction of virus-specific membranes is dose dependent, and requires a certain level of expression of viral products (Mackenzie *et al.*, 2001).

Cells infected with brome mosaic virus (BMV) can be extracted to yield membrane-bound, RNA-dependent RNA polymerase activity (Miller and Hall, 1983). After detergent solubilization, this BMV RNA-dependent RNA polymerase activity copurifies with an immunoprecipitable complex of viral proteins 1a and 2a and several host factors (Quadt *et al.*, 1993, 1995). An equivalent, initially membrane-bound BMV RNA polymerase activity can be isolated from yeast expressing the BMV 1a and 2a proteins and replicating BMV RNA derivatives (Quadt *et al.*, 1995). The BMV 2a protein contains a central RNA polymerase domain and an N-terminal domain that interacts with the 1a helicase domain (Kao and Ahlquist, 1992). Since 1a and 2a proteins lack obvious membrane-spanning domains, the nature of their association with membranes has been investigated. In BMV-infected protoplasts, 1a, 2a, and viral-specific RNA synthesis show colocalization in cytoplasmic spots surrounding the nucleus. Detection of organelle markers shows that BMV replication complexes are tightly associated with the ER, but not the medial Golgi or later compartments of the secretory pathway (Restrepo-Hartwig and Ahlquist, 1996). In addition, the 1a protein has been shown to associate with the ER when expressed in the absence of other viral factors, indicating that this protein may be responsible for ER localization and retention of the BMV RNA replication complex (Restrepo-Hartwig and Ahlquist, 1999). The 1a protein is located on the cytoplasmic face of the ER, and mapping experiments of the targeting determinant indicated that a large region at the N-terminus of this protein is required for membrane association and ER targeting, but the exact mechanism of insertion is unknown (den Boon *et al.*, 2001). In contrast, the predicted RNA-dependent RNA polymerase 2a depends on 1a protein for recruitment to the site of replication (Kao and Ahlquist, 1992; O'Reilly *et al.*, 1995, 1997). The recruitment of 2a protein is based on a direct interaction between the N-terminus of 2a and the C-terminus of 1a and is reflected in a 1a-induced increase of 2a accumulation (Chen *et al.*, 2001; Ishikawa *et al.*, 1997). The 1a protein also recruits viral RNA templates into replication, resulting in dramatically increased RNA stability but reduced translation (Janda and Ahlquist, 1998; Sullivan and Ahlquist, 1999).

Recently, a model for the assembly of the BMV RNA replication complex has been proposed (Schwartz *et al.*, 2002). The 1a protein alone, in the absence

of other viral components, can induce ER membrane invaginations into the ER lumen, forming 50- to 70-nm diameter spherular vesicles or spherules. The interior of these spherules remains connected to the cytoplasm via a membranous neck contiguous with the ER membrane. The 1a protein is also able to promote membrane lipid accumulation at an intracellular level (Lee and Ahlquist, 2003). When viral RNA is coexpressed with the 1a protein, it becomes protected inside the spherule in a membrane-associated, nuclease-resistant state. When viral polymerase 2a is coexpressed, it associates with the spherules through interaction with the 1a protein, and viral replication takes place in close association with the spherules, possibly in their interior, from which plus-strand RNA is released to the cellular cytoplasm.

The functions of membranes in BMV RNA replication have been emphasized by genetic results in yeast. A screen for host genes essential for BMV RNA replication has identified a partial loss-of-function mutation in the OLE1 gene. This mutation severely inhibits BMV RNA replication (Lee *et al.*, 2001). OLE1 is an essential gene encoding the delta9 fatty acid desaturase required for synthesis of unsaturated fatty acids. Under restrictive conditions, the 1a protein can still normally recruit viral RNA and 2a protein to membranes, but replication is strongly inhibited (Lee *et al.*, 2001). Limiting synthesis of unsaturated fatty acids does not reduce membrane synthesis, but a low ratio of unsaturated-to-saturated fatty acids in membrane phospholipids can affect membrane fluidity (Stuckey *et al.*, 1989). Furthermore, using the yeast OLE1 mutant, it has been shown that perinuclear ER spherules induced by 1a are locally depleted of unsaturated fatty acids, suggesting that 1a preferentially binds with one or more types of membrane lipids (Lee and Ahlquist, 2003).

BMV has also been useful to identify host genes involved in viral replication in a genome-wide screening in the yeast *Saccharomyces cerevisiae*. Using engineered BMV derivatives and approximately 4500 yeast deletion mutants, nearly 100 genes implicated in RNA replication and/or gene expression have been identified (Kushner *et al.*, 2003).

Tobacco mosaic virus (TMV), another positive-strand RNA virus, encodes two proteins (126K and 183K) involved in viral genome replication (Ishikawa *et al.*, 1986). TMV also encodes a movement protein (MP) required for cell-to-cell movement (Deom *et al.*, 1987), and a 17.5K protein (CP). These two latter proteins are dispensable for replication (Meshi *et al.*, 1987). Biochemical fractionation of TMV-infected protoplasts has shown that membrane fractions contain active replication complexes (Osman and Buck, 1996). Cytological analyses of TMV-infected cells have shown virus replication complexes associated with cytoplasmic inclusions or viroplasms, consisting of membrane rearrangements and amorphous proliferation of the ER, which expand throughout the infection. These inclusions contain ribosomes, microtubules, viral RNA, MP, and 126/183-kDa replication

proteins (Hills *et al*., 1987; Saito *et al*., 1987). Recent studies with TMV expressing a fusion GFP:MP protein showed fluorescent irregularly shaped structures derived from the ER membranes, colocalization of the TMV RNA with BiP, and disruption of fluorescent structures by BFA (Heinlein *et al*., 1998; Mas and Beachy, 1999). Immunostaining with tubulin provided evidence of colocalization of TMV RNA with microtubules, and disruption of the cytoskeleton with cytochalasin D generated severe changes in TMV RNA distribution (Mas and Beachy, 1999; Reichel and Beachy, 1998).

Ectopic expression studies have shown that the 126K protein associates with the ER in the absence of other viral proteins, and it has been suggested that this association may take place via either membrane-bound host proteins or membrane insertion of a region of 21 amino acid amphipathic helix detected within its primary structure (dos Reis Figueira *et al*., 2002). The MP behaves as an intrinsic membrane protein, promotes the formation of ER aggregates, and probably facilitates the establishment of TMV replication complexes (Heinlein *et al*., 1998; Reichel and Beachy, 1998). It has been proposed that the phosphorylation state of MP is important for altering the structure of the protein and its association with the ER and/or microtubules (Kahn *et al*., 1998).

Arabidopsis thaliana TOM1 and TOM2A have been recently indicated as host factors involved in RNA replication of TMV. The inactivation of either the TOM1 or TOM2A gene results in a decreased viral multiplication (Ishikawa *et al*., 1993; Ohshima *et al*., 1998). TOM1 and TOM2A are predicted to be seven-pass and four-pass transmembrane proteins, respectively, but neither of them possesses well-known sorting signals to specific organelles (Tsujimoto *et al*., 2003; Yamanaka *et al*., 2000). TOM1 has been shown to interact with the TMV-encoded 126K/183K proteins (Yamanaka *et al*., 2000), and recent studies have suggested that TOM2A interacts both with itself and with TOM1 (Tsujimoto *et al*., 2003; Yamanaka *et al*., 2000). It has been proposed that TOM1 and TOM2A are critical parts of the assembly of TMV RNA replication complex on cellular membranes where they colocalize (Hagiwara *et al*., 2003).

B. Viral Replication Associated with Membranes Derived from Endosomes-Lysosomes

The nonstructural proteins of alphaviruses are synthesized as a polyprotein precursor (P1234). This precursor is cleaved by a viral protease located in the carboxy-terminal half of nsP2 to produce several intermediate polyproteins, and four mature proteins nsP1, nsP2, nsP3, and nsP4 (Schlesinger and Schlesinger, 2001). The various nonstructural proteins form RNA replication complexes and nsP4 is the RNA-dependent RNA polymerase.

Electron microscopic analyses of alphavirus-infected cells have shown that viral replication and transcription take place in association with host-cell membranes (Schlesinger and Schlesinger, 2001). Semliki forest virus-infected cells show the presence of several cytoplasmic structures designated cytopathic vacuoles (CPV), which are absent in uninfected cells. Their size varies from 600 to 2000 nm, and their surface consists of small vesicular invaginations or spherules, of homogeneous size, with a diameter of about 50 nm (Grimley *et al.*, 1968; Peranen and Kaariainen, 1991). For Semliki forest virus-infected cells, CPVs have been shown to be modified endosomes and lysosomes with colocalization of all viral nonstructural proteins (Froshauer *et al.*, 1988). These structures are the sites of RNA replication (Kujala *et al.*, 2001). Moreover, CPVs costain with late endosomal markers [lysobisphosphatic acid (LBPA) and rab7], and lysosomal markers (lysosomal-associated membrane proteins: Lamp-1, Lamp-2). Interestingly, nsPs proteins are also found outside of the CPVs, indicating that only a fraction of the nsPs proteins is present in the replication complexes.

The membrane-binding properties of alphavirus nonstructural proteins have been studied by ectopic expression in homologous and heterologous systems. From these studies, it has been found that only nsP1 displays a specific association with cellular membranes (Peranen *et al.*, 1995). Biochemical and genetic experiments have shown that Semliki forest virus nsP1 is highly associated with cellular membranes by two mechanisms: (1) palmitoylation of cysteine residues within nsP1 (Laakkonen *et al.*, 1996) and (2) a membrane-binding domain of approximately 20 conserved amino acids (Lampio *et al.*, 2000). However, palmitoylation of nsP1 is not essential for virus replication, although virus release is delayed when palmitoylated residues are mutated (Ahola *et al.*, 2000). In addition, both enzymatic activities of Semliki forest virus nsP1 (methyltransferase and guanylyltransferase) are inactivated by detergents and reactivated by anionic phospholipids, like phosphatidylserine (Ahola and Ahlquist, 1999). Thus, binding to anionic phospholipids causes a conformational change, which activates nsP1 protein. Mutagenesis of putative essential amino acid residues interacting with membranes leads to alteration of virus production (Salonen *et al.*, 2003).

Studies of Semliki forest virus nonstructural proteins expressed individually or as a polyprotein have shown that these proteins need to be synthesized as a polyprotein precursor to assemble a replication complex (Salonen *et al.*, 2003). Uncleaved polyproteins containing nsP1 are membrane bound and palmitoylated, and those containing nsP3 are phosphorylated, reflecting properties of authentic nsP1 and nsP3, respectively (Salonen *et al.*, 2003). Interestingly, uncleaved P12 precursor is localized almost exclusively to the plasma membrane and filopodia, like nsP1 expressed alone, whereas uncleaved P123 and uncleaved P1234 are found on cytoplasmic vesicles, some of which contain late endosomal markers. Thus, the nsP1 domain

alone is responsible for the membrane association of the nonstructural polyprotein, whereas the nsP1 domain together with the nsP3 domain targets the nonstructural proteins to the intracellular vesicles (Salonen *et al*., 2003). It has therefore been proposed that the polyprotein is attached to membranes first by the nsP1-binding domain, which adopts an α-helical conformation (Salonen *et al*., 2005). Thereafter, the nsP1 domain of the polyprotein undergoes a conformational change to activate both methyltransferase and guanylyltransferase activities (Ahola *et al*., 1999). The palmitoylation of cysteine residues on nsP1 will then anchor the polyprotein irreversibly to the membrane. The polyprotein has a half-life of about 15 min (Kujala *et al*., 2001). The initial cleavage releases nsP4 from the polyprotein precursor, giving rise to the minus-strand polymerase complex. During this time, a replication complex likely synthesizes only minus-strand RNA before it is transformed into a stable plus-strand polymerase, which operates as the unit of replication within the spherule.

Replication complexes have been identified in rubella virus-infected cells as cytoplasmic membrane-bound structures (Lee *et al*., 1992). These structures comprise vacuoles, which are lined internally with membrane-bound vesicles measuring approximately 60 nm in diameter. These vesicles contain thread-like inclusions and are usually attached to the surrounding vacuole membrane via a membranous neck. These vesicles are observed in infected cells as early as 8 h pi. Peak numbers of rubella virus replication complexes occur at 24 h pi, coinciding with maximum viral titers (Lee *et al*., 1992). Electron microscopy analyses have shown the localization of replicating viral RNA in the membrane-bound vesicles, which contour the rubella virus replication complexes (Lee *et al*., 1994). Other studies have shown colocalization of replicating viral RNA and two lysosomal markers (Lamp-1 and acid phosphatase) indicating that rubella virus replication complexes involve virus-modified lysosomes (Magliano *et al*., 1998). In addition, rubella virus replicase has been shown to be associated with the spherules (Kujala *et al*., 1999).

C. Viral Replication Associated with Mitochondria and Chloroplasts

Turnip yellow mosaic virus is a small spherical plant virus. Its genome encodes two nonstructural proteins, 69K and 206K (Morch *et al*., 1988; Weiland and Dreher, 1989). The 206K precursor is required for genome replication (Weiland and Dreher, 1989), and possesses a domain organization of methyltransferase, proteinase, NTPase/helicase, and RNA-dependent RNA polymerase activities (Bransom and Dreher, 1994; Gorbalenya *et al*., 1989; Kamer and Argos, 1984; Morch *et al*., 1988; Rozanov *et al*., 1992, 1995;

Weiland and Dreher, 1989). The cysteine protease is responsible for the proteolytic cleavage of the 206K protein (Bransom *et al.*, 1991; Morch *et al.*, 1989), leading to the release of an N-terminal protein (140K) containing the methyltransferase, proteinase, and NTPase/helicase domains, and a C-terminal protein (66K) encompassing the RNA-dependent RNA polymerase domain (Bransom *et al.*, 1996; Kadare *et al.*, 1995). Both the 140K and the 66K viral proteins are essential for turnip yellow mosaic virus RNA replication (Prod'homme *et al.*, 2001; Weiland and Dreher, 1989).

Besides providing enzymatic functions for RNA replication, the 140K protein seems to be a key organizer of the assembly of turnip yellow mosaic virus replication complexes, which are associated with membrane vesicles present at the chloroplast envelope (Prod'homme *et al.*, 2001). The 140K protein localizes to the chloroplast envelope in the absence of any other viral factors, and thus seems to be a major determinant for chloroplast localization and retention of viral replication complexes (Prod'homme *et al.*, 2003). In contrast, the 66K protein, encompassing the RNA-dependent RNA polymerase domain, has a cytoplasmic distribution when expressed alone and depends on the 140K protein for recruitment to the sites of replication (Prod'homme *et al.*, 2003). The recruitment of the 66K protein to the replication complexes involves protein–protein interactions with the membrane-bound 140K protein (Jakubiec *et al.*, 2004). Interestingly, using a two-hybrid system and coimmunoprecipitation, the interaction domains were mapped to the proteinase domain of the 140K protein and to a large region encompassing the core polymerase domain within the 66K protein (Jakubiec *et al.*, 2004). Since many viral families assemble their replication complexes via protein–protein interactions of helicase and polymerase domains (Johansson *et al.*, 2001; Kao and Ahlquist, 1992; Kim *et al.*, 2002; O'Reilly *et al.*, 1995, 1997; Van Der Heijden *et al.*, 2001), turnip yellow mosaic virus follows a new pathway to assemble its RNA replication complex on cellular membranes.

Flock house virus is a small nonenveloped virus infecting insects. Previous studies with flock house virus have suggested that intracellular membranes are involved in RNA replication. Viral RNA-dependent RNA polymerase activity is associated with a membrane fraction from lysates of *Drosophila* cells infected with flock house virus (Wu and Kaesberg, 1991). Moreover, the membrane and phospholipids dependence of flock house virus RNA positive-strand synthesis imply that membrane association is crucial for at least some steps of genome replication (Wu *et al.*, 1992). Electron microscopy studies after viral infections demonstrate the appearance of vesicular bodies in the cytoplasm of infected cells (Garzon *et al.*, 1990). The vesicular bodies contain RNA, have morphological characteristics of mitochondria at early stages of infection, and are associated with virus particles at later stages of infection (Garzon *et al.*, 1990).

The localization of flock house virus RNA replication in infected cells has been analyzed by biochemical and ultrastructural methods. Mitochondria have been identified as the key cellular organelle for genome replication of this virus. Flock house virus protein A, the RNA-dependent RNA polymerase, has been shown to be tightly associated with the outer mitochondrial membranes, and colocalizes with sites of genome replication. Flock house virus infection induces the formation of membrane-bound spherules of 40–60 nm in diameter in the mitochondrial intermembrane space (Miller *et al.*, 2001). Expression in heterologous systems defined flock house virus protein A as an outer mitochondrial transmembrane protein with an N-terminus located in the intermembrane space or matrix, and the C-terminus exposed to the cytoplasm. The N-terminal 46 amino acids contain sequences sufficient for mitochondrial localization and membrane insertion (Miller and Ahlquist, 2002).

IV. Interactions Between Virus Proteins and Host Cell Membranes During Virus Assembly and Budding

Viral particles of enveloped viruses contain a lipid bilayer derived from a cellular membrane. These particles contain an outer layer of proteins, usually glycoproteins, which are anchored in the membrane as integral membrane proteins. These proteins function to attach the virion to target host cell receptors and facilitate the entry and fusion of the viral membrane with that of the host cell. A complex of protein and nucleic acid is contained on the interior of the lipid bilayer. This complex is usually referred to as a nucleocapsid core.

Although viral particles of different virus families have the same objective of transferring viral genetic information from one cell to another, strategies for enclosing and protecting viral genomes vary widely. Production of infectious virus particles requires spatially and temporally coordinated interaction of components that make up an infectious virion. While naked viruses are usually released from infected cells by disruption of the plasma membrane, enveloped viruses contain a host cell-derived lipid bilayer, which surrounds the nucleocapsid core and which is acquired during budding. Budding requires the selection of an assembly site—the plasma membrane or an intracellular membrane of the secretory pathway—where viral components are transported. Assembly of the viral components leads to initiation of the budding process, growth of the bud, and finally completion of the bud with the release of the virus particle. These different steps in the budding process are complex and require involvement of both host and viral components. Various types of interactions are engaged in budding. They include

interactions of viral proteins with the lipid bilayer as well as protein–protein interaction of viral components. Interactions between viral proteins and host cell membranes involved in budding of some well-characterized viruses are discussed in this section.

The roles played by the viral membrane glycoproteins in the formation of the viral envelope vary among different viruses (Fig. 7). For some viruses, these proteins are not required at all. Indeed, viruses such as rhabdoviruses and retroviruses bud normally in the absence of their glycoproteins to form the characteristic bullet-shaped and rounded particles, respectively (Garoff *et al.*, 1998). This viral shape is determined by their matrix protein and the nucleocapsid. For some other viruses, the viral membrane proteins are all

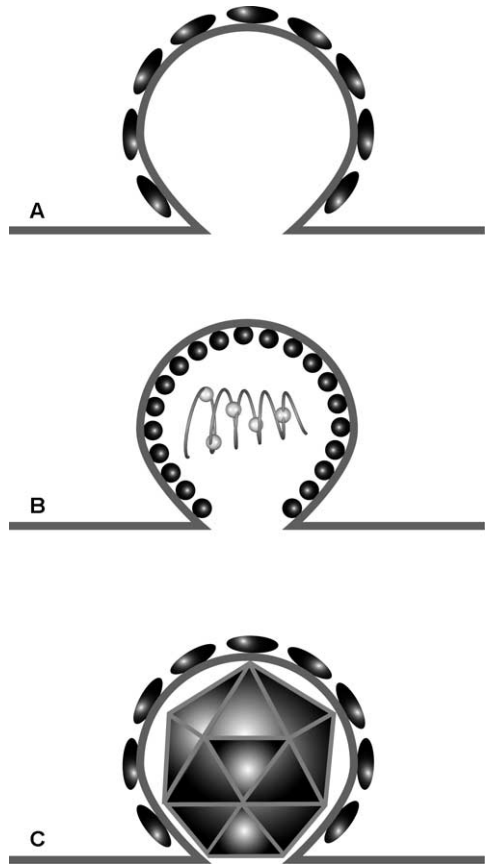

FIG. 7 Viral proteins that drive budding. Viruses budding can be driven by the viral envelope proteins (A), by a matrix protein or Gag polyprotein (for retroviruses) that associates with the inner leaflet of the membrane (B), or by both the envelope proteins and the nucleocapsid (C).

that is required for envelope formation. In this case, these proteins have the ability by themselves to carry out the budding of particles devoid of a nucleocapsid. This is the case for the coronaviruses (Vennema *et al*., 1996), the flaviviruses (Ferlenghi *et al*., 2001), and the hepadnaviruses (Bruss, 2004). The dimensions of these "empty" particles can perfectly match those of normal virions in the case of coronaviruses, whereas such subviral particles are smaller for the flaviviruses and hepadnaviruses. Intermediate between these extremes are the viruses for which the membrane proteins are essential but not sufficient to form the viral envelope. This is the case for the alphaviruses (Garoff *et al*., 2004).

A. Selection of Assembly Sites

1. Subcellular Localization Signals in Envelope Glycoproteins

Viral envelope glycoproteins are synthesized using the same pathway as cellular membrane glycoproteins. They must therefore be transported through the secretory pathway of the cell to their final destination. Viral envelope glycoproteins largely contribute to determine the site of virus assembly and budding. This notion comes from the fact that viral envelope glycoproteins generally accumulate at the site of virus budding even when they are expressed alone. Many enveloped viruses are believed to assemble at the cytoplasmic face of the plasma membrane and bud out of the cell (Garoff *et al*., 1998). The envelope proteins of these viruses are synthesized in the secretory pathway and accumulate at the plasma membrane. However, other enveloped viruses assemble intracellularly, obtaining their lipid envelope from intracellular compartments. These viruses bud into the lumen of intracellular compartments and exit the cell by exocytosis. The envelope proteins of viruses that assemble in intracellular compartments possess signals that direct them to the site of viral assembly. These signals mimic those used by endogenous cellular proteins and utilize the cellular machinery for localization.

a. Targeting Viral Envelope Glycoproteins to a Pre-Golgi Compartment Several viruses acquire their envelope in a pre-Golgi compartment. They include the hepadnaviruses (Ganem and Schneider, 2001), the coronaviruses (Lai and Holmes, 2001), the flaviviruses (Lindenbach and Rice, 2001), and the spumaviruses (Delelis *et al*., 2004). Two ER sorting signals have been well defined for transmembrane proteins (Teasdale and Jackson, 1996). Type II transmembrane proteins possess a motif consisting of two arginines near the cytoplasmic N-terminus. Type I transmembrane proteins contain lysines at positions −3 and either at position −4 or −5 in relation to the cytoplasmic C-terminus (dilysine or KKXX motif). Interestingly, a functional dilysine

motif is commonly found at the C-terminus of primate spumaviruses (Goepfert *et al.*, 1997). This localization directs budding of these spumaviruses to intracellular membranes (Shaw *et al.*, 2003). Although the dilysine motif of spumaviruses imposes a partial restriction on the site of viral maturation, it is not necessary for virion formation (Goepfert *et al.*, 1999).

Coronaviruses bud at the ER-to-Golgi intermediate compartment (ERGIC) and this requires accumulation of the viral envelope proteins at this location in the secretory pathway (Lai and Holmes, 2001). The coronavirus particle contains at least three envelope proteins. The first is the spike (S) glycoprotein, a large type I transmembrane protein, which plays a major role in virus entry. The second (M) is a type III triple-spanning membrane protein, which plays an essential role in virus assembly. The third critical membrane-bound constituent of the virion is the small hydrophobic envelope E protein. Some coronaviruses also contain an additional envelope protein, the hemagglutinin–esterase (HE) protein.

The S protein from group 1 and 3 coronaviruses, as well as SARS coronavirus, also contains a dibasic ER retrieval signal in its cytoplasmic tail (Lontok *et al.*, 2004). These dibasic signals likely play a role in accumulation of S proteins near the site of virus assembly and could serve to limit surface expression. Dilysine signals have been shown to direct retrieval of escaped proteins from post-ER compartments back to the ER. Proteins with a dilysine signal bind the coatomer complex (COPI) and are recruited into vesicles that travel in a retrograde direction (Cosson and Letourneur, 1994; Gaynor *et al.*, 1998). The efficiency of binding to COPI is influenced by the context surrounding the dilysine motif, which contributes to steady-state localization of proteins bearing this signal to the ER, ERGIC, or Golgi complex (Teasdale and Jackson, 1996). Contrary to the proteins of group 1 and 3 coronaviruses, the cytoplasmic tail of the S protein from group 2 lacks an intracellular localization signal.

Although the presence of a dibasic signal at the C-terminus of the S protein might play a role for its incorporation into the virus particle, the other envelope proteins, M and E, also need to be incorporated into the particle. The mature M protein accumulates in the Golgi apparatus and is not transported to the plasma membrane (Lai and Holmes, 2001). The information for the intracellular localization of M resides within the first transmembrane domain (Swift and Machamer, 1991) and, additionally, in the carboxyl-terminal portion (Locker *et al.*, 1994). The small hydrophobic membrane protein E has also been reported to transiently reside in a pre-Golgi compartment (Lim and Liu, 2001) before it progresses to the Golgi apparatus (Corse and Machamer, 2000, 2002). Interestingly, the M protein is able to interact with itself and with S and E proteins (Corse and Machamer, 2003; de Haan *et al.*, 1999, 2000; Lim and Liu, 2001). These interactions likely contribute to retaining all the envelope proteins at the site of budding.

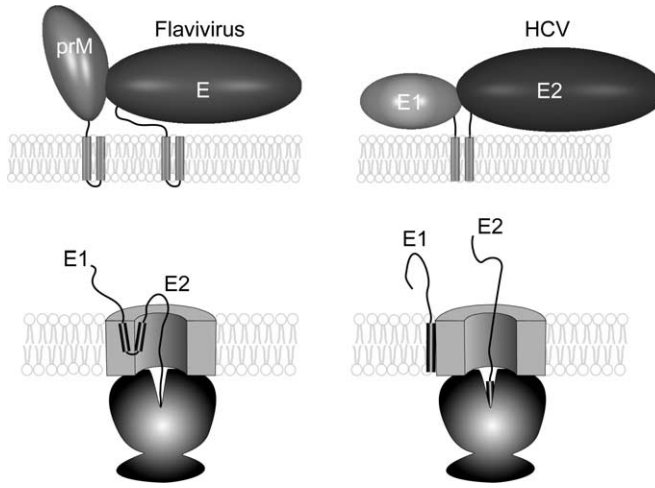

FIG. 8 Transmembrane domains of the envelope proteins of flaviviruses and hepatitis C virus (HCV). The immature flavivirus particle contains two envelope proteins, prM and E, that are associated as a heterodimer. The C-termini of prM and E form two antiparallel transmembrane α-helices. HCV envelope glycoproteins, E1 and E2, also form a heterodimer at the surface of virions, mediated by interactions between the C-termini of E1 and E2. During translation and translocation, the transmembrane domains of HCV envelope glycoproteins form a hairpin structure before cleavage of the polyprotein by a host signal peptidase, and a reorientation of the second hydrophobic stretch occurs after cleavage to produce a single membrane-spanning domain (lower panels).

The immature flavivirus particle contains two envelope proteins, prM and E, which are associated as a heterodimer. Virion morphogenesis of the flaviviruses occurs in association with ER membranes, suggesting that there should be accumulation of the virion components in this compartment (Mackenzie and Westaway, 2001). ER localization signals have been identified in the transmembrane regions of flavivirus envelope proteins (Op De Beeck *et al*., 2004). These domains also play an essential role in the formation of the flavivirus envelope (Op De Beeck *et al*., 2003). The C-termini of prM and E form two antiparallel transmembrane α-helices potentially making coiled-coil structures, leaving the C-terminus of each protein in the lumen of the ER (Zhang *et al*., 2003) (Fig. 8). Interestingly, the first transmembrane passage contains enough information for ER localization (Op De Beeck *et al*., 2004). It has been proposed that in the absence of dominant luminal or cytosolic associations, proteins distribute based on interactions between their transmembrane domain and the surrounding lipid environment (Bretcher and Munro, 1993; Munro, 1995). A similar transmembrane-based sorting might exist in the ER and might lead to ER retention of protein having a short transmembrane domain (Pedrazzini *et al*., 1996;

Szczesna-Skorupa and Kemper, 2000; Yang *et al.*, 1997). A similar mechanism seems to be involved in ER retention of the flavivirus envelope proteins. Indeed, the first transmembrane passage of E contains enough information for ER localization and increasing the length of this transmembrane passage leads to export out of the ER (Op De Beeck *et al.*, 2004).

Interestingly, ER retention signals have also been identified in the transmembrane domains of the envelope proteins of the related HCV (Dubuisson *et al.*, 2002), indicating some conservation in the functions of the transmembrane domains of the envelope protein in the Flaviviridae family. Sequence analyses indicate that these domains are all composed of two hydrophobic stretches separated by a small connecting segment containing one or more charged residues (Cocquerel *et al.*, 2000). However, they have different structures (Op De Beeck and Dubuisson, 2003) (Fig. 8). Indeed, the transmembrane domains of HCV envelope glycoproteins form a hairpin structure before cleavage of the polyprotein by a host signal peptidase, and a reorientation of the second hydrophobic stretch occurs after cleavage to produce a single membrane-spanning domain (Cocquerel *et al.*, 2002). Interestingly, the charged residue(s) present in the middle of these transmembrane domains have been shown to be essential for ER retention (Cocquerel *et al.*, 2000), as in the case of some other ER proteins (Bonifacino *et al.*, 1990, 1991; Letourneur and Cosson, 1998; Yang *et al.*, 1997).

b. Targeting Viral Envelope Glycoproteins to the Golgi Apparatus Viruses of the Bunyaviridae family are characterized by budding in the Golgi apparatus due to the accumulation of the two viral glycoproteins, Gn and Gc, in this organelle (Schmaljohn and Hooper, 2001). When Gn of most viruses of this family is expressed on its own, it localizes to this organelle. In contrast, the Gc glycoprotein remains in the ER when it is expressed alone and is transported to the Golgi only after interaction with Gn (Schmaljohn and Hooper, 2001). The mapping of the Golgi retention signal has been done for several bunyaviruses. However, no consensus motif has been delineated. It seems that different viruses in this diverse family have developed their own specific strategy for Golgi trafficking rather than displaying a consensus Golgi retention motif. The signal for Golgi localization has been mapped to the cytoplasmic tail of the Gn protein for Uukuniemi virus (Andersson and Pettersson, 1998; Andersson *et al.*, 1997). A region including the transmembrane domain and part of the cytoplasmic tail is responsible for Golgi localization of Gn for the Punta Tora (Matsuoka *et al.*, 1996) and the Rift Valley fever (Gerrard and Nichol, 2002) phleboviruses. The signal for retention in the Golgi apparatus has been mapped to the transmembrane domain of the Bunyamwera virus (Shi *et al.*, 2004). No specific Golgi localization signal has been found in Hantavirus glycoproteins. In this case, localization of the glycoproteins in the Golgi apparatus requires the coexpression of both

Gn and Gc. Interestingly, these proteins are retained in the ER when they are expressed separately (Shi and Elliott, 2002; Spiropoulou *et al*., 2003).

Rubella virus is another example of a virus that buds in the Golgi apparatus (Hobman *et al*., 1993). Like the other members of the Togaviridae family, rubella virus encodes two envelope glycoproteins, E1 and E2, which interact to form a heterodimer. However, unlike the envelope proteins of other Togaviridae, rubella virus envelope glycoproteins are mainly retained in the Golgi apparatus. A Golgi retention signal has been identified in the transmembrane domain of E2 (Hobman *et al*., 1995). Interestingly, when expressed alone, E1 glycoprotein is retained in the ER, indicating that E1 needs to interact with E2 to leave the ER compartment (Hobman *et al*., 1997). An ER retention signal has been mapped in the transmembrane and cytoplasmic domain of E1.

c. Targeting Viral Envelope Glycoproteins to the Endocytic Pathway Retroviruses are generally assumed to bud at the plasma membrane. However, it has recently become apparent that some of these viruses use the endocytic pathway to coordinate their assembly and release (Pelchen-Matthews *et al*., 2004). HIV-1 envelope (Env) glycoprotein is synthesized as a precursor and is processed during its passage through the secretory pathway by a host cell protease to yield the surface subunit (SU) and the transmembrane (TM) subunit. The TM subunit contains a C-terminal cytoplasmic domain of more than 150 amino acids. Newly synthesized HIV-1 Env undergoes endocytosis after its arrival at the cell surface. Internalization of HIV-1 Env is mediated by interaction of the AP-2 clathrin adaptor complexes with a membrane-proximal, tyrosine-based signal in the cytoplasmic domain of the TM subunit (Berlioz-Torrent *et al*., 1999; Boge *et al*., 1998; Ohno *et al*., 1997). Additional determinants downstream of the proximal tyrosine-based sorting signal are also implicated in HIV-1 Env endocytosis (Berlioz-Torrent *et al*., 1999). The cytoplasmic domain of HIV-1 Env has been shown to interact with AP-1 adaptor complexes (Berlioz-Torrent *et al*., 1999; Wyss *et al*., 2001), and a dileucine motif at the C-terminus of the cytoplasmic domain of the TM subunit is implicated in the recruitment of AP-1 complexes. This dileucine motif, together with the membrane-proximal tyrosine-based motif, helps to control expression of Env at the cell surface (Wyss *et al*., 2001). In addition, two other amino acid sequences, which inhibit Env surface expression, have also been identified in the cytoplasmic domain of the TM subunit (Bultmann *et al*., 2001). Recently, a diaromatic motif located in the cytoplasmic tail of HIV-1 Env has been shown to interact with TIP47, a cellular protein that drives its retrograde transport from endosomes to the trans-Golgi network (Blot *et al*., 2003). Interestingly, retrograde transport of Env is implicated in the optimization of fully infectious HIV-1 production.

d. Targeting Viral Envelope Glycoproteins in Polarized Epithelial Cells Viruses that assemble and bud from the plasma membrane do not bud randomly, but bud asymmetrically from the surface of polarized epithelial cells (Compans, 1995). For infection of host organisms, epithelial cells of the respiratory or the gastrointestinal tract are often the primary targets for replication. Epithelial cells at mucosal surfaces establish polarity and develop two distinct membrane domains. These membrane domains are exposed to very different physiological environments because the apical membrane faces the lumen, while the basolateral membrane is in contact with the underlying stratum of the epithelia. The two poles of the cell exhibit distinct profiles of proteins and lipids. The apical plasma membrane is enriched in sphingolipids, whereas the basolateral membrane predominantly contains glycolipids, phosphatidylcholines. Tight junctions in polarized cells prevent lateral diffusion of these lipids. At the end of the infectious cycle, some viruses are released preferentially from the apical surface, thus favoring the establishment of a localized infection. Conversely, viruses that are released from the basolateral membrane find access to the underlying tissue and the blood system, facilitating the development of a systemic infection. For instance, orthomyxoviruses and paramyxoviruses have been found to bud preferentially from the apical membrane of polarized cells, whereas VSV and Marburg virus bud almost exclusively from the basolateral surface (Schmitt and Lamb, 2004). For many viruses that exhibit polarized budding in epithelial cells, it has been found that the viral envelope glycoproteins are targeted intrinsically to the same membrane from which a virus buds. However, altering the subcellular localization of viral envelope glycoproteins does not necessarily lead to the selection of another budding site in polarized cells (Nayak *et al.*, 2004). Signals for apical sorting have been mapped in the transmembrane domains of the two influenza virus glycoproteins HA and NA (Schmitt and Lamb, 2004). The basolateral sorting signal in glycoprotein G of VSV has been identified as a short tyrosine-containing sequence in the cytoplasmic tail of this protein (Thomas and Roth, 1994). Similarly, a tyrosine motif has also been implicated in the basolateral sorting of the Env protein of some retroviruses (Lodge *et al.*, 1997).

e. Targeting Viral Envelope Glycoproteins to Lipid Rafts Among the host components that are intimately involved in regulating the budding process, there is growing evidence that lipids play an important role. The lipid composition of viruses budding from the plasma membrane such as apical or basolateral membranes of polarized epithelial cells is not necessarily the same as the average lipid composition of these membranes. This suggests that viruses bud from specific microdomains present within these membranes. Accumulating evidence suggests that sphingolipids and cholesterol can become segregated from other membrane lipids to form ordered lipid

microdomains, called rafts, floating in a glycerophospholipid-rich environment (Simons and Ikonen, 2000). Raft lipids are probably held together weakly, establishing a dynamic equilibrium of raft and nonraft regions within the plasma membrane. There is mounting evidence that lipid rafts play a role in the assembly pathway of some enveloped viruses (Chazal and Gerlier, 2003). Indeed, some viral envelope glycoproteins have been found to be enriched within membrane rafts of infected cells. For instance, in influenza A-infected cells, the envelope glycoproteins HA and NA are found to be associated with rafts (Schmitt and Lamb, 2004). In addition, HA and NA from purified influenza virions were found to be associated with a membrane that had solubility properties characteristic of membrane rafts, suggesting that the virion envelope is composed of a raft membrane and that the virus buds through rafts. However, assembly of viral proteins on raft membranes does not appear to be used by all the viruses that bud from the plasma membrane. Indeed, the viral proteins of some viruses like VSV and rabies virus are excluded from raft membranes in infected cells. In addition, not all the viral proteins that assemble at raft domains in infected cells possess specific raft-targeting signals. For instance, the measles virus F glycoprotein is associated with rafts when expressed alone, but not the H glycoprotein (Vincent *et al.*, 2000). However, the H glycoprotein is associated with raft membranes in measles virus-infected cells (Manie *et al.*, 2000; Vincent *et al.*, 2000). Interestingly, when coexpressed with the F glycoprotein, H is recruited to rafts. The association of viral envelope glycoproteins with rafts occurs during Golgi maturation (Chazal and Gerlier, 2003). It has indeed been proposed that raft assembly occurs in the Golgi apparatus (Simons and Ikonen, 1997). Signals for addressing HA and NA into lipid rafts have been mapped to the transmembrane domains, particularly the amino acids predicted to span the outer leaflet of the lipid bilayer (Nayak *et al.*, 2004). Interestingly, signals for apical transport of these proteins in polarized epithelial cells also reside in the transmembrane domains. However, the two types of signals are not identical. Indeed, it is possible to mutate the signal for raft association in influenza virus glycoproteins without altering apical targeting (Barman and Nayak, 2000; Barman *et al.*, 2001; Lin *et al.*, 1998). The cytoplasmic tails of influenza virus glycoproteins have also been found to play a role in the association with rafts (Zhang *et al.*, 2000a).

Although they do not possess an envelope, rotaviruses have been shown to associate with membranes during their assembly (Delmas *et al.*, 2004). In a polarized and differentiated intestinal cell line, rotaviruses are released from the apical surface through a nonconventional pathway that bypasses the Golgi apparatus (Jourdan *et al.*, 1997). It has been shown that rafts may be involved in this atypical pathway (Sapin *et al.*, 2002). Indeed, an important proportion of VP4, the most external viral protein that forms the spikes of mature virions, associates with lipid rafts and is targeted to the apical

membrane early. Later on, other structural viral proteins and viral infectivity also cosegregate with the raft fractions (Cuadras and Greenberg, 2003; Sapin *et al.*, 2002). This suggests that lipid rafts may serve as a platform for the final step of rotavirus assembly.

2. Interaction of the Matrix Protein with Membranes

Some enveloped viruses (Mononegavirale, Orthomyxoviridae, and Retroviridae) encode a protein, called a matrix protein, that interacts with the inner leaflet of cellular membranes and links other viral components to the matrix–membrane complex to form the viral particle (Garoff *et al.*, 1998). Matrix proteins have intrinsic membrane-binding properties, and they have been shown to play a critical role in virus assembly and budding (Garoff *et al.*, 1998). To play their role in virus morphogenesis, virus matrix proteins must be transported through the cytosol to the underside of the plasma membrane where budding occurs. Although matrix proteins of different virus families show functional analogy, they share no sequence or structural homology (Timmins *et al.*, 2004).

In the Retroviridae family, the matrix protein is initially part of the Gag polyprotein (Morita and Sundquist, 2004). Expression of Gag is largely sufficient to induce virus assembly and budding at the plasma membrane, which leads to the release of virus-like particles (VLPs). In immature viral particles, proteolytic processing generates several distinct products, including MA (matrix protein), CA (capsid protein), and NC (nucleocapsid protein), thus producing mature infectious particles. The membrane-binding domain of the MA protein directs the association of Gag with membrane, typically through a bipartite motif consisting of a covalently attached myristic acid moiety and a highly basic domain (Yuan *et al.*, 1993; Zhou *et al.*, 1994). There is considerable evidence that the N-terminal myristyl group of MA protein plays a role in regulating membrane binding (Tang *et al.*, 2004). The affinity of MA expressed alone is substantially lower than when expressed in the context of Gag precursor. It has been shown that myristate exposure is coupled with trimerization, with the myristyl group sequestered in the monomer and exposed in the trimer. The equilibrium constant is shifted toward the trimeric, myristate-exposed species in Gag, indicating that exposure is enhanced by Gag subdomains that promote self-association (Tang *et al.*, 2004) (Fig. 9).

The matrix protein of other virus families generally does not appear to be modified by fatty acids. They also lack hydrophobic stretches that would indicate membrane-spanning domains. The membrane binding of these matrix proteins does not display typical features of peripheral membrane proteins but rather is found to be very stable to salt or high-pH treatments (Schmitt and Lamb, 2004). Even if membrane association is a common

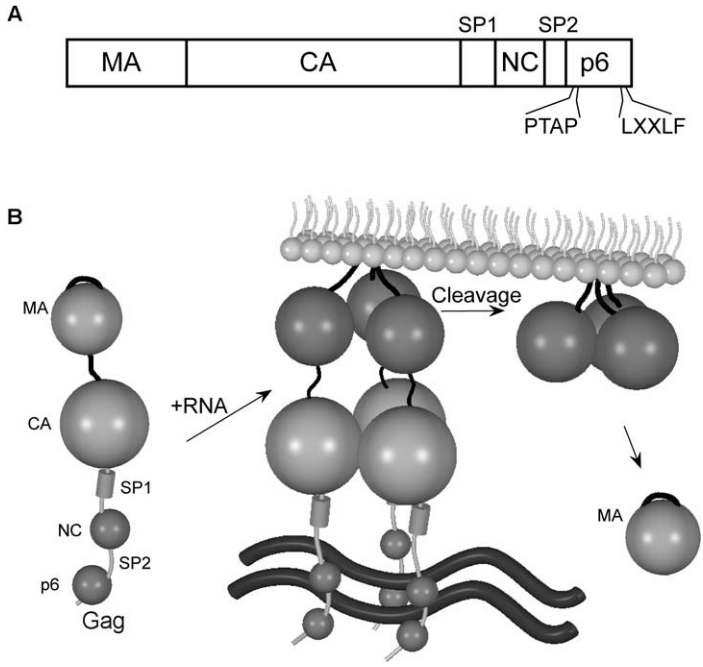

FIG. 9 Retroviral Gag polyprotein and interaction with cellular membranes. (A) Major domains of retroviral Gag polyproteins are matrix (MA), capsid (CA), nucleocapsid (NC), and p6. In addition, spacer peptides separate the CA and NC domains (SP1) and the NC and p6 domains (SP2). On p6, amino acid sequences of conserved viral late motifs are shown. (B) The membrane-binding domain of the matrix protein MA directs the association of Gag with membrane, typically through a bipartite motif consisting of a covalently attached myristic acid moiety and a highly basic domain. The myristyl group is sequestered in the monomer and exposed in the trimer. After cleavage, the affinity of MA for the membrane is substantially lower than in the context of Gag precursor. Importantly, MA protein needs to dissociate from the membrane during virus disassembly upon infection. Although the high concentration of myristylated MA in mature virions is expected to favor protein self-association and membrane binding, the equilibrium will shift toward the monomeric MA species in the diluting environment of the cell being infected, allowing MA to dissociate from the membrane.

feature of matrix proteins, there is no striking common motif evident for membrane interaction. This is likely due to the lack of structural homology between matrix proteins of different virus families (Timmins *et al.*, 2004). In the case of Ebola virus, the matrix protein is composed of two related β-sandwich domains, which are connected by a flexible linker (Dessen *et al.*, 2000). The C-terminal domain has been shown to mediate membrane association (Timmins *et al.*, 2004). More recently, it has been shown that the C-terminal 18 amino acids of Ebola matrix protein play a major role in oligomerization and interaction with lipid rafts at the plasma membrane,

suggesting that this sequence might directly interact with components of rafts at the plasma membrane (Panchal *et al.*, 2003).

B. Initiation and Formation of the Bud

Virus particle assembly is a complex phenomenon requiring concerted actions of many viral and host components. After selection of the budding site, viral components need to accumulate and their assembly leads to initiation of the budding process, growth of the bud, and finally completion of the bud with the release of the virus particle. Various types of interactions are engaged in budding. They include interactions of viral proteins with the lipid bilayer as well as protein–protein interactions of viral components. The driving forces leading to the budding process can derive from outside or inside the viral membrane by protein–protein interactions between the envelope glycoproteins or between matrix or Gag proteins. Some examples of these processes are discussed in this section.

1. Envelope-Driven Assembly of Viral Particles

In addition to production of infectious viral particles, cells infected with flaviviruses also release subviral particles containing the envelope proteins but lacking the nucleocapsid (Lindenbach and Rice, 2001). Similar secreted subviral particles have also been produced by coexpression of prM and E envelope proteins in cell culture, demonstrating that these proteins are intrinsically capable of forming specific particulate structures in the absence of other viral components. The size of these particles is about two-thirds that of the whole virion. They are assembled in an immature form in the ER and undergo the same maturation process as whole virions (Lorenz *et al.*, 2003). In addition, these subviral particles are functionally active; they induce membrane fusion in both cell–cell and liposomal assays (Corver *et al.*, 2000; Schalich *et al.*, 1996).

Structural analyses indicate that these particles are icosahedrally symmetric and capsidless (Ferlenghi *et al.*, 2001). The budding and symmetry of the subviral particles are determined by regular, lateral interactions among the envelope protein subunits. However, the arrangements of the proteins making the envelope of the full-size particle are different (Kuhn *et al.*, 2002). Biological and structural studies suggest that flavivirus assembly is driven by lateral interactions between heterodimers of the envelope glycoproteins E and prM (Mukhopadhyay *et al.*, 2005).

By being inserted in a cellular membrane, the transmembrane domains of these proteins also participate in the budding process. Their anchor function is indeed necessary to isolate a fraction of a cellular membrane that becomes

part of the viral envelope. Interestingly, the transmembrane domains of prM and E have been shown to play a crucial role in the biogenesis of the flavivirus envelope (Op De Beeck *et al.*, 2003). As mentioned in Section IV.A.1.a, the C-termini of prM and E form two antiparallel transmembrane α helices potentially making coiled-coil structures (Zhang *et al.*, 2003). Disrupting these structures by alanine insertion altered the formation of the envelope in the context of viral and subviral particles (Op De Beeck *et al.*, 2003).

In the context of infectious viral particles, the nucleocapsid core needs to be incorporated into the particle. Nucleocapsid assembly appears to take place on the cytoplasmic face of membranes with which prM and E proteins are associated (Khromykh *et al.*, 2001). However, there seems to be no direct interaction between the capsid protein and the envelope proteins (Zhang *et al.*, 2003), suggesting that the capsid protein might interact directly with the inner leaflet of the envelope lipid bilayer (Ma *et al.*, 2004). The mechanism by which encapsidation occurs is not understood, but may require participation of additional viral proteins as well as capsid protein (Kummerer and Rice, 2002; Liu *et al.*, 2003).

Hepatitis B virus contains a nucleocapsid surrounded by an envelope containing three membrane proteins: the large (L), the middle (M), and the small (S) envelope proteins (Ganem and Schneider, 2001). These envelope proteins are expressed from a single open reading frame by differential translation initiation. As a result, the sequence of S is repeated at the C termini of M and L. These envelope proteins show a complex transmembrane topology (Fig. 10). Curiously, the transmembrane topology of about half of the L protein changes drastically after translation (Ganem and Schneider, 2001). This alternative topology leads to the exposure of the preS domain of the L protein on the luminal side of the ER (e-preS), whereas the other keeps the preS domain in the cytosol (i-preS). Importantly, the two topologies provide separate functions to the L protein. Indeed, the e-preS domain is exposed on the surface of the virion and participates in virus receptor binding (Urban and Gripon, 2002), whereas the i-preS domain is important for interaction with the nucleocapsid (Bruss, 2004). The envelope proteins of hepatitis B virus play a major role in virus budding. Patients infected by this virus release subviral particles that contain predominantly the S protein with variable amounts of M and only trace quantities of L subunits (Ganem and Schneider, 2001). In addition, expression of S protein alone also leads to the production of subviral particles, indicating that the viral information necessary for this assembly process resides in the S domain. These subviral particles are assembled in a pre-Golgi compartment. The envelopment of the hepatitis B virus nucleocapsid depends on viral envelope proteins. The S and L proteins, but not the M protein, are required for this process (Bruss, 2004). The nucleocapsid is incorporated into particles by interaction of the i-preS domain of the L protein.

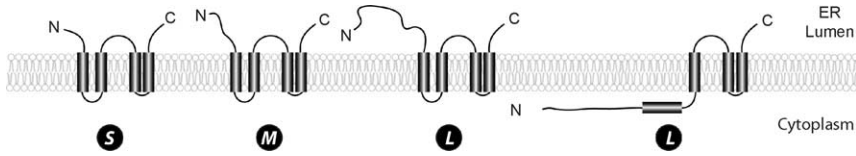

FIG. 10 Topology of hepatitis B virus envelope proteins. Hepatitis B virus contains three membrane proteins: the large (L), the middle (M), and the small (S) envelope proteins, which are expressed from a single open reading frame by differential translation initiation. As a result, the sequence of S is repeated at the C termini of M and L. The transmembrane topology of about half of the L protein changes drastically after translation. This alternative topology leads to the exposure of the preS domain of the L protein on the luminal side of the endoplasmic reticulum (ER), whereas the other keeps the preS domain in the cytosol.

The coronavirus particle is composed of a nucleocapsid containing the genomic RNA associated with the nucleocapsid (N) protein, surrounded by an envelope containing three to four envelope proteins (S, M, and E, and HE for some viruses) (Lai and Holmes, 2001). Coronaviruses have been shown to assemble in a pre-Golgi compartment, and as for the flaviviruses, the envelope proteins drive particle assembly. Indeed, coexpression of both M and E has been shown to induce the production of VLPs, which are similar in size and appearance to authentic virions (Baudoux *et al.*, 1998; Bos *et al.*, 1996; Corse and Machamer, 2000; Vennema *et al.*, 1996). Although the coronavirus envelope can form itself, the nucleocapsid can stabilize the virion structure (de Haan *et al.*, 1998; Kuo and Masters, 2002). Virus budding is most likely triggered by interactions between E and M proteins. The M protein plays a central role in coronavirus assembly. Indeed, in addition to its essential role in envelope formation, M protein interacts by its C-terminus with the nucleocapsid (Escors *et al.*, 2001; Kuo and Masters, 2002; Narayanan *et al.*, 2000). This interaction likely triggers the packaging of the nucleocapsid into the virion. In addition, due to its subcellular localization, the budding site of coronaviruses is likely dictated by the M protein. Interestingly, the M protein is able to interact with itself and with S, E, and HE proteins (Corse and Machamer, 2003; de Haan *et al.*, 1999, 2000; Lim and Liu, 2001; Nguyen and Hogue, 1997). These interactions likely contribute to the formation of the envelope and help to incorporate the other envelope proteins into the coronavirus envelope.

The alphaviruses particle consists of a nucleocapsid containing the genomic RNA enclosed in a shell formed by the capsid protein, surrounded by an envelope containing the envelope glycoproteins E1 and E2. Interestingly, structural studies reveal two shells in the alphaviruses (Schlesinger and Schlesinger, 2001). One is present on the internal side of the viral membrane, which is formed by the capsid protein, and the other one on the external side, which is formed by the glycoproteins. Both shells are organized

according to a T = 4 symmetry. Budding of the alphaviruses occurs at the plasma membrane, and the envelope glycoproteins of alphaviruses have also been shown to play a major role in this process (Garoff *et al.*, 2004). Lateral interactions within the "skirt" region of the glycoprotein layer have been shown to participate in assembly (Forsell *et al.*, 2000). However, when expressed alone, the envelope glycoproteins do not induce budding (Suomalainen *et al.*, 1992). Indeed, budding and particle formation require interaction of the E2 glycoprotein endodomain with the capsid protein (Forsell *et al.*, 2000). Although the alphavirus glycoproteins play a major role in virion assembly, the real contribution of the preassembled nucleocapsid shell in the process remains unclear (Garoff *et al.*, 2004).

2. Matrix- or Gag-Driven Assembly of Viral Particles

The importance of matrix proteins for virus budding has been shown by the demonstration of budding of VLPs from cells expressing matrix proteins alone (Schmitt and Lamb, 2004). In many cases, additional viral components, when coexpressed with the viral matrix proteins, have been found to be incorporated into VLPs. In addition, in some cases, coexpression of matrix proteins along with additional viral components can substantially increase the efficiency with which particles bud. Interestingly, production of a rabies virus, which lacks the entire matrix gene, led to a 5×10^5-fold reduction in viral particle production (Mebatsion *et al.*, 1999), confirming the major role played by the matrix protein in the context of a viral system. The mechanism by which matrix proteins drive budding at the plasma membrane is not known. Matrix proteins interact with the inner leaflet of cellular membranes and the curving of the membrane may result from a self-assembly process of matrix proteins or may be induced by interactions with cellular structures, such as the cytoskeleton (Takimoto and Portner, 2004). Experimental data on the matrix protein of several viruses suggest that matrix proteins are assembled as a layer beneath the plasma membrane of infected cells and induce other viral components to gather at this location, from which virus budding can then occur (Schmitt and Lamb, 2004). Determination of the atomic structure of some matrix proteins is beginning to shed some light on the potential implication of these proteins in virus assembly (Timmins *et al.*, 2004). As discussed in section IV.A.2, the matrix protein of Ebola virus is composed of two related β-sandwich domains, which are connected by a flexible linker (Dessen *et al.*, 2000). Early work showed that this conformation is metastable and allows an easy transition into oligomeric ring-like structures *in vitro* (Ruigrok *et al.*, 2000; Scianimanico *et al.*, 2000). The ring structures are either octamers or hexamers (Timmins *et al.*, 2003). Interestingly, SDS-resistant octamers have been shown to be present in Ebola VLPs and in virus particles (Panchal *et al.*, 2003). The N-terminal domain of Ebola

matrix protein is involved in oligomerization, whereas the C-terminal domain has been shown to mediate membrane association (Timmins *et al.*, 2004). Interestingly, a single point mutation that abolishes RNA binding and octamer formation does not affect VLP formation, indicating that octameric matrix protein is dispensable for VLP formation (Hoenen *et al.*, 2005). However, when the mutation was introduced into the Ebola virus genome, no virus was rescued, indicating that RNA binding and octamer formation are essential for the Ebola virus life cycle.

Specific interactions between viral glycoproteins and matrix proteins can help in concentrating matrix proteins at the budding site. When expressed alone, the matrix protein of influenza virus does not bind to detergent-resistant membranes (Ali *et al.*, 2000). However, in the presence of viral envelope glycoproteins, the matrix protein of influenza interacts with HA and NA localized in rafts, and the matrix protein associated with membranes becomes detergent resistant (Ali *et al.*, 2000; Zhang *et al.*, 2000b). Thus the envelope glycoproteins, which are targeted to rafts, can drag other viral components so as to promote assembly within rafts, a location where influenza virus has been shown to assemble and bud (Scheiffele *et al.*, 1999). However, this is not the case for measles virus for which both the F glycoprotein and the matrix protein contain signals for independent targeting to rafts (Vincent *et al.*, 2000). Although interactions between matrix proteins and cytoplasmic tails of viral envelope glycoproteins seem to play a role in the budding of many negative-strand RNA viruses, incorporation of glycoproteins into viral particles can occur independently of specific signals as observed for glycoprotein G of VSV (Schnell *et al.*, 1998). The nucleocapsid is an essential additional viral component that also needs to accumulate at the budding site to form infectious viral particles. Interactions between matrix proteins and viral nucleocapsids are well documented and are presumed to be critical for efficient incorporation of genomes into budding virions (Schmitt and Lamb, 2004).

In the Retroviridae family, the matrix protein is initially part of the Gag polyprotein (Morita and Sundquist, 2004). Expression of Gag is largely sufficient to induce virus assembly and budding at the plasma membrane. Gag is a complex polyprotein containing different functional domains. In HIV, Gag is organized into four distinct regions, which carry out different primary functions in the coordinated process of particle formation: the N-myristylated MA domain targets Gag to the plasma membrane, CA makes important protein–protein interactions that are required for particle assembly, NC captures the viral RNA genome and couples RNA binding to particle assembly, and p6 recruits cellular proteins that function in the final stages of virus release (Adamson and Jones, 2004). In addition to these domains, spacer peptides separate the CA and NC domains (SP1) and the NC and p6 domains (SP2) in Gag polyprotein. Although Gag is processed by

the viral protease to produce infectious virions, extracellular particles are produced in the absence of this protease. This indicates that the Gag polyprotein can be considered as the element that drives virus budding. Assembly is therefore a result of ordered oligomerization in which neighboring regions on adjacent Gag monomers provide the necessary molecular contacts. Like the matrix proteins of negative-strand RNA viruses, MA associates with membranes and is found beneath the inner layer of the virion membrane. However, unlike other matrix proteins, the MA domain does not play a major role in driving particle assembly. Indeed, deletion of the MA domain does not affect the ability of other Gag assembly domains to drive particle assembly (Adamson and Jones, 2004). The predominant domains involved in Gag assembly have been identified in the C-terminal third of the CA domain and in its adjoining spacer peptide SP1. In addition, the NC domain has also been shown to promote Gag assembly after binding of the viral genome to the NC domain. In conclusion, correct particle assembly occurs via Gag multimerization and is driven by multiple cooperative interprotein contacts involving several Gag domains. Although multimerization of Gag may be initiated in the cytosol, binding to the plasma membrane facilitates the concentration of Gag in an oriented way, which further aids multimerization, assembly, and budding (Morikawa *et al*., 2000; Nermut *et al*., 2003; Ono *et al*., 2000). A molecular reorganization of Gag takes place upon proteolytic maturation of the polyprotein. This maturation is considered to take place concomitant with, or shortly after, release of the immature particle and is mediated by the virion-encoded protease. After cleavage between MA and CA proteins, the matrix protein MA remains associated with the membrane; however, the affinity of this interaction is considerably weaker than in the context of uncleaved Gag polyprotein (Hermida-Matsumoto and Resh, 1999; Zhou and Resh, 1996). Importantly, MA protein needs to dissociate from the membrane during virus disassembly upon infection. As discussed in Section IV.A.1.a, the N-terminal myristyl group of MA protein plays a role in regulating membrane binding and dissociation (Tang *et al*., 2004). Indeed, the myristyl group is sequestered in monomeric MA and exposed in the trimeric form of the protein. Although the high concentration of myristylated MA in mature virions is expected to favor protein self-association and membrane binding, the equilibrium would shift toward the monomeric MA species in the diluting environment of the cell being infected, allowing MA to dissociate from the membrane (Tang *et al*., 2004) (Fig. 9). Although Env is not required for budding of the retroviruses, it is essential for the formation of infectious particles. Env can be concentrated at the site of virus budding through specific interactions between the cytoplasmic tail of the TM subunit and the MA protein of Gag (Pelchen-Matthews *et al*., 2004). However, the fidelity of Env incorporation is not stringent.

C. Late Steps in Virus Budding and Release

The last stage of budding requires a final pinching off step to release the assembled virion from the cell. Recently, it has become apparent that some viruses use the endocytic pathway to coordinate their release from infected cells. These viruses have been shown to exploit the machinery that generates the internal membranes of multivesicular bodies (MVB) (Pelchen-Matthews *et al.*, 2004; Pornillos *et al.*, 2002). It has been known for a while that a deletion of the C-terminal region of HIV Gag (p6 protein) causes a defect in viral particle release (Gottlinger *et al.*, 1991). Electron microscopic studies of such mutants show particles that fail to pinch off the plasma membrane. It has also been shown that a highly conserved PTAP sequence motif, termed late domain, is playing a crucial role in viral budding (Huang *et al.*, 1995). Several classes of viral late domains have now been identified: P(T/S)AP, YXXL, LXXLF, and PPXY (Timmins *et al.*, 2004). These motifs are present in the Gag proteins of retroviruses and in the matrix proteins of some negative-strand RNA viruses. Importantly, these viral late domains function by recruiting cellular factors that are normally involved in intraluminal vesicle formation of MVB (Pelchen-Matthews *et al.*, 2004; Pornillos *et al.*, 2002). Proteins destined for delivery to intraluminal vesicles are frequently found to be monoubiquitinated on their cytoplasmic domain. This allows them to be recognized by protein complexes called endosomal sorting complexes required for transport: ESCRT-I, ESCRT-II, and ESCRT-III. In addition to recognizing ubiquitinated proteins, the ESCRT machinery is thought to drive the outward vesiculation and scission of the intraluminal vesicles, a process similar to virus budding. Interestingly, viral late domains function by recruiting the same factors that are involved in intraluminal vesicle formation (Fig. 11). It is now recognized that late domain interactions with cellular factors most likely recruit the ESCRT machinery to the site of budding where the components of this machinery are utilized by the viruses in a mechanistically analogous manner to their normal cellular function to mediate virus budding (Morita and Sundquist, 2004; Pelchen-Matthews *et al.*, 2004; Pornillos *et al.*, 2002).

V. Concluding Remarks

Viruses interact with cellular membranes during all major steps of their life cycle. The nature of the interactions between viral proteins and cellular membranes is complex, and often not completely understood. While significant progress has been made at biochemical, structural, and ultrastructural levels of viruses and viral proteins, crucial aspects of virus–host interactions

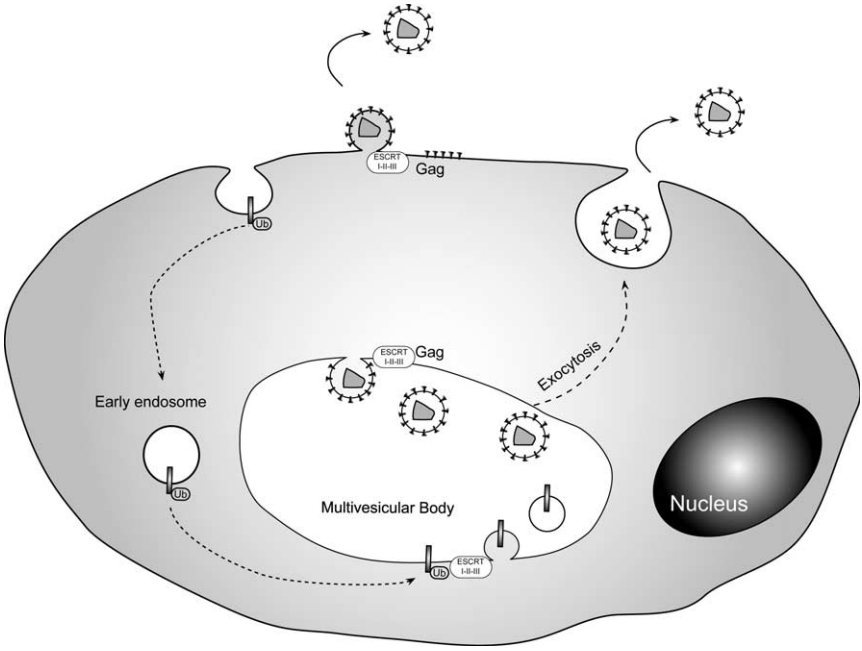

FIG. 11 Virus budding and the machinery of multivesicular body formation. Proteins destined for delivery to intraluminal vesicles are frequently found to be monoubiquitinated on their cytoplasmic domain. This allows them to be recognized by protein complexes called endosomal sorting complexes required for transport (ESCRT-I, ESCRT-II, and ESCRT-III) that drive the outward vesiculation and scission of the intraluminal vesicles in the multivesicular body. Viral late domains that are present in Gag polyprotein are involved in recruiting the ESCRT machinery to the site of budding (plasma membrane or multivesicular body) where the components of this machinery are utilized by the viruses for budding.

remain to be elucidated. The concerted interactions of viral proteins, lipid membranes, and host factors result in a variety of conformational changes leading to the establishment of different protein associations. These distinct protein associations mediated and supported by membranes provide viruses with the ability to cross the plasma membrane for particle entry, modify different intracellular organelles for genome replication, and acquire envelope proteins for virion assembly. In each of these processes, specific cell membranes are targeted by distinct viral protein signals to create either a microenvironment or a compartment on which appropriate host factors will assist. Although, in general, the major steps of viral replication are known, the specific requirements for establishing interactions and functions through particular membranes are matters of active research. This not only will contribute to a better understanding of virus replication but will also advance the understanding of membrane–protein interactions in the cell.

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