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Minireview

More than one door – Budding of enveloped viruses through cellular membranes

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Abstract **Enveloped viruses exit their host cell by budding from a cellular membrane and thereby spread from one cell to another. Virus budding in general involves the distortion of a cellular membrane away from the cytoplasm, envelopment of the viral capsid by one or more lipid bilayers that are enriched in viral membrane glycoproteins, and a fission event that separates the enveloped virion from the cellular membrane. While it was initially thought that virus budding is always driven by viral transmembrane proteins interacting with the inner structural proteins, it is now clear that the driving force may be different depending on the virus. Research over the past years has shown that viral components specifically interact with host cell lipids and proteins, thereby adopting cellular functions and pathways to facilitate virus release. This review summarizes the current knowledge of the cellular membrane systems that serve as viral budding sites and of the viral and cellular factors involved in budding. One of the best studied cellular machineries required for virus egress is the ESCRT complex, which will be described in more detail.**

Keywords: Virus envelopment; ESCRT; Budding

1. Introduction

Replication of viruses is intrinsically tied to their host cell. Viral spread, however, depends on an extracellular step in the replication cycle, involving the release of virus particles from an infected host cell and subsequent infection of a target cell. Most non-enveloped viruses exit their host cells by lysis, which involves breakdown of the cell membrane and death of the host cell. In contrast, enveloped viruses are released via budding at a host cell membrane, thereby acquiring a cell-derived membrane containing viral (and sometimes cellular) proteins. Virus egress by budding maintains the integrity of the host cell membrane and thus allows regulated production without necessarily killing the producing cell. Furthermore, enveloped viruses can enter target cells via fusion with cellular membranes. Thus, the ability of enveloped viruses to cross lipid bilayers during virus exit (by budding) and entry (by fusion) without compromising membrane integrity increases the efficacy of viral spread and gives directionality to the viral release-entry process that can be temporally regulated at various stages. This confers evolutionary advantages on enveloped animal viruses, since plant viruses and bacteriophages have to cross a cell wall for exit and entry and therefore benefit less from the advantages of membrane fusion.

Virus budding can be defined as the envelopment of a viral core by a cellular membrane containing viral glycoproteins and subsequent membrane fission to release the particle from the membrane. When budding occurs at the plasma membrane, virions are directly released into the extracellular space, and many viruses such as retroviruses, alphaviruses, rhabdoviruses, and ortho- and paramyxoviruses have been shown to bud predominantly at the surface of infected host cells. However, virus budding can also occur on intracellular membranes, resulting in the accumulation of particles in the lumen of cellular organelles. A wide variety of intracellular membranes such as the nuclear envelope (NE), rough and smooth endoplasmic reticulum (ER), endosomes, intermediate or pre-Golgi compartment (IC), Golgi cisternae and the *trans*-Golgi-network (TGN) have been proposed to serve as platforms for virus budding (Fig. 1). When budding occurs intracellularly, virus release requires the subsequent transport of virus-filled vesicles towards the cell surface and their fusion with the plasma membrane. This viral secretion step depends on cellular secretory transport, which can be controlled and regulated by the virus at various stages, allowing directed virus release, for instance in a specific host cell environment.

2. Cellular membrane platforms for virus budding

Besides the plasma membrane, various intracellular membrane systems have been shown to serve as viral budding platforms (Fig. 1). Budding sites have not been identified unequivocally for all viruses, however, and the choice of budding site may also be cell-type dependent. For instance, the retrovirus Human Immunodeficiency Virus type-1 (HIV-1) buds almost exclusively from the cell surface of T cells and many cell lines, while several lines of evidence indicated intracellular budding in macrophages: (i) the observation of intracytoplasmic vacuoles into which HIV buds and accumulates (ii) localization of endosomal marker proteins to both the limiting membrane of these vacuolar structures and the viral envelope and (iii) immunoprecipitation of infectious virus derived from

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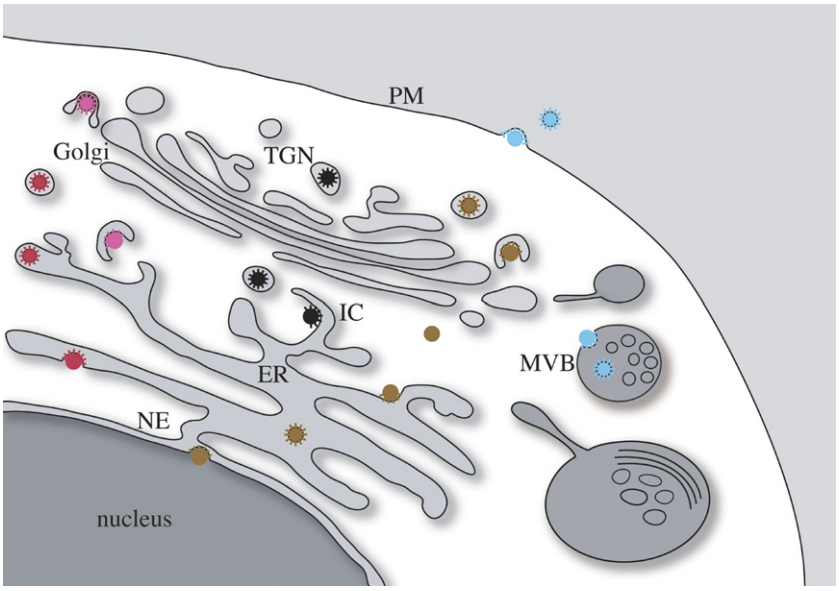

Fig. 1. Various membrane systems are implicated in budding of enveloped viruses. Many enveloped viruses (e.g. retroviruses, paramyxoviruses, orthomyxoviruses, arenaviruses, filoviruses, coronaviruses and some rhabdoviruses) bud from the plasma membrane of infected cells (depicted in light blue). In the case of the retrovirus HIV-1, the limiting membrane of multivesicular bodies has also been implicated in virus budding, and it was proposed that viruses exit cells in an exosome-like fashion. Many other viruses bud into the lumen of organelles along the secretory pathway (ER, IC, Golgi and TGN). In such cases, viruses are often transported within secretory vesicles to the plasma membrane, where they are finally released from the cell (red, brown, black). Members of the Flaviviridae family bud on membranes of the ER or ER-to-Golgi IC, resulting in accumulation of enveloped virus particles in the lumen of the IC, followed by maturation in the Golgi or TGN, respectively (black). Similarly, Foamy Viruses, 'unusual' retroviruses, were proposed to bud into the lumen of the ER, from where enveloped particles are transported through the Golgi and TGN to the plasma membrane (red). Most DNA viruses replicate in the nucleus, and therefore have to cross the NE prior to budding at cellular membranes. In the case of Herpesviruses (brown), the viruses exit the nucleus by budding on the NE, followed by release of non-enveloped viral capsids from the ER into the cytoplasm. In a second budding step, herpesviruses may bud on TGN-derived vesicles, from which viruses are finally released. Vaccinia virus (pink), the prototype member of the Poxvirus family, is consecutively enveloped by several membrane layers, which are presumably derived from the ER and the TGN, respectively. NE-nuclear envelope. ER-endoplasmic reticulum. IC-intermediate compartment. TGN-trans-Golg network. MVB-multivesicular body.

macrophages with antibodies to endosomal proteins [1,2]. Based on this, it was proposed that HIV buds into multivesicular bodies (MVBs) of macrophages. This appeared particularly intriguing in view of the parallel identification of the cellular ESCRT-machinery, which mediates budding of intralumenal vesicles into the MVB, as being essential for HIV release (see below). Intracellular budding and storage of infectious virus could also allow for delayed and controlled virus release, triggered by contact with a target cell [3]. More recently, however, the role of intracellular compartments in HIV budding has been questioned and it was argued that the intracellular pool of viruses mainly represents re-internalized particles that have originally budded from the plasma membrane [4]. Recent work from our laboratory revealed that the cell surface of macrophages displays deep invaginations into which HIV particles can bud and where they accumulate, supporting the notion that the plasma membrane is the primary site of HIV budding also in macrophages [5]. Since macrophages are a major target for HIV infection in vivo, this clearly remains an issue that needs further investigation.

Membrane envelopment of viral cores is more complex in case of some DNA viruses such as the prototype member of the Poxviridae family, Vaccinia Virus, which employs two different cellular membrane systems for primary and secondary envelopment, respectively [6]. Intracellular mature viruses (IMVs) are formed by envelopment of the viral core with membranes that may be derived from the ER [7,8], whereas in a second round of envelopment, some IMVs acquire additional membranes derived from the TGN [9]. These intracellular enveloped viruses are released from the cell mainly via fusion with the plasma membrane.

Most DNA viruses replicate in the nucleus, and their egress therefore requires crossing of the nuclear envelope and, subsequently, the cell membrane. The first step can be achieved by transport of viral genomes – usually in a complex with viral and cellular proteins – through nuclear pore complexes, followed by budding on a cellular membrane in the second step. The nucleocapsids of herpesviruses such as Herpes Simplex Virus (HSV) are too large for nuclear export and herpesviruses therefore leave the cell by consecutive envelopment, de-envelopment and re-envelopment processes [10]. HSV nucleocapsids traverse the nuclear envelope by budding from the inner nuclear membrane into the perinuclear cleft and subsequently lose their temporary envelope by fusion with the outer nuclear membrane (Fig. 1). Naked cytoplasmic nucleocapsids then acquire their final envelope by budding at a cellular membrane, which may be derived from early endosomes, the IC or the TGN ([10] and references therein).

Virus budding and its analogy to cellular envelopment processes is of central interest for cell biology, since the identification of viral factors involved- and how they usurp cellular machinery-yields important insights into cellular functions. Furthermore, interfering with virus release is also a potential approach for therapeutic intervention against viral infections, thus warranting study of these processes also from a medical point of view.

3. The minimal 'driving force' of budding

Early studies in Semliki Forest virus (SFV)-infected cells have shown that SFV budding requires recruitment of the viral core by envelope glycoproteins. This led to the hypothesis that the specific interaction of viral membrane glycoproteins with inner structural proteins (matrix, core or capsid) is the driving force for bud formation [11] (Fig. 2C). It was predicted that this principle should apply to release of all enveloped viruses, but later studies indicated that budding mechanisms vary for different virus families and that budding may be dependent on viral envelope glycoproteins, inner core proteins or both. Expression of coronavirus or flavivirus glycoproteins without any other viral components led to release of enveloped particles morphologically similar to complete virions [12,13]. Furthermore, Hepatitis B Virus (HBV) surface glycoproteins can be secreted as subviral particles in the absence of any other viral component [14]. On the other hand, budding of most retroviruses (with the exception of Foamy Virus) occurs independent of membrane glycoproteins and is exclusively driven by the major structural core protein, Gag [15]. Accordingly, expression of Gag alone leads to the formation of enveloped, morphologically normal virus-like particles (VLPs) lacking viral glycoproteins at the plasma membrane. Later studies showed that this also applies to other viruses that bud at the plasma membrane, where expression of the viral matrix protein – forming a protein structure that supports the inner face of the virion membrane – suffices for release of enveloped VLPs (see below).

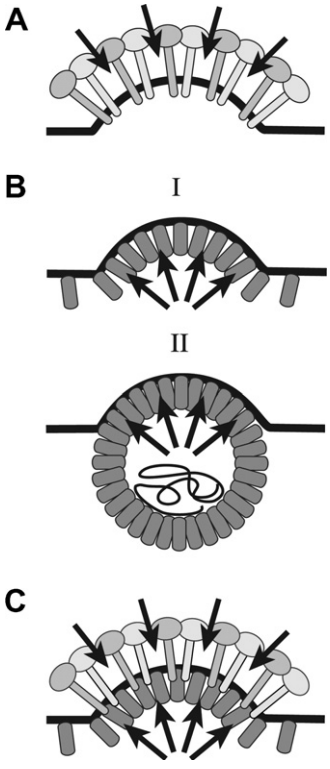

Fig. 2. Enveloped virus budding is driven by 'pull'- or 'push'-mechanisms. (A) Viral membrane glycoproteins assemble on the cellular membrane that serves as budding site, thereby creating a 'pulling' force that drives membrane curvature and bud formation. (B) Inner viral structural proteins (I) or pre-assembled viral nucleocapsids (II) attach to the cytoplasmic side of the budding site and create a 'pushing' force that drives membrane curvature and bud formation. (C) Pushing and pulling forces can act in concert to drive virus budding.

Taken together these data indicate that there is no common principle that governs budding of all viruses. It is clear that formation of fully infectious viruses always requires the presence of all components including glycoproteins, inner structural proteins and viral genomes, but the minimal requirements that drive bud formation can be assigned to either one of three classes: (i) Budding is primarily driven by viral membrane glycoproteins. In such cases, glycoproteins serve as 'pulling' forces assembling on the nascent viral membrane (Fig. 2A). They often form a symmetric (e.g. icosahedral) lattice that drives viral morphogenesis of, for instance coronaviruses and flaviviruses [12,13]. Expression of other viral glycoproteins can lead to formation of extracellular vesicles without a symmetrical structure [16], although this may be due to induction of cellular vesiculation rather than an active budding process. (ii) Budding is driven by inner structural proteins. As discussed above, this was initially observed for the Gag protein of retroviruses. In this case, the driving force assembles on the cytoplasmic side of the membrane and can therefore be regarded as a 'pushing' force (Fig. 2B). This mechanism also applies to members of the families of rhabdoviruses [17], filoviruses (reviewed in [18]), arenaviruses [19] and paramyxoviruses (reviewed in [20]), where expression of the viral matrix protein is sufficient for release of VLPs resembling the complete virion. (iii) Budding requires both viral core proteins and glycoproteins, as is the case for SFV and most other alphaviruses [21] (Fig. 2C). There are, however, exceptions: Budding of the retrovirus Foamy virus and of SARS coronavirus, for instance, depends on expression of the nucleocapsid together with the viral glycoproteins [22,23]. Herpesviruses appear to differ in their budding requirements, as they do not contain typical viral matrix proteins (forming a lattice on the cytosolic face of the membrane), but utilize viral tegument proteins (forming a largely amorphous layer between the nucleocapsid and the membrane) to interact with the membrane [24]. The identity of these tegument proteins differs for the first and second envelopment step, consistent with budding occurring at different cellular membranes [10].

4. Membrane association of viral components

A prerequisite for budding of infectious enveloped virus particles is the assembly of all virus components at a conjoint cellular membrane. Viral membrane glycoproteins are, as a rule, co-translationally inserted into ER membranes [25] and often modified during their passage through the secretory pathway, where they may be retained by specific signals. Endocytosis motifs in the cytoplasmic tails of viral glycoproteins can mediate re-localization from the cell surface to endosomal membranes. For instance, retroviral Env glycoproteins were shown to localize predominantly to intracellular endosomal membranes at steady state due to highly conserved endocytosis motifs [26,27].

The inner structural proteins of enveloped viruses that form the viral core or capsid, on the other hand, generally lack membrane-spanning domains and have to be anchored in or associated with membranes containing the viral transmembrane proteins prior to or during budding. Co-localization of

glycoproteins and core proteins can be achieved by different mechanisms: a simple approach is consecutive translation of one single structural polyprotein that is inserted into a lipid bilayer. Proteolytic cleavage of the polyprotein then yields individual capsid and glycoproteins, both of which remain associated with the same membrane. For instance, all structural proteins of members of the *Flaviviridae* family including Hepatitis C Virus are synthesized as a single polyprotein, which is co-translationally inserted into the ER membrane (reviewed in [28]). Upon proteolytic cleavage, both the E1/E2 glycoproteins and the RNA-binding core protein remain associated with the ER, thus ensuring co-localization of all components prior to budding. Similarly, the structural proteins of alphaviruses such as SFV are synthesized as a single polyprotein on ER membranes [21]. The SFV capsid protein C is, however, released into the cytoplasm upon proteolytic cleavage, whereas the E1/E2 glycoprotein is targeted through the secretory pathway towards the plasma membrane, where C binds specifically. In this case, independent targeting of C to the plasma membrane is required. This also applies to most other viruses, whose inner structural proteins are commonly translated from individual mRNAs on free polysomes in the cytoplasm and subsequently recruited to their respective budding membrane by specific targeting signals.

Such recruitment of inner structural proteins to the budding site may be facilitated by their specific interaction with the cytoplasmic tail of viral transmembrane proteins. Pre-assembled cytosolic capsids of Foamy Virus or the retrovirus Mason-Pfizer Monkey Virus (M-PMV) specifically traffic to intracellular membranes enriched in viral glycoproteins, where budding occurs [29,30]. Direct interaction of core and membrane glycoproteins is likely to occur during budding of all enveloped viruses in order to assure formation of a complete virion, but specific binding may be weak and contribute to budding only in multimeric complexes. Incorporation of glycoproteins into VLPs may even occur without specific interaction. Striking examples are retroviruses, which can be functionally pseudotyped (i.e. incorporate heterologous membrane proteins) with glycoproteins from completely unrelated virus families (summarized in [31]), making a specific interaction of inner structural and viral membrane proteins highly unlikely. Moreover, the predominant steady-state localizations of inner viral structural proteins and glycoproteins often only partially overlap. In summary, specific interaction of viral membrane glycoproteins and inner core proteins are rarely sufficient to anchor core proteins to the budding membrane and trigger virion formation. Other mechanisms are likely to be required to target viral proteins towards selected membranes as described below.

5. Membrane targeting of inner viral structural matrix proteins

Many viral structural proteins acquire covalent modifications that allow interaction with cellular membranes or membrane microdomains. For instance, the major structural Gag proteins of many retroviruses, as well as Z proteins of arenaviruses, are modified by myristic acid, which is covalently attached to their N-terminus. N-terminal acylation has long been proposed to promote membrane attachment of otherwise soluble proteins in a reversible and regulated manner by adopting an exposed or sequestered conformation ('myristyl switch'; [32]). A substantial amount of studies indicates that this mechanism applies to the myristoylated matrix protein of HIV [33–36]. Mutational analysis has shown that membrane association is abrogated by deletion of the myristyl anchor of Gag, confirming the importance of acylation for membrane binding. The relatively weak binding energy provided by a myristyl anchor alone (8 kcal/mol, corresponding to a dissociation constant of 10^{-4} M) [37] is, however, insufficient to stably bind a soluble protein to a lipid bilayer and additional binding forces are needed to provide sufficient binding energy. Several retroviral Gag polyproteins have been proposed or shown to contain a cluster of basic amino acids in addition to N-terminal acylation [36,38]. Basic patches appear to be a conserved feature of retroviral matrix proteins, including those that are not myristoylated [39,40]. Generally, basic residues can serve to strengthen membrane binding by ionic interaction with the head groups of acidic phospholipids. This interaction contributes a binding energy of approx. 1.4 kcal/mol per lysine, and the total binding energy of these basic patches therefore generally equals or exceeds that of the acyl group. In addition, positively charged residues also appear to determine specificity of plasma membrane attachment, as HIV Gag is redirected towards intracellular membranes upon mutation or deletion of the basic patch [41,42]. In summary, acylation confers membrane binding ability to proteins, whereas basic clusters increase membrane affinity and binding specificity [37,43].

Recent findings have shown that the binding specificity mediated by the basic cluster may be due to interaction with specific host cell phospholipids. Depletion of phosphatidylinositol–(4,5) bisphosphate (PI(4,5)P_2), which contains four acidic charges and is concentrated on the plasma membrane, led to the relocalization of HIV Gag to intracellular membranes [44]. PI(4,5)P_2 serves as plasma membrane targeting factor for various cellular proteins and it may thus not be surprising that viral matrix and core proteins make use of this mechanism as well. N-terminal acylation in combination with a basic cluster appears to be the common feature of this targeting mechanism with a recent report showing that several myristylated proteins with N-terminal basic patches, including HIV Gag, specifically interact with PI(4,5)P_2 and PI(3,4,5)P_3, but not with phosphoinositides that concentrate on endosomal or Golgi membranes [45]. Structural analysis of the N-terminal domain of HIV-1 Gag, bound to a truncated version of PI(4,5)P_2, suggested that the specificity of interaction – at least in this case – not only depends on ionic interactions of lysine residues with multiple acidic charges, but also involves insertion of a lipid chain of PI(4,5)P_2 into a hydrophobic groove of the viral protein. This interaction may not only be important for plasma membrane binding in general, but also contribute to the sorting of viral structural protein into liquid-ordered membrane microdomains ([46], see below). It will be interesting to see whether other viral matrix proteins with basic clusters employ a similar mechanism or if plasma membrane binding relies primarily on ionic interactions with PI(4,5)P_2 in such cases.

6. Viral lipids and role of membrane microdomains

An important determinant for the lipid composition of the viral envelope is the composition of the cellular membrane the virus buds from [47,48]. It has long been known, however, that the lipid composition of the viral and cellular membrane

can differ significantly [49–54] and it was therefore proposed that some viruses selectively bud from membrane microdomains. Such microdomains may serve as budding platforms where viral transmembrane and core proteins are concentrated. Obvious candidates for such microdomains are the so-called lipid rafts, highly enriched in cholesterol, sphingolipids, and phospholipids with saturated side chains [55]. Several viruses were proposed to bud from lipid rafts [56,57], although these data suffer from the fact that the in vivo existence of raft microdomains has not been unequivocally proven. Instead, resistance to extraction with cold detergent is often taken as proxy for lipid rafts. Accordingly, sorting of proteins into detergent-resistant membranes (DRM; [58]) was suggested to indicate their raft localization. Incorporation of DRM markers into the envelope of, e.g. Influenza, Sendai-, Measles, Ebola virus and several retroviruses including HIV-1 (or co-localization of viral glycoproteins with DRM markers) has been taken as evidence for virus budding from rafts [57]. DRM may, however, comprise a heterogeneous population of microdomains, clustered together by extraction with cold detergent, since budding of viruses supposed to bud from lipid rafts as well as from non-raft regions of the plasma membrane is affected by cholesterol depletion [59–61]. Recently, the complete lipid composition of purified HIV-1 particles has been determined without any detergent treatment [62]. The viral lipidome was shown to be very similar to the suggested composition of lipid rafts with a strong enrichment of cholesterol, sphingolipids including the unusual lipid dihydro-sphingomyelin as well as phospholipids with saturated side chains. Interestingly, HIV-1 particles excluded a bona fide DRM marker (flotillin 1) indicating that the virus buds from a certain subset of cellular microdomains. It is currently not known, however, whether these microdomains are preexisting or virus-induced, and analyses of the lipid composition of viruses with different budding preferences and derived from different host cells will be important to further our understanding.

Little is currently known about the sorting of viral structural proteins into lipid microdomains. Cellular signal transduction proteins that may be associated with lipid rafts are often found to be myristoylated as well as palmitoylated (reviewed in [63,64]). Double acylation has however not been reported for viral proteins involved in budding from DRM, indicating that a different sorting mechanism is involved. Structural analysis of the HIV-1 matrix domain in a complex with a soluble derivative of PI(4,5)P_2 provided an intriguing suggestion: in this structure the 2′ acyl chain was buried within a hydrophobic groove of the protein, while the 1′ acyl chain, which extends in the opposite direction, would be free to insert into a lipid bilayer [46]. Since the 2′ chain is usually unsaturated while the 1′ chain is saturated, sequestration of the 2′ acyl chain by matrix could promote the association of PI(4,5)P_2 with liquid ordered lipid microdomains. Thus, interaction with PI(4,5)P_2 may help to explain binding of the viral Gag protein to the plasma membrane (see above) as well as its sorting into microdomains with a high content of saturated fatty acids.

Lipid microdomains may also be organized by cellular proteins, and viral budding platforms could thus be defined by interaction of viral structural proteins with these organizers. The so-called tetraspanins (proteins with four membrane-spanning domains) have been proposed to interact laterally to form tetraspanin-enriched microdomains (TEM) within the plasma membrane and some intracellular membranes (reviewed in [65]). Several tetraspanins have been shown to be incorporated into HIV-1 particles [1,66] and a recent study showed co-localization of HIV-1 Gag with TEM [67], suggesting that TEM may serve as platforms for virus budding. In summary, there is accumulating evidence that virus budding – at least in some cases – occurs from specialized membrane microdomains, which may be preexisting or virus-induced and may be organized by their own lipid composition or by viral and/or cellular proteins. Most likely, a combination of these mechanisms may commonly apply, with differences between virus families. Future studies of the composition, regulation and function of virus budding microdomains will probably yield important insights into the organization of such cellular membrane microdomains.

7. Cellular factors involved in virus budding and release

While it was initially believed that virus budding is entirely driven by viral factors, more recent work has shown a requirement for cellular functions in this process. Initial evidence for the involvement of cellular activities came from studies, which showed that mutation of short peptide motifs in retroviruses lead to an arrest of virus replication at the late stage of budding [68,69]. These motifs were termed late domains, as late domain defective variants commonly exhibited almost completely budded virions which remained tethered to the cell by a thin membrane stalk. Various late domain motifs were identified in retroviruses, filoviruses, arenaviruses, rhabdoviruses, paramyxoviruses, reoviruses and flaviviruses and in most instances functionally confirmed (reviewed in [70,71]). Two different types of late domains (consensus sequences PS/TAP and YPDL) are known to interact with components of the endosomal sorting complex required for transport (ESCRT; TSG101 and AIP1/ALIX, respectively), while a third late domain (consensus sequence PPxY) was shown to interact with ubiquitin ligases of the NEDD4 family. These results suggested that both ESCRT and mono-ubiquitination play a role in virus budding. Studies in paramyxoviruses revealed a fourth type of late motif (sequence FPIV; [72]), but its interaction partner is currently unknown.

ESCRT was originally identified in the budding yeast *Saccharomyces cerevisiae*, where it functions in sorting of proteins destined for degradation into the intralumenal vesicles (ILV) of MVB upon sequential recruitment of ESCRT-I, -II and -III subcomplexes to endosomal membranes (Fig. 3). ESCRT function is conserved in mammalian cells, where it mediates sorting of mono-ubiquitinated cell surface receptors into MVB (Fig. 3; [73]). Knockout of ESCRT components in yeast and blocking of ESCRT function in mammalian cells led to formation of an enlarged vacuole or MVB lacking ILV (class E compartment) [74,75], indicating that ESCRT predominantly functions in budding of ILV into the lumen of MVB. For details on the composition and function of ESCRT complexes the reader is referred to several recent reviews [73,76–78].

HIV-1 mutants defective in recruiting the ESCRT-I component TSG101 exhibit a late budding phenotype, suggesting that ESCRT provides a cellular budding machinery for HIV-1 [79]. Further studies using dominant negative ESCRT mutants and siRNA-mediated knockdown indicated a requirement for components of ESCRT-I and -III in budding of HIV-1 and several

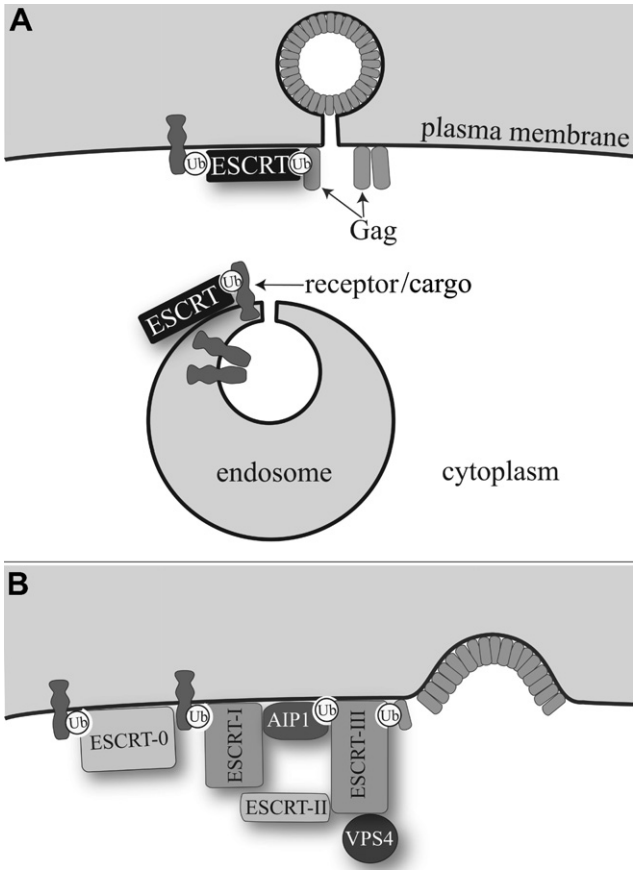

Fig. 3. ESCRT plays a role in the formation of intralumenal MVB vesicles and in retroviral budding. (A) The endosomal sorting complex required for transport (ESCRT) functions in sorting of ubiquitinated cell surface receptors destined for degradation into the ILV of MVB. For several retroviruses (as well as other viruses – see text), the topologically equal process of virus budding (away from the cytoplasm) was shown to depend on ESCRT as well. Viral Gag proteins were shown to bind ESCRT, thereby recruiting it to the budding site. (B) ESCRT subcomplexes are sequentially recruited to the retroviral budding site. ESCRT-0 binding to membranes is facilitated by the binding to PI(3)P and ubiquitinated proteins such as retroviral Gag, respectively. This is followed by recruitment of ESCRT-I and/or AIP1/ALIX by retroviral proteins. AIP1/ALIX binds both ESCRT-I and -III, which finally recruits the ATPase VPS4. VPS4 ATPase activity is believed to be required for the release of ESCRT-III into the cytosol and to facilitate budding of the virus. The ESCRT-II subcomplex is clearly required for ILV formation, but its involvement in virus budding is unclear. Ub, ubiquitin. MVB, multivesicular body.

other enveloped viruses. ESCRT-II – which is essential for ILV formation at the MVB – appears to be dispensable for HIV-1 budding [80], indicating that enveloped viruses have usurped only part of the cellular ESCRT machinery for their budding. The extensive amount of primary data on the role of ESCRT in virus budding is summarized in a number of excellent reviews [70,71,81–83]. In general, ESCRT-I is required for budding of viruses with a PS/TAP late domain, whereas it appears to be dispensable for viruses containing a PPxY late domain. In contrast, budding of almost all viruses with known late domain functions appears to depend on the ATPase VPS4, believed to resolve the ESCRT-III complex as a final step in vesicle budding [75,84,85]. This differential dependence suggests different or partially overlapping entry points of various viral late domains into the ESCRT pathway. The PPxY late domain has been shown to interact with cellular E3 ubiquitin ligases of the NEDD4 family, and mono-ubiquitination is believed to be important for virus release in this case (reviewed in [70,83]). Mono-ubiquitination as well as ESCRT-function are also essential for sorting of cell surface molecules into MVB with several ESCRT components being ubiquitinated and/or containing ubiquitin interaction motifs. These observations suggest that reversible ubiquitination in MVB sorting may serve to stabilize protein-interactions to form a transient protein web. A similar role for mono-ubiquitination may also be envisaged for viral budding: Ubiquitinated viral proteins may interact with ubiquitin-binding components of the ESCRT pathway, and this interaction may serve as entry point into the ESCRT pathway for viruses that lack direct ESCRT-interacting motifs. Mono-ubiquitination also occurs for viruses lacking a known E3-ligase recruitment motif and may aid in virus budding in these cases as well [86,87]. It should be noted, however, that the relative importance of ubiquitination and of individual ubiquitination sites has not been assessed in detail for any virus so far.

Several viruses harbour multiple closely spaced or overlapping late motifs. In these cases, the PPxY motif always appears to be dominant, while the other late motif(s) contribute to release to a lesser extent [88–90]. Interestingly, mutation of the PT/SAP motif in case of Mason-Pfizer Monkey Virus (M-PMV) and Human T-Cell Leukemia Virus type I caused the typical late budding arrest described above, while mutation of the closely spaced PPxY late domain motif led to formation of membrane-proximal complete capsids which apparently failed to induce membrane curvature. These results suggest a participation of ESCRT in the induction of curvature – i.e. as a membrane scaffold – in these cases, which may be supported by the recently determined structure of the ESCRT-III component CHMP3 [91], which was shown to multimerize as a curved protein lattice. In the case of HIV-1 and other viruses, on the other hand, ESCRT appears to be important for membrane fission with no apparent effect of a late domain mutation on curvature induction. It is difficult to envisage a differential contribution of the same cellular factors to budding of different viruses, and further studies will hopefully allow generating a unified picture. Late domain motifs have also been identified in Foamy Virus and HBV [92–94], which are believed to bud into the ER or pre-Golgi compartment, respectively, suggesting that ESCRT may be recruited to and function at membranes where it is normally not present. Finally, the lack of known late domains in many enveloped viruses raises the question whether they use other entry points into the same pathway or whether entirely different mechanisms of budding exist. This clearly warrants further studies.

8. Conclusions and open questions

Early models of enveloped virus budding suggested that (i) budding is generally driven by the interaction of viral transmembrane proteins with the inner core structure, (ii) membrane bending occurs mainly by wrapping the cellular bilayer around a pre-formed or concomitantly assembling viral core or matrix layer, and (iii) fission of viral and cellular membranes occurs by default. Evidence accumulated over the past years indicated, however, that the process of budding is much more complex: Depending on the virus, the driving forces may act

on either side, or on both sides, of the membrane and there is some evidence for cellular (scaffolding) proteins also being involved in this process. Targeting of virion components to specific membrane systems or lipid microdomains as well as the organization of microdomains (featuring a high concentration of viral proteins) by cellular or viral factors may promote assembly of complex structures, based on the synergy of multiple weak interactions. The nature and role of such microdomains remained largely elusive due to a lack of suitable tools for their investigation. However, recent technology advances like fluorescence resonance energy transfer, high resolution light microscopy and cryo-electron tomography are likely to overcome some of these difficulties.

The mechanisms of membrane distortion early in virus budding are poorly understood, but are also likely to require cooperation of viral and cellular factors. Quantitative analyses in conjunction with mathematical modelling and simulation of membrane bending and curvature induction will shed more light on this process in the future. While it is reasonable to assume that lateral interaction between viral structural proteins can provide energy for membrane bending, experimental evidence also points to a requirement for cellular machineries at least in some viral systems. For example, BAR domain proteins such as endophilins or amphiphysins, implicated to induce membrane curvature, were recently proposed to play a role during virus budding [95]. Moreover, specific lipids may play a role in membrane bending: the ESCRT protein AIP1/ALIX – recruited by some viral late domains – was found to specifically interact with the cone-shaped lipid LBPA in vitro [96] and LBPA was therefore suggested to modulate membrane curvature during ILV formation at the MVB. It should be noted, however, that LBPA appears to be restricted to endosomal membranes and cone-shaped lipids have not been reported in the plasma membrane so far.

Different studies have indicated a role of ESCRT both very early during virus budding as well as during the last pinching-off step: mutations of late domain motifs in Human T-cell Leukemia Virus and M-PMV arrested budding at a stage before induction of membrane curvature [89,97], whereas other late domain mutations arrested virus budding at a very late stage. An important functional role of ESCRT in both membrane bending and membrane fission is consistent with its involvement in the topologically equivalent process of vesicle budding at the MVB. From what is currently known it can be proposed, however, that mechanistic differences between viral and cellular pathways, as well as between pathways used by different viruses, exist. Detailed analyses of these pathways and comparative studies of the envelopment of viral and cellular structures are likely to yield many more important insights.

Historically, cell biology and virology were closely connected fields with an immense mutual benefit, but this association has weakened over the years. Studying viral membrane envelopment may serve to re-establish a fruitful co-operation of these two fields in the future.

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