Budding of enveloped viruses from the plasma membrane

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Summary

Many enveloped viruses are released from infected cells by maturing and budding at the plasma membrane. During this process, viral core components are incorporated into membrane vesicles that contain viral transmembrane proteins, termed 'spike' proteins. For many years these spike proteins, which are required for infectivity, were believed to be incorporated into virions *via* a direct interaction between their cytoplasmic domains and viral core components. More recent evidence shows that, while such direct interactions drive budding of alphaviruses, this may not be the case for negative strand RNA viruses and retroviruses. These viruses can bud particles in the absence of spike proteins, using only viral core components to drive the process. In some cases the spike proteins, without the viral core, can be released as virus-like particles. Optimal budding and release may, therefore, depend on a 'push-and-pull' concerted action of core and spike, where oligomerization of both components plays a crucial role.

Introduction

Viral budding and release is one of the least well characterized processes in the life cycle of enveloped viruses. Because of its obvious importance for the production of progeny virus, the budding process may provide an effective target for anti-viral therapy. Defects in viral budding have also been implicated in altered viral pathogenesis[1-3]. In the mammalian cell, viruses can bud from the nuclear membrane (herpesviruses), into the lumen of the endoplasmic reticulum (flavi- and hepadnaviruses), into the pre-Golgi compartment (coronaviruses and poxviruses), into the Golgi (bunyaviruses) or at the plasma membrane (PM[†]). This review will focus on budding at the PM. Viruses that bud at the PM include arena-, alpha-, rhabdo-, paramyxo-, orthomyxo- and retroviruses. Very little is known about arenavirus budding, and therefore we will restrict our discussion to the other virus groups.

The budding and maturation process involves the interaction of many components. Viral capsid proteins bind to the nucleic acid of the virus, forming the nucleocapsid (NC). The NC may interact directly with viral transmembrane (TM) proteins, or with viral matrix proteins lining the inner leaflet of the membrane. Binding of matrix proteins to the membrane may be catalyzed by covalent addition of lipids to the pro-

teins and/or by interaction with TM proteins. Ultimately, the PM bends around the viral core, pinching off an enveloped viral particle, and releasing it into the extracellular medium. Although release of core-containing particles can, in some cases, occur in the absence of TM envelope (spike) proteins, inclusion of spikes in the virion is required for making the particle infectious. In addition to core-spike interactions, lateral interactions between spike components in the plane of the membrane may play an important role in the life cycle of the viruses. Such lateral interactions may serve to facilitate the budding process by catalyzing patch formation (i.e. the formation of aggregates of budding components, including lipids and proteins), and by formation of multiple presentation motifs for binding to the core. The lateral interactions also ensure that correct combinations of spike proteins for assembly and infectivity are formed.

Viral budding may not be a simple task, since in most cases virion membrane curvature is extensive, leading to large differences in surface areas of the inner and outer leaflet of the virion envelope (e.g. the outer leaflet of the alphavirus envelope is 40% larger than the inner leaflet). Another complexity is how the viral bud rapidly and accurately pinches off from the PM. Recent data suggest that virus release is dependent on the state (composition, fluidity, etc) of the membrane, which may be modulated by viral and host proteins. The lipid content also seems to play a role in these processes. While the viruses which bud from

the PM utilize different strategies for budding, the basic principles of assembly and release seem to be universal. In this review we have categorized the viruses according to three basic budding strategies, as indicated in Fig. 1 and Table 1.

The alphaviruses

The replication and structure of the positive strand alphaviruses has been extensively studied (reviewed in refs 4 and 5), and it is within this group of viruses that the mechanism of budding is best understood. The alphavirus particle contains a single molecule of positive-strand RNA packaged into an NC consisting of 240 copies of the capsid monomer (C), arranged in pentamer and hexamer rings, forming a T=4 symmetry core. The viral envelope also has a T=4 icosahedral symmetry, containing 240 copies each of the spike proteins E1 and E2 which, in three pairs of E2E1 heterodimers, form 80 spike structures[6,7]. While the C protein and the spike proteins occur in a 1:1 molar ratio, the spike complexes are positioned between the capsomeres such that each trimeric spike interacts with C monomers of three separate capsomeres (Fig. 2A). This arrangement may also serve to stabilize the core structure by crosslinking capsomeres to each other.

The alphaviruses encode four 'structural' proteins C, p62, 6K and E1, all of which are involved in virus budding and release from the infected cell. The C protein is entirely cytoplasmic. The three type I TM proteins, p62, 6K and E1, form a complex which migrates to the cell surface where budding occurs. Before reaching the PM the p62 protein is cleaved by furin-type host proteases to its mature form, E2. The p62 form is needed to chaperone E1 during transport to the cell surface so that the fusion peptide located in E1 is not prematurely exposed; an E1 trimer is the fusion-active form[8]. The 6K protein is not incorporated into new virions but plays a role in virus release (discussed below). Budding of

alphaviruses is entirely dependent on the presence of both C and the E2E1 spike complex[9].

The cytoplasmic (tail) domain of E2 is responsible for the interaction of the spike with the NC[10]. This highly conserved stretch of about 30 amino acids contains three conserved Cys residues, all of which are palmitylated and all of which are important for virus assembly[11]. The N-terminal half of the tail has a direct repeat (consensus LTPYALTPGA), involved in interaction with the NC. Within this repeat, substitution of the Tyr residue by Phe or Trp results in reversions selected at a rapid rate, suggesting a Tyr requirement for optimal budding[12]. The Leu residue in position +2 from the Tyr is also important for budding[13,14]. A hydrophobic pocket on the capsid protein is involved in binding the Tyr and Leu residues of the E2 tail[13,15]. Within this pocket, a Tyr and Trp pair interact with the Tyr side chain of the E2 tail, docking it into the pocket (Fig. 2B,C). The (E2E1)₃ spikes may form patches on the PM of infected cells[16,17], possibly providing a multiple binding surface facilitating the budding process.

When the C protein is released from the nascent chain it transiently associates with the ribosomal large subunit, from whence it is recruited into newly forming NC structures. Until recently, the prevailing belief was that preformed cytoplasmic NCs are precursors in budding. More recent results, however, indicate that another pathway may be used as well. When the spike binding pocket was mutated to block entry of the E2 tail Tyr side-chain, C became deficient in stable cytoplasmic NC formation. Nevertheless, this did not abolish budding of virus, which still occurred with substantial efficiency[15]. Other studies, in which mutations within the central region of C were generated, also yielded variants which were deficient in stable cytoplasmic NC formation but nevertheless could bud quite efficiently[13,18]. Recent X-ray data of C protein crystals have provided a possible explanation for these observations[13]. In the crystals a C protein monomer binds to a neighboring C monomer via

Fig. 1. Three basic budding strategies outlined in this review. (A) Alphaviruses bud by binding cytoplasmic NCs (yellow) to spike complexes at the PM (green). The spikes consist of three copies each of the viral TM proteins E1 (pink) and E2 (blue). E1 does not participate in NC binding. The cytoplasmic tail of the E2 protein binds to the NC (see Fig. 2). No foreign proteins can be incorporated into alphavirus particles, which form very condensed structures with defined spike-NC interactions. (B) The negative strand viruses (here exemplified by VSV) accumulate the matrix protein (MA, yellow) and the RNP (blue) beneath the PM. This binding is sufficient to curve the membrane and may in itself lead to release of virus-like particles. Viral spike proteins (pink) accumulate at the bud but it is unclear whether spikes and MA have direct interactions. Host proteins may be incorporated into virus particles. (C) Retroviruses direct the Gag-protein precursor (yellow) to the PM by virtue of an N-terminally located myristic acid (red). The MA domain of the GAG polyprotein presumably interacts directly with the cytoplamic tail of the Env spikes. Retroviruses also readily incorporate foreign (host) proteins into their envelope. The possible role of lipid sorting as part of patching and budding is indicated by a darker green colour of the membrane of the buds.
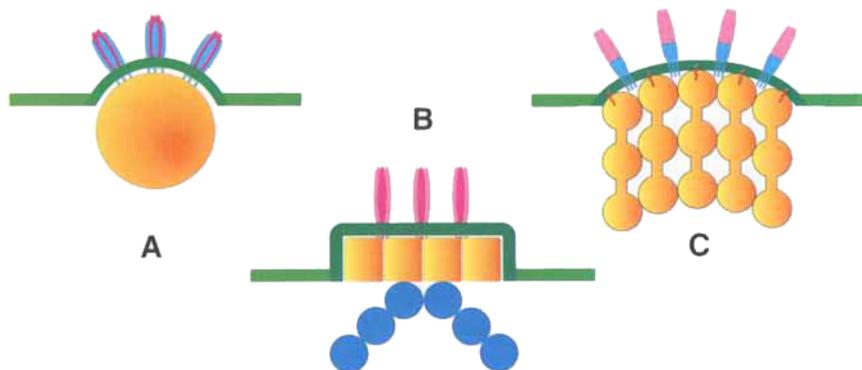
Table 1. *Viral structural components involved in budding*

| Virus | Core | | Matrix Protein | Spike protein | | |
	Protein	Symmetry		Precursor	Mature	Oligomer state
Alphavirus	C	Icosahedral	None	p62E1	E2E1	(E2E1)$_3$
Rhabdovirus	N	Helical	M	G	G	G$_3$
Paramyxovirus	N	Helical	M	F0;HN	F1; HN	F1$_3$; HN$_4$
Orthomyxovirus	NP	Helical	M1	HA0, NA	HA1/2; NA	(HA1/2)$_3$; NA$_4$
Retrovirus (HIV)	Gag	Quasiicosahedral	MA	gp160	gp120-gp41	(gp120-gp41)$_3$

an 'arm' extension containing a Leu-X-Leu motif, which is docked in the spike-binding pocket. Mutation of this motif in the middle region of C results in aggregation of the C protein. Thus, mutation of either the arm or the arm (spike) binding pocket results in inability to form NCs, but does not abolish budding. This suggests that binding of the arm serves some assembly function, and implies that budding, even for wild-type virus, may occur by two pathways using either preassembled or co-assembled NCs.

While NC-spike interactions seem to be defined for alphaviruses, the role of the small 6K TM protein is still an enigma. This palmitylated protein is transported to the PM in complex with the E2E1 heterodimer but little of the 6K is incorporated into new virions. However, when the gene encoding 6K is deleted from the genome, virus budding is reduced by as much as 100-fold, and NCs accumulate underneath the PM suggesting that an initial interaction with the spike has occurred[19]. The few virions produced from the 6K deletion appear to be of wild-type size and shape, and properties such as binding to new cells, entry and fusion appear to be unaltered[20]. This indicates a specific role for 6K in virus release. Several observations suggest that 6K plays a role in conditioning the membrane for budding. For example, the effects of the 6K deletion mutant are less marked in cells with a more fluid (less viscous) membrane such as insect cells[20], where the sphingomyelin and phosphatidylcholine contents are low (9% and 14% respectively) compared to the PM of mammalian cells (e.g. 25% and 27%, respectively in BHK cells), and where the cholesterol to lipid ratio is very low (1:6) compared to that in BHK cells (1:1). Mutation of 6K, which abolishes palmitylation, leads to impaired envelopment and production of multicored virions[21], suggesting that lack of palmityl groups obstruct bending of the membrane and/or vesicle fission. Another potential mode of action of 6K is related to its ability to increase membrane permeability for ions[22]. Indeed, wild-type virus growing in cells which are in medium of low ionic strength mimic the 6K deletion mutant phenotype. In such cases, NCs accumulate underneath the membrane; this block in virus release can be rapidly reversed by change to isotonic medium[4]. Thus, 6K may function to keep the cytosolic and medium ion concentrations in balance, thereby allowing greater flexibility for the membrane to pinch off virus particles.

Negative strand RNA viruses

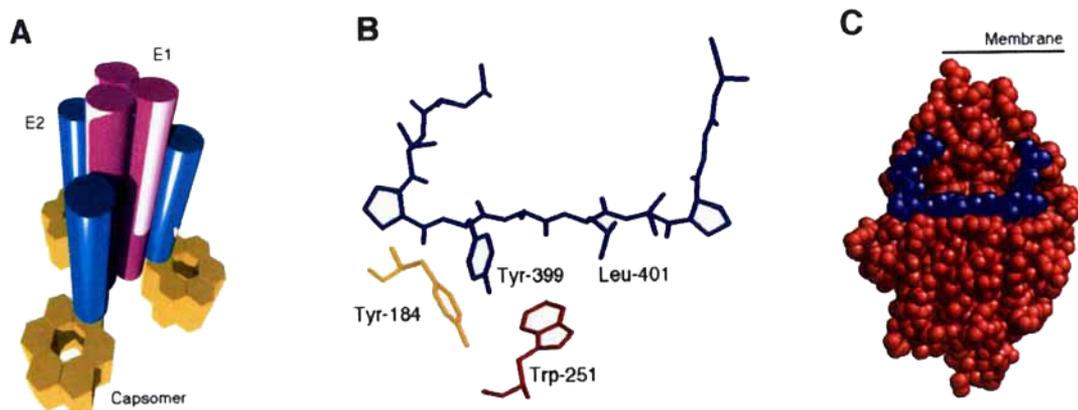The negative strand rhabdo-, paramyxo- and orthomyxo-

Fig. 2. Schematic representation of alphavirus spike-capsid interactions. (A) In the trimeric spike structure, centrally located E1 proteins (pink) are surrounded by the E2 proteins (blue), which interact with three separate capsomeres (yellow)[6]. The membrane has been omitted for clarity. (B) A model of how the tail repeat motif (LTPYALTPGA) may interact with core structures. The two proline residues provide bending of the motif to allow proper presentation of the Tyr-399 and Leu-401 residues (Semliki Forest virus coordinates) into the hydrophobic cavity of the C protein. In the cavity, the C protein residues Tyr-184 and Trp-251 interact with the Tyr-399 of the E2 tail[14,15]. Recent work suggests that the Leu-401 residue also docks into the pocket and thus would contribute to the binding[13,14]. (C) Space-filling model of the capsid monomer molecule (in red) onto which the tail peptide (in blue) of the E2 spike protein has been docked. The position of the viral membrane is indicated. Note that the presentations here are very schematic and the reader is referred to original publications for more accurate details.

viruses appear to utilize similar budding strategies. The simplest of them are the rhabdoviruses (e.g. vesicular stomatitis virus, rabies), which have a nonsegmented genome and encode one TM spike protein G. The paramyxoviruses (e.g. parainfluenza virus, respiratory syncytial virus, Sendai virus, mumps virus and measles virus) also have a nonsegmented genome, but encode the two spike proteins HN (hemagglutinin-neuraminidase) and F (fusion). The orthomyxoviruses (e.g. influenza), have a segmented genome and neuraminidase (NA) and hemagglutinin (HA) spike proteins. All three families encode a matrix protein (M), which is believed to be the key protein involved in virus budding[23].

In the infected cell the M protein binds to RNP (comprising the genomic RNA and N protein) and to the inner surface of the PM. It has been shown that the M protein, in the absence of spike, has the ability to promote vesiculation of the membrane, releasing M-containing particles into the extracellular medium[24,25]. Coexpression of spikes, however, significantly stimulates M binding[25] and budding[25-27]. One would expect, therefore, that M and spike would interact directly, and indeed mutation of the cytoplasmic tail of influenza NA aborts stimulation of M membrane binding[26]. Although it has been possible to recover influenza virus where the cytoplasmic tail of HA[28] or NA[29,30] was deleted, virus particles formed with low efficiency, and revertants of tailless HA occurred readily[28]. The morphology of double (HA/NA) tailless particles also differed greatly from wild-type virus, being both more elongated and irregular in shape[31]. Studies involving chimeric spike protein or pseudotyping (packaging of viral particles with heterologous spike proteins) are also illustrative. Pseudotyping of rabies with the envelope protein of HIV-1 was possible only when the cytoplasmic domain of HIV Env was swapped for the vesicular stomatitis virus (VSV) G tail[32].

Other results point to a role in budding for the TM anchor domain of the spike protein. Budding of spikeless rabies is stimulated sixfold by coexpression of tailless G protein[25]. A chimeric HA having the TM domain of herpes simplex virus and the HA external and cytoplasmic domains was hardly incorporated into virions at all[33]. On the other hand, efficient inclusion of native foreign proteins into VSV has been reported[34], indicating that inclusion of VSV anchor or tail sequences are not always necessary. One possible explanation for these apparently conflicting results could be the formation of membrane patches induced by G (and M) rather than protein protein interactions. VSV virions are known to harbor a different lipid composition from its host membrane. G- and M-induced patching results in formation of microdomains which, compared to normal PM distribution, have a high phosphatidic acid, phosphatidylserine, cholesterol and sphingomyelin content and are low in phosphatidylcholine and phosphatidylethanolamine content[35]. The G and M proteins alter the lateral distribution of these lipids, suggesting that enrichment is part of the budding

process. While expression of either M or G separately results in formation of lipid patches, co-expression of both proteins has a synergistic effect leading to greater condensation of the patch[35]. M exists in both a phosphorylated and unphosphorylated form but, when it is locked into the phosphorylated state (by reducing Ca^{2+}-levels), normal patch formation is abolished and M becomes dispersed in the membrane[36]. The negatively charged headgroups of the lipids appear to be more important than the fatty acids in M protein-induced patch formation[35], providing a possible explanation for the importance of the unphosphorylated state of M.

The retroviruses

Budding of mammalian C-type retroviruses such as Moloney murine leukemia virus (Mo-MLV) and most other retroviruses such as Rous sarcoma virus (RSV), human and bovine T-cell leukemia virus (HTLV and BLV) and lentiviruses (e.g. human and simian immunodeficiency viruses, HIV and SIV) is dependent on the assembly and attachment of the viral Gag (group specific antigen) protein to the membrane[37]. In contrast, type B and D retroviruses preassemble the core in the cytoplasm. However, a single substitution in the matrix (MA) of Mason-Pfizer monkey virus (type D) makes this virus core assemble at the PM[38], demonstrating its close relationship to C-type retroviruses. The viral TM spike protein, Env, carries both receptor binding and fusion functions and forms trimers, which are activated by furin cleavage to form a TM component and a surface component (gp41 and gp120 in HIV-1, respectively).

The Gag polyprotein alone, i.e. the precursor of matrix (MA), capsid (CA) and NC proteins, is capable of forming virus-like particles[39,40]. The N-terminal domain of the MA protein is necessary and sufficient for membrane targeting of Gag[41,42]. This region contains an N-terminal myristic acid and a cluster of basic residues along the first helix of the protein (Fig. 3). Myristylation needs to be completely blocked to block assembly, suggesting that relatively few molecules are sufficient for PM targeting[43]. The membrane-binding signal of MA is regulated in the context of Gag. Within full-length Gag, the membrane-targeting signal is exposed, but in the mature, virion-associated form of MA, this signal is less exposed allowing release of MA from the membrane during entry into new cells[44]. Thus, it is the Gag precursor which initiates budding. Cleavage of the precursor by the viral protease to form the mature structural proteins occurs later, during or after budding.

The 3-D structures of HIV, SIV, BLV and HTLV-II MA proteins have recently been determined. These studies show that the MA proteins, although diverse by primary sequence, are structurally very similar. The crystal forms of HIV and SIV MA form trimers (Fig. 3B), which may have relevance for the higher-order structure of the core. The CA protein also participates in the organization of the core (see
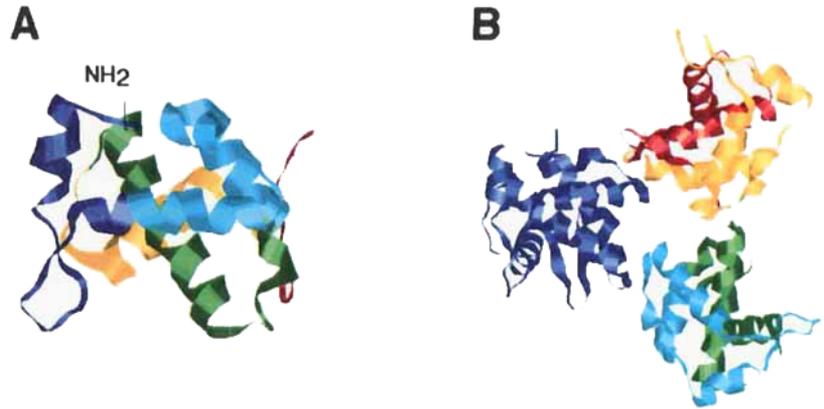
A

NH2

B

Fig. 3. Crystallographic structures of simian immunodeficiency virus MA protein[49]. (A) The monomer structure of MA, with its first helix in purple and the N terminus is indicated. The N-terminally located myristic acid molecule is not shown. (B) Crystallographic trimer of MA, which has been suggested to be part of a higher-order fullerene-type structure.

ref. 45 and references therein), and partial structures of HIV CA have been determined[46,47]. A fullerene-type higher order organization of the Gag particle has been suggested[48], and the trimeric MA structures can be placed in an orientation compatible with the proposed fullerene-model[49]. Recently, it was found that the CA protein on its own can form hexameric rings[45] somewhat similar to those previously reported for HIV-Gag particles[48]. Although some minor differences were found, the overall similarity suggests a fullerene-like structure for the retrovirus core.

Although the core particle is able to bud on its own, incorporation of spike proteins is, of course, necessary for virions to become infectious. This suggests that there may be specific interactions between the cytoplasmic domain of the Env protein and the Gag protein. The close association of the matrix protein (MA) with the membrane suggests that it is the MA domain of the Gag polypeptide which is involved in Env binding. Indeed, mutations blocking Env incorporation have been mapped within the first 40 N-terminal residues of MA[50,51]. A direct Env-MA interaction is also supported by the observation that Env can mediate polarized budding by HIV-1 Gag which, in the absence of Env, is non-polarized in epithelial cells[52,53]. Perhaps the most compelling evidence for a direct MA-Env interaction comes from recent in vitro binding studies, which showed that the last 67 C-terminal residues of HIV-1 Env tail interact with MA[54]. However, while certain Env tail mutations block its incorporation into virions, others have no effect[55-57] or even increased incorporation[58-60]. Interestingly, an HIV-1 Env protein lacking its entire cytoplasmic domain is efficiently incorporated into budding virus. Such tailless Env forms tighter patches at the PM than full-length protein, providing a possible explanation for this phenomenon[61]. This result also raises the possibility that Env incorporation may not be by direct Env-spike interactions.

Vpu is an HIV-1 derived TM protein which enhances virus release from infected cells[62,63]. Vpu has been suggested to form homo-oligomers which function as ion channels[64,65], but its structure indicates that it may also have a membrane destabilizing activity. Vpu of HIV-1 can act in trans and stimulate the release of Gag particles of other

retroviruses such as HIV-2 and Mo-MLV, which themselves lack a Vpu-like protein[66].

The cytoplasmic domains of lentivirus Env proteins also have some interesting features relevant to this discussion. The distal part of the tail contains two amphipatic helical segments, which can perturb and even form pores in the cell membrane[67-69]. By analogy with Vpu and 6K, this could result in enhanced virus release due to destabilization of the membrane and/or change in intracellular ion concentration. The amphipatic segments are docked to the membrane by two conserved palmitate residues[70]. While HIV-2 does not have a Vpu protein, its Env protein has recently been found to harbor a Vpu-like factor in its cytoplasmic domain which is responsible for efficient virus release. Removal of this factor can be compensated for by co-expression of Vpu from HIV-1[71,72].

Conclusions

Budding and the host connection

In a recent budding model[25], internal membrane-associated components, such as M, were proposed to provide a 'push' function which would result in the bending of the membrane. This bending would be further stimulated by a pulling force exerted by the spike proteins. Many of the results reviewed here do indeed support such a 'push' role for M and Gag. The observation that expression of VSV G or HIV Env on their own can result in release of G- or Env-containing vesicles[61,73] is compatible with the 'pull' concept. It may be that the structure of the large external domain of a spike protein complex imparts an asymmetry that can induce bending of the membrane. For example, a tsE2 mutant of Sindbis virus (an alphavirus) accumulates NCs under the PM under nonpermissive conditions (similar to a 6K deletion mutant phenotype), suggesting that an altered structure of E2 can interfere with the pulling function of the spike, thus hampering membrane curvature and budding.

Virus envelopment and budding strongly resemble cellular processes of vesicular transport, where traffic within the various cellular compartments is dependent on specific

interactions between soluble and TM protein components. Such interactions provide the signals to load specifically proteins for transport into vesicles which bud from one compartment and fuse with another. The basic principles of how these reversible protein-protein interactions are designed may well be utilized by viruses.

Vesicular transport is also exploited by some viruses to ensure viral budding from specific plasma membrane domains of polarized cells. This has obvious implications in promoting normal viral dissemination in the host and access to sensitive cell types. These membrane domains (e.g. apical and basolateral) have different lipid and protein compositions[74,75]. The differential distribution of membrane proteins is generated by a sorting of these proteins into distinct vesicles during exit from the Golgi apparatus[76]. Sorting to the basolateral membrane appears to be frequently mediated by a tyrosine-based signal, for example in the envelope glycoproteins of both HIV-1[52] and VSV[77]. Signals which mediate specific apical transport are less defined. The expression of influenza hemaglutinin, neuraminidase and M2 proteins is restricted to the apical membrane in polarized cells[78]; however this signal, at least in the case of influenza HA, can be overcome by the addition of a basolateral sorting signal[79]. A glycosylphosphatidyl-inositol (GPI) anchor has been proposed to act as a dominant apical membrane sorting signal[80]; addition of a GPI anchor to the VSV envelope protein redirects its expression to the apical membrane[81]. An intact microtubule network may also be more important for maintenance of apical sorting than basolateral sorting[82]. Sendai virus, which normally buds from the apical membrane, exhibits non-polar budding in the pantropic mutant F1-R[1], which is implicated in the altered pathogenesis of the mutant. In this study, the microtubule network becomes disrupted upon expression of the matrix protein of the mutant virus. The fusion protein of the mutant, when expressed on its own or with wild-type M protein, is restricted to the apical surface; however, this restriction is lost upon expression with the mutant M protein.

Vesicular traffic in the mammalian cell also involves specific lipids in both budding and fusion[83]. It is striking how many similarities can be found between vesicular trafficking and viral budding events. While a high content of phosphatidylcholine impairs exit of vesicles from the Golgi, transient increase in negatively (acidic) charged lipids such as phosphatidic acid and phosphatidylserine change the physical character of the bilayer, forming microdomains, which allow vesicle formation by changing membrane curvature and recruitment of proteins required for vesicle budding[84-86]. Ion concentration (Ca^{2+}) also plays a role in budding of intracellular vesicles which, as we have seen, appears to be true for viral budding as well.

In the infectious process, efficient fusion of the virus membrane with the host membrane may require both cholesterol and sphingomyelin[87-89]. By analogy to cellular processes of vesicle fusion, it may be that patching of nega-

tively charged lipids during budding may not only serve to facilitate assembly, but may also help catalyze virus entry and disassembly. Thus, lipid patching perhaps, in concert with changes in local ion concentration and with the aid of the pushing and pulling forces of internal and TM components, may induce membrane curvature and virus release.

Acknowledgements

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