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REVIEW

Role of multivesicular bodies and their components in the egress of enveloped RNA viruses

A. Calistri[1], C. Salata[1], C. Parolin[2], G. Palù[1]*

[1]*Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy*
[2]*Department of Biology, University of Padova, Padova, Italy*

SUMMARY

As an enveloped virus buds, the nascent viral capsid becomes wrapped in a plasma membrane-derived lipid envelope, and a membrane fission event is thus necessary to separate the virion from the host cell. This membrane fission event is well characterised in the case of enveloped RNA viruses, where it is promoted by late assembly domains (L-domains) present at the level of specific viral structural proteins. Research conducted over the past 10 years has demonstrated that L-domains represent docking sites for cellular proteins essential for the biogenesis of a cellular organelle, the multivesicular body (MVB). In this way, enveloped RNA viruses hijack the MVB components to the cellular site where the budding is executed. This review will focus on the cellular machinery exploited by enveloped RNA viruses in order to be released from infected cells. The role of ubiquitin and lipids in viral budding will also be discussed. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

Enveloped viruses acquire their envelope by budding through cellular membranes of different origin. A productive infection requires that all the components necessary for the formation of infectious particles localise to the membrane at the site where budding will take place. Several enveloped RNA viruses such as retroviruses, rhabdoviruses, filoviruses, arenaviruses and paramyxoviruses contain, at the level of structural proteins, amino acidic motifs, known as late domains (L-domains), essential in the late steps of viral replication. L-domains are binding sites for cellular factors that facilitate viral budding. Recent evidence has clearly identified involvement of the endocytic pathway in retroviral budding. The eucaryotic endosomal system is a complex network of vesicles and organelles, surrounded by membranes, which coordinates protein transport from the plasma membrane to the trans-Golgi network (TGN) and/or to the lysosomes. A central role at this level is played by the multivesicular bodies (MVBs). The MVBs are organelles that consist of a limiting membrane enclosing many internal vesicles 40–90 nm in diameter. MVBs derive from early-endosomes and thus contain molecules that have been internalised through endocytosis. They also receive biosynthetic cargos from the TGN including precursors of lysosomal enzymes. The MVB

*Corresponding author: Giorgio Palù, Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Via Gabelli, 63, 35121-Padova, Italy.
E-mail: giorgio.palu@unipd.it

Abbreviations used

AIP4, Atrophin-1 interacting protein 4; AMSH, SH3 domain of STAM; CHMP, chromatin modifying proteins; DOA, Degradation of alpha 2; EAPs, ELL-associated proteins; EGFR, Epidermal growth factor receptor; EIAV, Equine Infectious Anemia Virus; ERAD, Endoplasmic reticulum-associated degradation; ESCRT, Endosomal sorting complex required for transport; HECT, Homologous to E6AP COOH terminus; HRS, Hepatocyte growth factor-regulated tyrosine kinase substrate; ILVs, Intraluminal vesicles; JAB, Janus kinase-binding protein; K63, Lysine 63; LBPA, Lysobisphosphatidic acid; LCMV, Lymphocytic choriomeningitis virus; L-domains, Late domains; LFV, Lassa fever virus; MIIC, Specialised class II MHC compartment; MMuLV, Moloney murine leukaemia virus; MPMV, Mason-Pfizer monkey mirus; MPN, Mpr/Pad1/N-terminal; MVBs, Multivesicular bodies; Nedd4, Neural precursor cell expressed, developmentally downregulated gene 4; PI(3)P, Phosphatidylinositol 3-phosphate; POSH, Plenty of SH3s; RSV, Rous sarcoma virus; STAM, Signal-transducing adaptor molecule; SV5, Simian virus 5; TGN, Trans-Golgi network; Tsg101, Tumour susceptibility gene 101; Ub, Ubiquitin; UBPs, Ub-specific proteases; UBPY, Ubiquitin-specific processing protease Y; USP, Ubiquitin-specific peptidase; Vps, Vacuolar sorting protein; VSV, Vesicular stomatitis virus.

biogenesis requires at least four separate heteromeric protein complexes called ESCRT (endosomal sorting complex required for transport)-0, ESCRT-I, ESCRT-II and ESCRT-III. These protein complexes are transiently recruited from the cytosol to the endosomal membrane where they function sequentially in the sorting of transmembrane proteins into the MVB pathway and in the formation of MVB vesicles. The final step is topologically identical to budding of infectious particles away from the cytosol. In this review we will focus on the relationship between L-domain activity, release of enveloped RNA viruses and the host endosomal sorting machinery. The role of ubiquitin and lipids in viral egress from infected cells will also be discussed.

THE MULTIVESICULAR BODIES AND THEIR BIOGENESIS

The proteasome and the lysosome represent the two major cellular sites for protein degradation. Ubiquitin attachment to the target protein is a sorting signal for both these pathways [1]. Ubiquitination causes sorting to the lysosomes from different compartments, including the Golgi apparatus when the proteins need to be diverted to the endosomes, and the cell surface when the proteins need to be internalised. Moreover, ubiquitination triggers the sorting of proteins into the lumen of the late endosomal MVBs. MVBs are unique membrane-enclosed structures characterised by several intraluminal vesicles (ILVs) and are essential components of the endocytic pathway [2–4]. Protein sorting into the MVBs is the main mechanism for the degradation of post-Golgi integral membrane proteins in all eukaryotic cells. Indeed, while the proteasome is involved in degrading integral membrane proteins in the early secretory pathway, through the process known as endoplasmic reticulum-associated degradation (ERAD), it does not appear to play a role in the degradation of proteins that have travelled past the Golgi apparatus [1,5–7]. It has been demonstrated that mono-ubiquitination is a main signal throughout the MVB pathway [1]. Indeed, ubiquitin bound to the target proteins is recognised by several endosomal proteins, which act as receptors responsible for binding and directing protein cargos towards ILVs. Moreover, it has been shown that multiple mono-ubiquitination or short oligo-ubiquitin chains consisting of lysine 63 (K63)-linked ubiquitins increases MVB sorting efficiency [8–12]. Thus, integral membrane proteins, such as the activated epidermal growth factor receptor (EGFR), that need to be degraded, are delivered to late endosomes and lysosomes, by ubiquitination and incorporation into the ILVs that bud from the limiting membrane of the early endosomes giving rise to the MVBs. Fusion of MVBs with lysosomes brings about degradation of the ILVs and their content due to their susceptibility to the lysosomal hydrolases. Thus, ILV biogenesis is a crucial step in the pathway. It has been demonstrated that ILVs are created and they bud, thanks to the sequential recruitment of several cytoplasmic proteins, known as class E vacuolar sorting proteins (Vps) to the endosomal membrane. Most Vps proteins act in complexes named ESCRT-0,-I, -II and -III. In yeast, class E Vps mutants appear to be characterised by a common phenotype, displaying defects in endocytosis, mainly at the level of MVB structure and at the level of ILV formation [13,14].

Starting from the relevant role of ubiquitination in the pathway, the definition of ubiquitin interaction domains [8,15,16] has greatly facilitated the identification of Vps proteins also in mammalian cells. We can divide endosomal ubiquitin-binding proteins into two groups: peripheral receptors and ESCRT-core proteins [1]. The former group includes Vps27/Hrs/STAM complex, known as ESCRT-0. It is recruited to the endosomal membrane by binding of the lipid phosphatidylinositol 3-phosphate [PI(3)P], which is enriched on the cytosolic face of the early endosomes. Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) itself is a soluble, multidomain protein that is recruited to the endosomal membrane via an interaction between its FYVE domain and PI(3)P [17–19]. Hrs membrane localisation is also controlled by phosphorylation, with the dephosphorylated form predominating on the membrane. Moreover, this complex binds to the target protein by recognising ubiquitin [20]. In mammalian cells this step leads to the localisation of the cargo within endosomal membrane clathrin-rich subdomains. The second group includes Vps23/TSG101 and Vps36/EAP45 which are subunits of the ESCRT-I and ESCRT-II respectively [21]. ESCRT-0 recruits ESCRT-I to the membranes, followed by ESCRT-II. Finally the ESCRT-III subunits, also known as chromatin modifying proteins (CHMP) in mammalian cells, are recruited in order to form a large

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polymer on the surface of endosomes [22]. The ubiquitinated target protein is sequentially passed through the ESCRT complexes, first bound by ESCRT-0, then to Vps23/Tsg101 and finally to Vps36, which delivers it to the forming ILV with the help of ESCRT-III and associated factors. At the end of the cycle, an AAA-ATPase, Vps4, disassembles the ESCRT components, dissociating them from the endosomal membrane (Figure 1). Dominant negative forms of this protein and of ESCRT-III components block the entire pathway [23]. It is interesting to note that most of the interacting partners among the different ESCRTs have been identified [15]. For instance, it has been demonstrated that ESCRT-I is recruited through a direct interaction between Hrs and Tumor susceptibility gene 101 (Tsg101) and appears to involve two distinct protein–protein interaction sites [24–27]. One of these sites apparently serves primarily to recruit Tsg101, whereas the central PSAP motif within Hrs (residues 348–351) serves both to bind and activate Tsg101 to recruit downstream factors. Moreover, it has been demonstrated that all the endosomal ubiquitin-binding proteins undergo some level of ubiquitination in a mechanism that is known as coupled mono-ubiquitination [28]. It is not known whether coupled mono-ubiquitination is a form of regulation for switching off the protein function, stimulates the formation of large complexes of different ubiquitin-binding proteins or simply represents a side effect. What is clear is that the majority of ubiquitin is removed from the target protein, just prior to delivery into the ILVs. The deubiquitinating enzyme performing this task in yeast has been identified in Doa4/Ubp4. Doa4-like proteins with a similar function have still to be identified in mammalian cells. Two candidates are represented by Ubiquitin-specific processing protease Y/Ubiquitin-specific peptidase (UBPY/USP8) and SH3 domain of STAM (AMSH). It has been demonstrated that AMSH can interact with the MVB sorting machinery and that, under some conditions, loss of this protein slows down EGFR degradation [29, 30]. Interestingly, both AMSH and UBY appear to function also in early steps of MVB biogenesis, via interaction with STAM and clathrin [31], suggesting that ubiquitination is a dynamic process with target proteins undergoing multiple rounds of ubiquitination/deubiquitination while they travel towards the MVB lumen. Thus, the complete ubiquitination/deubiquitination cycle is considered essential, not just the initial ubiquitination of the cargo [16]. In this context, different ubiquitin ligases play a role in MVB biogenesis. In particular, EGFR downregulation requires the RING ubiquitin ligase Cbl [32]. Moreover, atrophin-1 interacting protein 4 (AIP4),

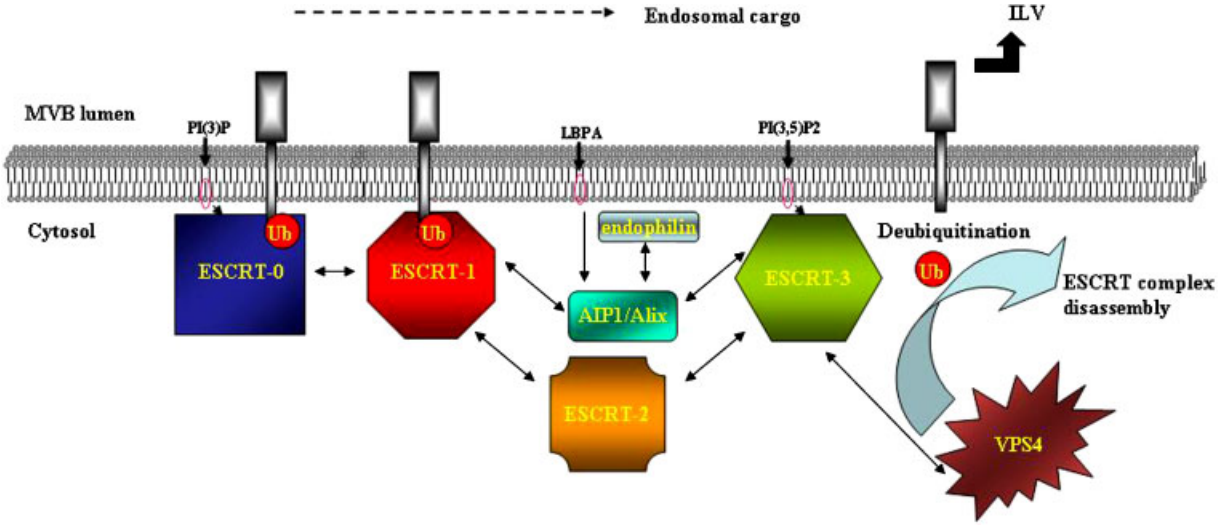

Figure 1. Schematic representation of ubiquitinated protein sorting through the MVB pathway. Ub-cargo is sorted in a sequential manner: firstly it is recognised by ESCRT-0 and then passed first to ESCRT-I and then to ESCRT-II before being incorporated into intraluminal vesicles. Before ILV budding, Ub-cargo is deubiquitinated and the ESCRT network is disassembled by the Vps4. Some of the characterised interactions between Vps proteins and specific lipids are also highlighted

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a HECT-type ligase, associates with Hrs and, like other Nedd4-like family members, seems to play a role in MVB biogenesis. Other ligases clearly linked to the ubiquitin sorting machinery are: Tsg101 associated ligase (Tal), Nedd4.1 and the Mahogunin ligases [33–36]. The main proteins involved in MVB biogenesis in yeast and mammalian cells and their reciprocal interactions are reported in Table 1.

Finally, it is worth noting that not all the proteins sorted into the MVB ILVs are destined for degradation. Proteins such as class II MHC and transpanins accumulate in MVBs and give rise to a specialised class II MHC compartment, named Specialised class II MHC compartment (MIIC). MIICs are secreted from the cells upon fusion of the MVB limiting membrane with the plasma membrane of a variety of cells, including neurons, epithelial cells, mast cells, melanocytes and different haematopoietic cells [37]. In dendritic cells the MIICs can fuse back to the limiting endosomal membrane, thus facilitating the antigen presentation process [38]. Moreover, MVBs can generate additional non-degradative endosomal compartments. In this context, it has been demonstrated that the von Willebrand factor is packaged into the ILVs of platelets and endothelial cells [39] and that CD63 enriched MVBs give rise to the azurophilic granules of the neutrophils. Furthermore, melanosomes are derived from endosomes [40, 41]. Up to now it is not clear whether these MVB-like structures derive from common precursor and how they avoid the fusion to the lysosome.

THE BUDDING OF ENVELOPED RNA VIRUSES

An enveloped virus needs to face several challenges before leaving the infected cells. In particular, for a virus budding from the cell surface, such as a retrovirus, the first challenge is represented by the actin cortex the virus has to by-pass in order to reach the plasma membrane. Then, the second challenge is represented by the need to acquire the envelope by wrapping the nascent virion in the cell membrane. Finally, in the last step, the mature enveloped particle has to free itself from the cellular surface in a process that is known as fission. All these late stages in the viral life cycle are not easy to accomplish.

Table 1. Proteins involved in the biogenesis of multivesicular bodies both in yeast and in mammalian cells. The characterised interacting partners are also reported

Complex	Yeast	Mammal	Binds to
ESCRT-0	Vps27	Hrs	Ub, PI(3)P, Vps23
	Hse1	STAM1, 2	Ub, Rsp5
ESCRT-I	Vps23	TSG101	Ub, Vps27
	Vps28	VPS28	Vps20, Vps36
	Vps37	VPS37A, B, C, D	—
	Mvb12	—	—
ESCRT-II	Vps36	EAP45	Ub, PI(3)P, Vps28
	Vps22	EAP30	—
	Vps25	EAP20	Vps20
ESCRT-III	Snf7	CHMP4A, B, C	Bro1, Vps4, PI(3)P
	Vps20	CHMP6	Vps28, Vps25, Vps4
	Vps2	CHMP2A, B	—
	Vps24	CHMP3	Did2, PI(3,5)P2
Other interactors	Bro1	Alix	Doa4, Snf7
	Rsp5	Nedd4	Hse1
	Doa4	AMSH, UBPY	Ub, Bro1
	Did2	CHMP1A,B	Vps4, Vps24, Vta1
	Vps60	CHMP5	Vta1
	Vta1	LIP5	Vps4, Did2, Vps60
	Vps4	VPS4A, B/SKD1	Did2, Snf7, Vps20, Vta1

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Retroviruses have historically served as important model systems for studying many aspects of viral replication including viral entry, reverse transcription, integration, transcription, splicing and assembly. More recently, retroviruses have become valuable models for studying how enveloped viruses can wrap themselves into cellular membranes, acquire an envelope and subsequently leave the infected cells [42–44]. Indeed, in addition to their important pathology and intriguing biology, another feature renders retroviruses an optimal tool for studying viral assembly and release: Gag protein, which is the major structural protein of the virus, can assemble and bud from cells in the absence of any other viral factors [42]. In most cases, with the exception of B- and D-type retroviruses, newly translated Gag molecules are first targeted to membrane sites of assembly, then coalesce into semispherical particles that distort the membrane and finally are released from the cell when the membrane neck is pinched off behind the assembled particle. The different steps of this process are driven by different conserved Gag domains (Figure 2): the N-myristoylated MA region targets the protein to cellular membranes, CA makes important protein–protein interactions during particle assembly, and NC captures the viral RNA genome and couples RNA binding with assembly. Moreover, Gag contains regions exemplified by the p6 domain of HIV-1 that are now known to recruit cellular proteins that facilitate virus budding [23,45–48]. Seminal studies performed by mutating and deleting HIV-1 Gag defined short proline rich motifs required for efficient particle release [49,50]. These sequence motifs were termed late domains (L-domains) because virus assembly arrested at late stages when they were mutated [49,51]. Starting from these studies, three different classes of L-domains have been well characterised to date (P(T/S)AP, PPxY or YPxL, Figure 2), although this list is probably not exhaustive. Moreover, we now know that these L-domains are not a unique feature of retroviruses [49,52–56], but they are present in both positive- and negative-strand enveloped RNA viruses such as rhabdoviruses [57], filoviruses [58–60], arenaviruses [61,62] and paramyxoviruses [63]. The finding of the widespread use of common late domains is particularly significant because it suggests that very different families of enveloped RNA viruses could bud via similar mechanisms and in particular could take advantage of the same cellular pathway (Table 2). Studies carried out in the past 10 years have clearly demonstrated that this common cellular pathway is the MVB biogenesis machinery and that enveloped RNA viruses employ MVBs as a platform to assemble their envelope and to exit from infected cells. It is not surprising that viruses, which are obligate intracellular parasites and have evolved in order to exploit cellular pathways to their advantage, utilise the ESCRT components to achieve egress from infected cells. Indeed, the final steps of MVB biogenesis that bring to the formation and

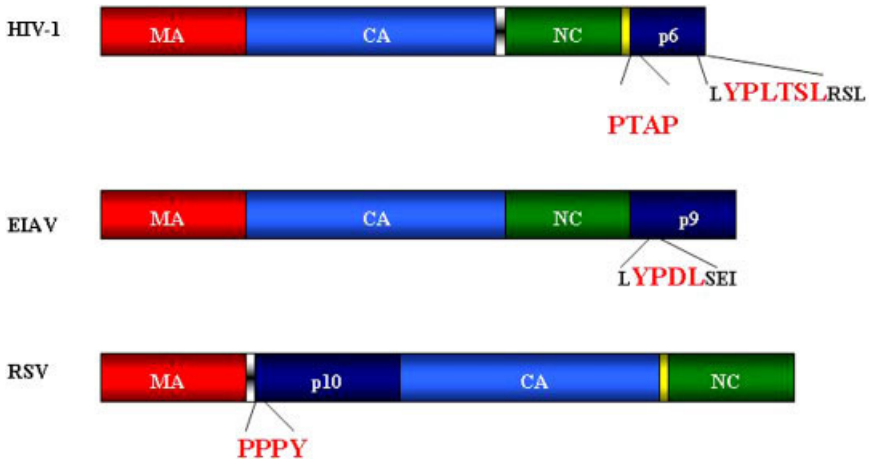

Figure 2. Organisation of the Gag protein in primate lentiviruses (HIV), non primate lentiviruses (EIAV) and oncoretroviruses (RSV). The specific location and the aminoacid sequence of the characteristic L-domains are indicated

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Table 2. Localisation and aminoacid sequence of the L-domains characterised in different enveloped RNA viruses

Family	Virus	Localised in	L-domain sequence
Retrovirus	HIV-1		PE**PTAP**PEE . . . . . YPLAS**LRSLF**G
	HTLV		DPQIP**PPPY**VE**PTAP**QV
	MLV	Gag	LLTED**PPPY**RD
	RSV		TASAP**PPPY**VG
	EIAV		TPQTQNL**YPDL**SEIK
Filovirus	Ebola	VP40	MRRVIL**PTAPPEY**MEAI
	Marburg		NTYMQYLN**PPPY**ADHS
Rhabdovirus	VSV	Matrix	LGIA**PPPY**EEDTSMEYA**PSAP**
	Rabies		DDLWLP**PPEY**VPLKEL
Arenavirus	LFV	Z	AA**PTAP**PTGAADSI**PPPY**SP
	LCMV		TAPSS**PPPY**EE
Paramyxovirus	SV5	Matrix	QSIKA**FPIV**INSDG

budding of the ILVs into the organelle lumen are topologically identical to the budding of viruses away from the cytosol. It is clear now, from several independent studies, that viruses have evolved at least two mechanisms for exploiting the MVB biogenesis machinery. The first one, that is well established, is based on a direct interaction of ESCRT components with the L-domains present at the level of structural viral proteins. In this context, any one of the three tetra-amino acidic sequences P(T/S)AP, PPxY or YPxL [43,64] can function by recruiting Tsg101 [65], Nedd4-like ubiquitin ligases [66] and Aip1/ALIX [23,47], respectively. The second mechanism, that is more controversial, involves the ubiquitination of specific virion components [43].

LATE DOMAINS AND BUDDING OF ENVELOPED RNA VIRUSES

The first late domain to be identified and characterised was the P(T/S)AP motif found in the carboxy-terminal region of the HIV-1 Gag [49,50]. The same type of tetra-amino acidic sequence has subsequently been characterised in the Gag of primate and non-primate lentiviruses, with the only exception of the equine infectious anemia virus (EIAV), as well as of oncoretroviruses, such as the Mason–Pfizer monkey virus (MPMV) [46]. Moreover, the same motif has been found in the VP40 protein of the Ebola virus [67,59], and in the Z proteins of some arenaviruses [61,62]. The PT/SAP late domains function by recruiting Tsg101 [59,65,68,69]. Indeed in their seminal work, Garrus and coworkers showed that depletion of Tsg101 arrests HIV-1 release at a late stage, indicating that Tsg101 performs essential functions in viral budding. As mentioned before, Tsg101 is a component of the ESCRT-I and it is characterised by an N-terminal UEV domain [70,71]. UEV domains are structurally similar to ubiquitin E2 ligases and can bind ubiquitin, but they lack the active site cysteine residue required for enzymatic ubiquitin transfer. The Gag PT/SAP motif binds in a groove of Tsg101 UEV domain that is created by the lack of two helices, normally found in canonical E2 enzymes. As described above, Hrs, a component of the ESCRT-0, recruits Tsg101 and thus ESCRT-I through its central PSAP motif (residues 348–351). Thus, HIV-1 and related viruses use their PTAP sequences to mimic the normal function of Hrs in Tsg101 recruitment and activation [27]. Indeed, there is no evidence that the Hrs complex is directly involved in retrovirus budding.

A second well characterised late domain is the YPDL motif, first identified in the p9 domain of the EIAV Gag protein [53]. Although this late domain was originally proposed to interact with the AP-2 adapter complex [72], it now appears more likely that it facilitates viral budding by binding the cellular protein AIP1/Alix [23,47,48,73], which is a link between ESCRT-I and ESCRT-III, at least in yeast. A related, but more

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complex, YPLTSL motif in the p6 domain of HIV-1 Gag also recruits AIP1. In this case, the cellular protein is not essential for HIV budding, but it clearly cooperates with Tsg101 to facilitate virus release [23]. In particular, it is interesting to note that, as it has been shown by Gruenberg and colleagues [74], AIP1 controls the formation of budding MVB ILVs, by interacting with the conical lipid, lysobisphosphatidic acid (LBPA). Thus, AIP1 may play an important role in the generation of membrane curvature, during the envelope formation. Moreover, Tsg101 and AIP1 can interact directly with each other via a PTAP motif within AIP1 [23,47,48].

Finally, the PPxY motif, (where x can be any amino acid, usually a proline), was initially identified as a late domain in the p2b portion of RSV Gag [51]. This sequence has been found in many other retroviruses, including the murine leukaemia virus (MLV) [56] and MPMV [55], as well as in rhabdoviruses [57], filoviruses [58] and some arenaviruses [61,62]. The interesting finding is that the PPxY sequence is present in a number of cellular proteins where it is a consensus binding site for WW protein recognition domains [75]. Indeed, it has been demonstrated that WW domain containing ubiquitin ligases, such as the Nedd4-like family members, can bind viral PPxY late domains. In agreement with a functional role of such interaction, several studies have shown a role for the Nedd4-like ubiquitin ligases, and in particular WWP1, WWP2 and AIP4, in the budding of PPxY containing viruses [64]. However, there are multiple Nedd4-like proteins in animal cells and there is not yet a general consensus as to which subsets actually function as late domain binding partners. Importantly, as described above, Nedd4-like ubiquitin ligase proteins also have a role in the MVB pathway. The three classes of late domains and the respective interacting cellular proteins are summarised in Figure 3.

Finally, it has to be mentioned that additional late domains may still remain to be identified. For instance, a novel late domain FPIV motif has been identified in structural proteins of paramyxoviruses [63]. Although the cellular binding partners for this late domain are not yet characterised, there are indications that the paramyxoviruses and maybe also orthomyxoviruses can utilise the MVB pathway for viral egress [63].

What is well established is that late domains are frequently present in tandem in viral structural proteins and that almost all the viruses have more than one late domain (Table 2) in their structural proteins. This may imply that different late domains may function in different stages of viral budding, executing, for instance, different tasks. In agreement with this hypothesis and with the evidence that they function as docking sites

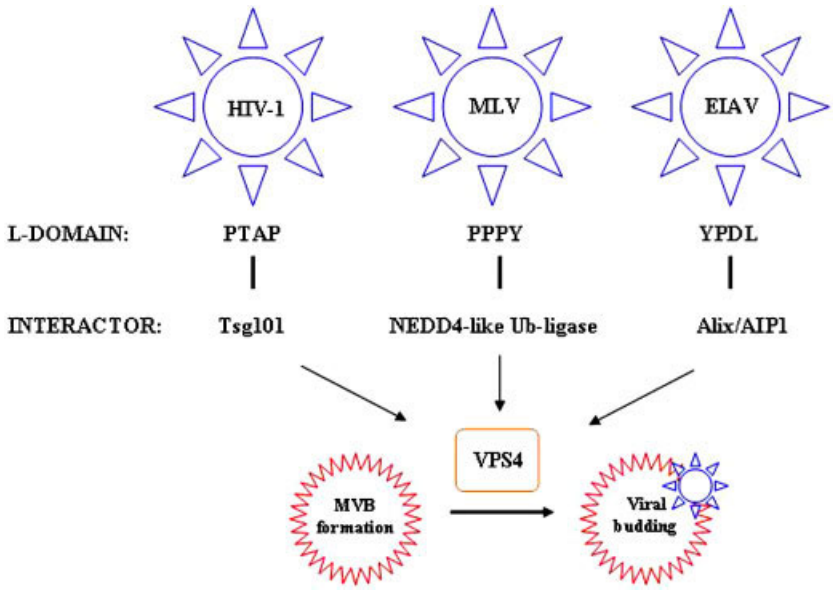

Figure 3. Schematic representation of the interactions between the different L-domains and specific cellular factors involved in the biogenesis of the MVBs

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for different components of the MVB biogenesis machinery, L-domains remain functional when moved to a different location in Gag or when swapped between viruses that use different types of L-domains [60].

THE ROLE OF UBIQUITIN IN EGRESS OF ENVELOPED RNA VIRUSES

The first indication of a potential role for ubiquitin in retroviral assembly and release came from the evidence that several retroviruses, including RSV, HIV, simian immunodeficiency virus (SIV) and MLV, incorporate high levels of unconjugated ubiquitin into their particles [76]. Moreover, the Gag proteins of HIV-1 and MLV are ubiquitinated at multiple sites [77] and the level of ubiquitination depends on the L-domain that is present [76]. Furthermore, Gag ubiquitination appears to be linked to L-domain functionality [78,79]. Interestingly, it has been demonstrated that late assembly domain function involves ubiquitin residues implicated in endocytosis [78,79]. Finally, proteasomal inhibitors, which deplete the cellular pools of free ubiquitin, also reduce RSV and HIV-1 Gag mono-ubiquitination and arrest virus budding at a late stage [60,80,81]. In addition to a possible involvement of ubiquitin in retroviral budding, Harty and coworkers have reported that ubiquitin may also play important roles in the release of filoviruses and rhabdoviruses [58,82].

Despite all the above evidence, the correlation between L-domain activity and Gag ubiquitination is still under debate. In particular, it is not clear whether the ubiquitination plays a functional role, or whether it is a side effect related to the fact that ubiquitin is a major signal throughout the MVB pathway. In this context, initial studies showed that lysines present in HIV p6 are not essential for L-domain activity [83]. However, a more detailed analysis of this region has shown that cumulative mutations of the ubiquitin acceptor sites in the NC-p2-p6 region arrest budding at a late stage [76]. These results would suggest that ubiquitination of lysines in the proximity of the L-domain may facilitate the interaction of Gag with some of the ubiquitin-binding proteins of the class E pathway, like Tsg101. In agreement with this conclusion, the inhibition of L-domain activity by proteasome inhibitors could be explained by a reduction of Gag ubiquitination and thus of its ability to interact with MVB components, as a consequence of the depletion of free ubiquitin in the cytoplasm [84]. However, considering the regulatory role of ubiquitin in the MVB pathway, the results of proteasome inhibitor experiments can also be interrupted as the relevant target for ubiquitination is not Gag itself, but a cellular protein perhaps an ESCRT component. As mentioned before, several ubiquitin-binding proteins are regulated themselves by mono-ubiquitination. Thus it may be that inhibition of the proteasome inhibits the MVB biogenesis pathway and viral budding by altering the ubiquitination status of ubiquitin-binding proteins in the pathway. This conclusion is supported by the fact that mutation of ubiquitin residues that are important for binding to Tsg101 also blocks HIV-1 budding [79,85]. Moreover, in agreement with this hypothesis is the fact that YPxL containing viruses, such as EIAV, are insensitive to the block of proteasome, perhaps because they bypass ubiquitin-binding proteins by recruiting AIP1, which is not thought to be regulated by ubiquitination.

A clear functional link between viral egress and ubiquitin is represented by the role of ubiquitin ligases belonging to the HECT-ubiquitin ligase or Nedd4-like ubiquitin ligase family in the budding of PPxY containing viruses such as oncoviruses, filoviruses and rhabdoviruses. Indeed, as discussed above, it is well established that PPxY motifs induce Gag ubiquitination and facilitate viral egress by recruiting a subset of HECT ubiquitin ligases and in particular WWP1, WWP2 and AIP4 [60,66]. A catalytically active HECT domain is required for optimal MLV release [66] but, also in this case, it remains unclear whether the relevant target of ubiquitination is a cellular cofactor or a structural component of the virus. On the other hand, HIV-1 and lentiviruses in general, which do not present such an aminoacidic motif within their Gag, would not be expected to exploit HECT ubiquitin ligases for their budding. However, two recent reports have clearly linked HIV-1 egress to Nedd4-2s a member of the Nedd4-like ubiquitin ligase family [86, 87]. The authors conclude that Nedd4-2s, even in the absence of a functional PPxY motif, may cooperate with other L-domains to enhance viral budding, maybe by leading to the ubiquitination and consequent activation of MVB components or by inducing Gag ubiquitination that may facilitate the interaction

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of the viral structural protein with some of the ubiquitin-binding proteins of the MVB pathway [86,87].

In addition to HECT-ubiquitin ligases, it has been demonstrated that other ubiquitin ligases may play a role in viral budding such as the Tsg101-associated ligase (Tal), which is characterised by a PTAP motif, and Mahoguin [33,36]. It is interesting to note that in both these cases the link between ubiquitin ligase-mediated Tsg101 ubiquitination, followed by the modulation of its ability to bind other ESCRT-I components, and the effect on viral budding seems to be strong. Finally, it has been reported recently that POSH (plenty of SH3s), a TGN-associated ubiquitin ligase, plays a role in HIV-1 egress from infected cells [88]. However, the lack of POSH does not induce a phenotype related to the L-domain activity, but most-likely to the inhibition of Gag transport to the plasma membrane.

THE ROLE OF LIPIDS IN THE LATE STEPS OF THE LIFE CYCLE OF RNA VIRUSES

One of the crucial steps in viral budding is represented by the membrane curvature. As for the other steps of budding, this phase of the process has been extensively studied in retroviruses [89]. Due to the ability of Gag to form virus-like particles in the absence of any other viral component, it was believed that the energy for the curvature was derived from Gag–Gag intramolecular interactions. However, the results obtained with MPMV and HTLV-I Gag suggested that late domains recruit cellular factors to help distort the membrane curvature [45,46,52]. In agreement with this hypothesis, a dominant negative form of AIP1 that inhibits EIAV budding also inhibits membrane curvature [23] and MLV Gag interacts with endophilin-2 [90]. These findings are extremely relevant because, as mentioned before, AIP1 controls inward fission process by interacting with LBPA [74]. On the other hand, endophilins can curve membranes towards themselves and can cooperate with the c-Cbl and HECT-ubiquitin E3 ligases in helping to generate membrane curvature during endocytosis [91,92]. Moreover, AIP1 can interact with endophilins [93] and this interaction has been reported to alter lipid curvature. Thus, during budding, the membrane distortion and the fission of viral envelope from cell membrane is likely to be the result of cellular/viral protein interaction. Specific lipids may also be involved. In this context, several studies indicate that some RNA-enveloped viruses, including HIV, utilise lipid rafts as platforms for assembly and budding [89]. Lipid rafts are detergent-resistant membrane microdomains composed of clusters of sphingolipids and cholesterol that can move laterally within the fluid bilayer [94]. Rafts have been proposed to function as platforms for the concentration and attachment of proteins during various membrane-associated events such as signal transduction. Data supporting raft involvement in HIV release can be summarised as follows: (i) virus particles are enriched in raft-associated molecules including sphingomyelin, phosphatidylethanolamine and cholesterol, as well as GPI-anchored proteins [95,96]; (ii) depletion of cholesterol inhibits virus release [97] and (iii) specific HIV-1 proteins accumulate in detergent-resistant membrane fractions characteristic of rafts [95,96, 98–101]. Besides this compelling evidence, it has to be mentioned that there is not universal agreement about the identification of these detergent-resistant portions of the plasma membrane enriched in HIV-1 proteins as lipid rafts. Moreover, the possible functional role of lipid rafts in HIV-1 assembly and budding is still under debate. However, supporting an involvement of specific lipids in viral egress from infected cells is the fact that phosphatidylinositols are essential in defining the identities of different intracellular membranes, and two specific phosphatidylinositols, PtdIns(3)P and PtdIns(3,5)P2, have been implicated in early and late stages of MVB biogenesis, respectively [102,103]. Interestingly, Hrs binds PtdIns(3)P, while the ESCRT-III component CHMP3 binds the phospholipid PI(3,5)P2 [104]. Note that highly phosphorylated phosphatidylinositols can promote the formation of native Gag assemblies *in vitro* [105]. Moreover, it has been recently reported that a selective inhibitor of PIKfyve, a member of the class III phosphatidylinositol phosphate kinase which synthesises PtdIns(3,5)P2, disrupts endomembrane transport and retroviral budding [106].

CONCLUDING REMARKS

Several studies conducted over the past 20 years have brought the conclusion that enveloped RNA viruses exploit the MVB pathway to build their envelope and/or to exit from infected cells. As

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described above, the viruses enter the pathway by following two main routes: (1) a direct interaction between viral structural proteins and components of the cellular ESCRTs and (2) ubiquitination of specific viral proteins. The first mechanism is well characterised. The structural viral proteins contain short proline rich domains, present also in cellular proteins, which mediate the binding to different factors essential for MVB biogenesis. Several important points can be underlined: (i) usually one virus has more than one late domain; (ii) different viruses enter the pathway at different levels; (iii) all the viruses enter the pathway at early stages (interaction with ESCRT-I, ubiquitin ligases, AIP1); (iv) the functional consequence is that the virus will be treated like a cargo on a vesicle built at the level of specific membranes where the virus brings the MVB machinery. These membranes are not necessary for the MVB membranes itself. For instance, in the case of HIV-1, it is clear that in cell types like lymphocytes, the virus hijacks the MVB components to the plasma membrane and this is the site where the virus acquires its envelope and buds from the cell. Instead, in the case of macrophages, MVBs are the site of HIV-1 budding and the virus is released when the MVBs fuse to the cell membrane as exosomes [107, 108]. Several questions remain to be answered: what is the difference between a T lymphocyte and a macrophage that results in this major difference in the mechanism of release? Why does the virus want to bud into a late endosome in the macrophage? Why does a late endosome sometimes fuse with the lysosome, but sometimes go to the plasma membrane? Can the virus control this decision?; (v) dominant-negative versions of most ESCRT-III components are potent inhibitors of release of enveloped RNA viruses [23,47,109]. Mutant Vps4 proteins are also potent inhibitors of budding. Importantly, the Vps4 mutants can inhibit the release of retroviruses that leave the cell via all the three known late domains [47,65,67]. Thus, the block imposed by the mutant Vps4 proteins is more general than that imposed by loss of Tsg101 or AIP1 (which strongly inhibits only viruses that rely upon PTAP and YP(x)nL late domains, respectively). This observation supports the idea that all retroviruses enter the MVB pathway upstream of Vps4 regardless of which Class E protein(s) they bind. However, Harty and colleagues have recently shown that the release of rhabdoviruses via PPxY late domain is not inhibited by dominant-negative Vps4A mutants, raising the possibility that not all viruses that utilise late domains require Vps4 ATPase activity [110] and (vi) a final important point is that the current list of yeast and human Class E proteins is probably not complete. Essentially, all of the known Class E genes were originally identified in yeast genetic screens [16,22], which may not have been saturating and would also only have picked up non essential genes.

As far as ubiquitination of structural proteins is concerned, the evidence for a functional role is not so striking as for late domains. However, (i) multiple retroviruses including RSV, HIV, SIV and MuMLV incorporate high levels of ubiquitin into their particles [83]; (ii) Gag of HIV-1 and Moloney murine leukaemia virus are monoubiquitinated at multiple sites [77]; (iii) levels of Gag ubiquitination are altered by the presence or absence of functional late domains [60,78]; (iv) specific mutations in ubiquitin can inhibit virus budding [79]; (v) ubiquitin plays an important role in the release of filoviruses and rhabdoviruses [58,82]; (vi) many oncoviruses, filoviruses and rhabdoviruses utilise PPxY late domains that can bind Nedd4-like ubiquitin ligases and (vii) ubiquitin ligases can cooperate with late domains to optimise lentiviral budding efficiency [86,87].

Finally, a new topic of research in this field is represented by the study of lipid, and in particular phospholipids, involvement in viral budding, starting from the consideration that lipids play a major role in MVB formation. Indeed, phosphatidylinositols are essential in defining the identity of intracellular membrane: PtdIns(3)P and PtdIns(3,5)P2 are implicated in early and late stages of MVB biogenesis, respectively [102]. Very recently it has been reported that inhibition of PtdIns(3,5)P2 blocks the retroviral life cycle at the level of mature particle release from infected cells [106]. Moreover, membrane distortion during virus budding is likely achieved through the combined interactions of viral, cellular proteins and specialised lipids. In this context, AIP1 seems to be emerging as a central mediator of the process. Indeed, the YPxL interacting protein can interact with endophilins [93] and LBPA [74], a conical lipid that can promote MVB vesicle formation *in vitro* [74]. Both these interactions alter lipid curvature.

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In conclusion, RNA-enveloped virus budding is an amazing example of how viruses exploit cellular pathways and in doing so they become extremely powerful tools to dissect fundamental cellular processes. Moreover, since it appears now that this process is widely spread and conserved among all viral families, including enveloped DNA viruses [111–113], it opens up the possibility of designing innovative potent antiviral approaches aiming to interfere with the viral replication cycle by disrupting fundamental viral/cellular protein interactions [114], modulating the ubiquitination process [115] and altering lipid metabolism [106].

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