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Review

The multivesicular body pathway: A newly discovered battlefield for gameplay between virus and host

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The main protein components of the multivesicular body (MVB) pathway include endosomal sorting complexes required for transport (ESCRT) and vacuolar protein sorting 4 (VPS4). Recent studies revealed that late domain motifs of enveloped viruses, such as PTAP, YPxL and PPxY (x indicates any amino acid residues), can bind separately to tumor susceptibility gene 101 (TSG101), ALG-2-interacting protein X (ALIX) and neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4), to directly or indirectly recruit ESCRT and VPS4 for budding and egress. Also discovered recently, MVB proteins can be regulated by the host cell to block virus budding, for example by conjugation of interferon-stimulated gene 15 (ISG15) protein to ESCRT III and NEDD4, phosphorylation of VPS37C by TANK-binding kinase 1 (TBK1) and phosphorylation of MVB12. Thus, the MVB pathway is emerging as a newly discovered battlefield for the gameplay between virus and host, opening an avenue to design novel antiviral therapeutics.

Key words: Multivesicular body, virus budding, late domain motif, ISGylation, TANK-binding kinase 1.

INTRODUCTION

The gameplay between virus and host has taken place over eons of evolution. The small genome of viruses reinforces the necessity to utilize as many host factors as possible for their own advantages. The multivesicular body (MVB) is a membrane trafficking and sorting station, formed by inward budding of late endosomes and driven by MVB proteins including ESCRTs and Vps4 (Wollert et al., 2010; Henne et al., 2011; Hanson et al., 2012). Recent studies suggest enveloped viruses can hijack MVB machinery via late domain motif (LDM)-mediated interactions for outward budding at the cell surface (Calistri et al., 2009; Martin et al., 2011; Ren et al., 2011), which is topologically and mechanistically similar to MVB budding. Interestingly, virus budding can be inhibited by conjugation of ISG15 protein to and phosphorylation of MVB proteins, uncovering a novel host defense response against viruses (Malakhova et al., 2008; Tsunematsu et al., 2010; Da et al., 2011; Kuang et al., 2011). Therefore, the MVB pathway is a newly discovered battlefield for the fight between virus and host, and has potential to develop as a target of novel antiviral therapeutics. In this review, we summarize the mechanism of the MVB pathway,

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Abbreviations: MVB, Multivesicular body; **ESCRT,** endosomal sorting complexs required for transport; **Vps,** vacuolar protein sorting; **ILVs,** intraluminal vesicles; **LDM,** late domain motif; ISG, interferon-stimulated gene; ISGylation ubiquitination-like modification by ISG15; **TBK1,** TANK-binding kinase1; **TSG101,** tumor susceptibility gene 101; **ALIX,** ALG-2-interacting protein X; **NEDD4,** neuronal precursor cell-expressed developmentally downregulated 4.

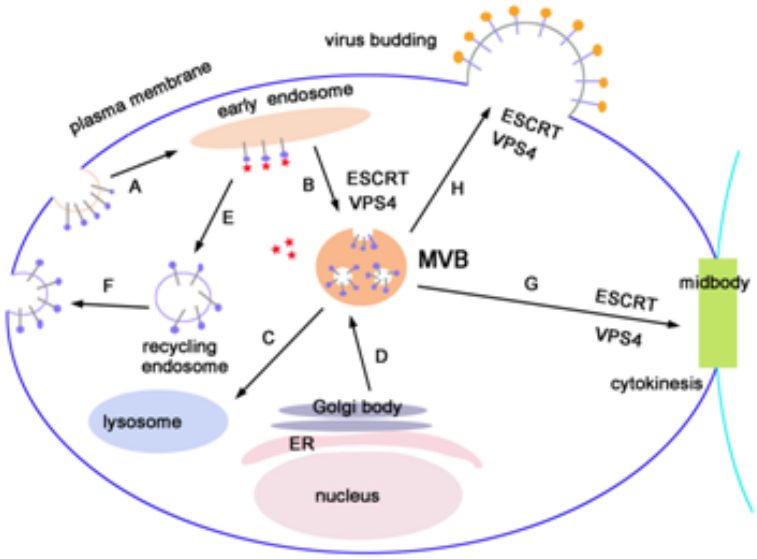

Figure 1. MVB pathway, cytokinesis and virus budding. Membrane proteins (blue dots and lines) on the cell surface are endocytosed to form the early endosome (A). Some are recycled back via the recycling endosome (E,F). Some are ubiquitinated (red star), sorted into MVB (B), deubiquitinated and released for degradation in lysosomes after membrane fusion with MVB (C). Some newly synthesized proteases are sorted into MVB (D) and lysosomes (C) to complete maturation. ESCRTs and VPS4 are involved in the MVB pathway, recruited to the cell surface for virus budding (H) and to the midbody for cytokinesis (G). The viral proteins are indicated by orange dots and blue lines.

protein interactions mediated by LDMs, ISGylation and phosphorylation of MVB proteins, and suggest future studies and perspectives.

MVB PATHWAY

The MVB is formed by invagination of the limiting membrane of late endosome, and concomitantly the incoming cargoes from endocytic and biosynthetic routes are sorted (Figure 1) (Woodman et al., 2008; Henne et al., 2011; Hanson et al., 2012). Some cargoes are recycled back to the plasma membrane, such as transferrin receptor. Some cargoes, such as epidermal growth factor (EGF) and EGF receptor (EGFR), are sorted into MVB and released for degradation following membrane fusion with a lysosome or yeast vacuole. Newly synthesized peptidases, such as carboxypeptidase S, undergo MVB sorting and final maturation in lysosomes or vacuoles in yeast. The yeast and human MVB pathways are most intensively studied, and have similar mechanisms (human nomenclature for proteins is used here, which is capitalized).

The MVB pathway is carried out by VPS proteins (Figure 2) (Wollert et al., 2010; Henne et al., 2011; Hanson et al., 2012; Hurley, 2010; Mayers et al., 2012). Many of them are recruited to the endosome membrane and assembled into ESCRTs, including ESCRT 0 (Hepatocyte growth factor-Regulated tyrosine kinase Substrate, HRS), Signal Transducing Adapter Molecule (STAM), I (TSG101, VPS28, VPS37, MVB12), II (VPS22, VPS25, VPS36), III (Charged Multivesicular body Protein 2, CHMP2), CHMP3, CHMP4, CHMP6). The ESCRTs are linked via sequential interactions, including HRS-TSG101, VPS28-VPS36, and VPS25-CHMP6. Other proteins include VPS4, LYST interacting protein 5 (LIP5), CHMP1, Increased Sodium Tolerance 1 (IST1), CHMP5, ubiquitin ligase (UBL), Neuronal precursor cell-expressed Developmentally Downregulated 4 (NEDD4), deubiquitinating enzyme (DUB), Ubiquitin isopeptidase Y (UBPY) and Associated Molecule of SH3 domain of STAM (AMSH), ALIX (ALG-2 interacting protein X), His domain phosphotyrosine phosphatase (HD-PTP) and BRO1 domain- and CAAX motif- containing protein (BROX) (Okumura et al., 2008; Malakhova et al., 2008; Dores et al., 2012; Doyotte et al., 2008; Ichioka et al., 2008). Some proteins have isoforms, such as STAM (1,2), VPS37 (A, B, C, D), MVB12 (A, B), CHMP2 (A, B), CHMP4 (A, B, C), CHMP1 (A, B), and VPS4 (A,B). Ubiquitination, catalyzed by UBLs including NEDD4, is an important signal for proteins entering MVB pathway, which are deubiquitinated by DUBs including

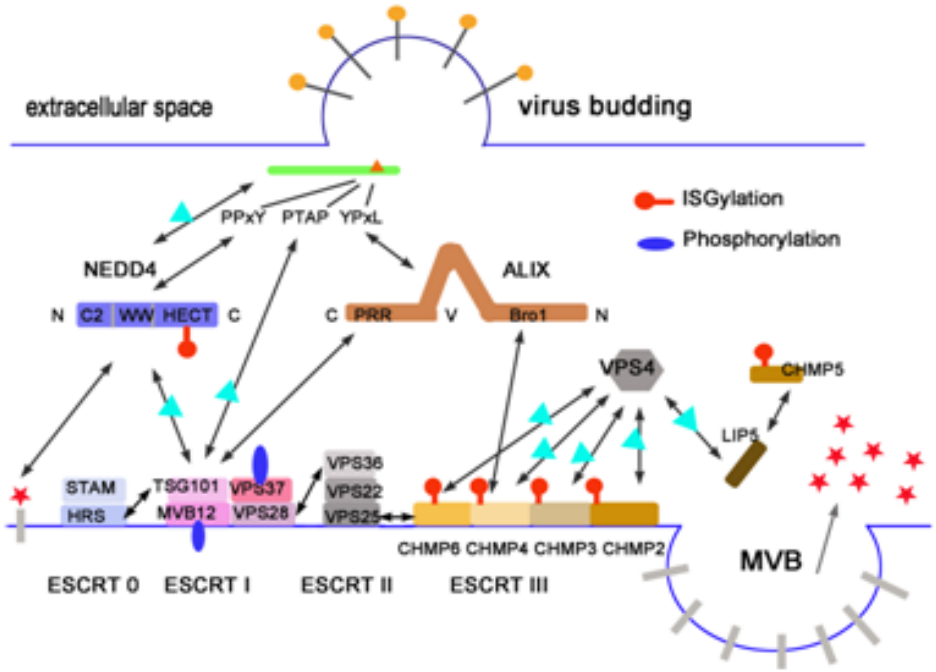

Figure 2. The gameplay of virus and host in the MVB pathway. ESCRTs are linked sequentially via interactions of HRS-TSG101, VPS28-VPS36, VPS25-CHMP6, and collectively promote MVB formation and protein sorting. Red star indicates ubiquitin. The ATPase VPS4 disassembles ESCRTs to recycle them, and is regulated by proteins including LIP5 and CHMP5. Enveloped viruses contain late domain motifs (PTAP, PPxY, YPxL) that can bind TSG101, NEDD4 and ALIX, respectively, to hijack MVB machinery for budding. The green rod indicates the virus structural protein, such as the Gag in HIV-1, and the orange triangle shows the relative location of late domain motifs. NEDD4 contains C2, WW and HECT domains, which can bind and ubiquitinate MVB substrate proteins, TSG101, ALIX, and virus structural proteins. ALIX contains Bro1 domain, “V” like domain and PRR that can separately bind CHMP4, viral YPxL motif and TSG101. ISGylation and phosphorylation can inhibit protein interactions (cyan triangles) to block virus budding. ISGylation can inhibit the activity of NEDD4, block interactions of CHMP2, CHMP3, CHMP4, CHMP6 with VPS4 and interaction of CHMP5 with LIP5. The kinase for phosphorylation of MVB12 is not known. Phosphorylation of VPS37C catalyzed by TBK1 can specifically inhibit PTAP motif-dependent virus budding.

UBPY or AMSH before being completely sequestered into MVB. ESCRT 0 binds membrane, marks the budding site for intraluminal vesicles (ILVs), clusters ubiquitinated proteins and recruits downstream ESCRT I and II, which are the main driving force of the MVB pathway. ESCRT III can cleave the bud neck to finish membrane abscission,the final stage of ILVs formation. Finally, the ATPase VPS4 is recruited to disassemble and recycle ESCRT III by hydrolyzing ATP to provide energy, and the upstream ESCRTs are also disassembled as the result of destabilized sequential interactions (Shestakova et al., 2010; Hill et al., 2012; Babst et al., 2011). The LIP5 can stabilize the dodecamer form of VPS4 and increase its ATPase activity. The IST1 can bind VPS4 to block the high-order oligomerization of VPS4. CHMP1 and CHMP5 can regulate LIP5 and VPS4 via unknown mechanisms. The tight spatiotemporal regulation of ESCRT and VPS4 is necessary for normal MVB progression. Dysfunction of them can block the MVB pathway and cause accumulation of incoming cargoes, resulting in abnormally enlarged endosomes called the class E compartment.

The MVB pathway is closely related to virus budding and cytokinesis (Hurley et al., 2010; Calistri et al., 2009; Caballe et al., 2011; Carlton et al., 2012; Carlton et al., 2007) (Figure 1). These three processes are topologically similar and require MVB proteins with certain preferences (Morita, 2012). Amazingly, they all require Vps4 and ESCRT III, the minimal membrane remodeling machinery, to finish membrane abscission by constricting and cleaving the bud neck (MVB pathway and virus budding) or the midbody connecting two daughter cells in cytokinesis, the last stage of these events (Adell et al., 2011; Hurley et al., 2010; Caballe et al., 2011). Thus, a conserved and parallel mechanism is functioning in these different processes.

VIRUS HIJACKS MVB PATHWAY FOR BUDDING

The viral late domain motifs are named for involvement in budding, the late stage of virus infection and replication. Most LDMs are classified into three types including PTAP, PPxY and YPxL(x represents any amino acid residues) that interact with TSG101, ALIX and NEDD4, respectively (Figure 2) (Ren et al., 2011). Some viruses contain one LDM, such as PTAP from Lassa virus. Some viruses have multiple LDMs, such as PTAP and YPxL for HIV-1; PTAP and PPxY for human T-cell leukemia virus-1 (HTLV-1) and Ebola virus (Chen et al., 2008). They are position-independent and interchangeable, acting as modular units (Zhadina et al., 2010).

PTAP motif

The PTAP motif mimicks PSAP, the only motif of ESCRT 0 interacting with ESCRT1 (Figure 2). The first step of sequential recruitment of ESCRTs is mediated by the interaction of the PSAP motif of HRS (ESCRT 0) with TSG101 (ESCRT I) in human, or the PSDP of Vps27 (homologue of HRS) with Vps23 (homologue of TSG101) in yeast (Ren et al., 2011; Im et al., 2010). The motif sequence may vary, but the binding mode is conserved. TSG101 is also required in cytokinesis, and was originally identified as a tumor susceptibility gene, consistent with its essential role in MVB pathway (Tanaka et al., 2008; Lee et al., 2008). Therefore, the TSG101-binding PTAP motif enables viruses to directly hijack MVB machinery for budding. The PTAP motif usually functions as the primary mode when co-existing with other LDMs, further supporting the importance of it in virus budding (Calistri et al., 2009; Bello et al., 2012). It is thus clear that TSG101 acts as the first decisive contact point for virus recruitment of MVB machinery.

YPxL motif

The YPxL motif binds a scaffold protein ALIX (Figure 2). It contains an N terminal CHMP4-binding Bro1 domain, a central “V” like and YPxL motif-binding domain, and a C terminal proline-rich region (PRR) that binds TSG101, apoptosis-linker gene 2 (ALG-2), endophilin, c-Cbl Interacting protein of 85 kDa (CIN85), Centrosomal protein of 55 kDa (CEP55), proline-rich tyrosine kinase 2 (PYK2) and receptor mediated endocytosis 1 (RME-1), suggesting ALIX has multiple functions (Shi et al., 2007; Lee et al., 2008; Ren et al., 2011). ALIX can bind CHMP4, TSG101 and NEDD4, and the respective yeast homologues Bro1, Vps32, Vps23 and Rsp5 have similar interactions. It is unknown whether they interact simultaneously or sequentially and how they are regulated.

Recent studies showed that ALIX may directly help viruses catch ESCRT I and III by binding to the viral YPxL motif (Fisher et al., 2007), or indirectly by recruiting NEDD4 to ubiquitinate viral or host proteins (Sette et al., 2010). Some viruses have no YPxL motif, but contain other ALIX-binding sequences, such as simian immunodeficiency virus (Zhai et al., 2011), indicating the important role of ALIX in virus budding.

Besides ALIX, HD-PTP and BROX also have Bro1 domains. They can prompt virus budding, but differently influence the MVB pathway (Popov et al., 2008; Ichioka et al., 2008; Doyotte et al., 2008; Dores et al., 2012; Mu et al., 2012). BROX can bind CHMP4 and CHMP5. ALIX can bind YPxL motif of a G protein coupled receptor protease activated receptor 1 (PAR1) and mediate its MVB degradation. It does not affect MVB degradation of EGFR, whereas HD-PTP does, suggesting they have different substrate requirements. The PRR of ALIX interacts with Bro1 and “V” like domain, rendering ALIX in an autoinhibited state (Zhou et al., 2010). The physiological mechanism of activating ALIX remains unknown.

PPxY motif

The PPxY motif can bind NEDD4 (Figure 2), which contains an N terminal membrane- or substrate-binding C2 domain, PPxY motif-binding WW (two highly conserved tryptophans) domain and a C terminal Homologous to the E6-AP Carboxyl Terminus (HECT) domain with ubiquitin ligase activity (Yang et al., 2010; Sette et al., 2010). NEDD4 belongs to the NEDD4 protein family, which includes NEDD4-1 (NEDD4), NEDD4-2(NEDD4L), WW domain containing E3 ubiquitin protein ligase 1 (WWP1), WWP2, ITCH (itchy E3 ubiquitin protein ligase), SMURF1 (SMAD specific E3 ubiquitin protein ligase 1), SMURF2, NEDD4-like ubiquitin protein ligase 1 (NEDL1) and NEDL2. Many of these proteins can facilitate virus budding.

Not unexpectedly, ubiquitination is also involved in virus budding (Shields et al., 2011; Gustin et al., 2011). Gag ubiquitination is required for budding of HIV-1 (Gottwein et al., 2006), but not HTLV-1, which nevertheless requires the interaction of WWP1 with Gag (Heidecker et al., 2007). Similarly, the budding of lysine-free Gag of prototypic foamy virus (PFV) is also promoted by WWP1, but the direct fusion of a ubiquitin molecule to this Gag can also promote budding (Zhadina et al., 2010). These studies suggest that ubiquitination of viral Gag or other factors by NEDD4 proteins are functioning in a context-dependent manner. HIV-1 Gag cannot bind NEDD4 for lack of PPxY motif, but overexpression of NEDD4 could rescue budding defects of HIV-1 Gag with PTAP mutations, which required interaction with ALIX, ALIX ubiquitination and the ALIX-YPxL pathway, suggesting NEDD4 may indirectly function in virus budding via ALIX interactions (Sette et al., 2010).

It seems that it does not matter whether the ubiquitination or protein interaction mediated by NEDD4 proteins are functioning, as long as MVB machinery can be recruited to the virus budding site (Weiss et al., 2010; Zhadina et al., 2010). Firstly, the C2 domain can target the HECT ubiquitin ligases to membrane but does not directly interact with HIV-1 Gag. However, the truncated C2 domain of a NEDD4 mutant could bind to HIV-1 Gag to enhance virus budding. This targeting capability could be transferred to other NEDD4 family proteins, yeast Rsp5 or isolated HECT domains, and could even be functionally replaced by CypA (cyclophilin A), a Gag binding protein unrelated to MVB (Weiss et al., 2010). Secondly, expression of a fusion of WWP1 with TSG101, the former containing C2 and WW but not the HECT domain and the latter lacking a PTAP-binding UEV (ubiquitin E2 variant) domain but still participating in ESCRT 1 formation, could prompt PPxY-dependent PFV budding, whereas the fusion of HECT domain from WWP1, ITCH or NEDD4L with UEV could stimulate PTAP-dependent PFV budding (Zhadina et al., 2010). This suggests that virus recruitment of ESCRTs can be directly mediated by protein interactions or indirectly via ubiquitination. Moreover, some HECT ligases could stimulate virus budding similarly but ubiquitinate Gag differently and some could ubiquitinate Gag but had no effect on virus budding (Weiss et al., 2010). Together, these studies demonstrate the important role of PPxY-NEDD4 interaction in recruiting MVB machinery for virus budding, as well as multiple and flexible ways of function of NEDD4 proteins, which is beneficial to enhance virus competitiveness.

Exosome

Some MVBs are routed to cell surface to fuse with plasma membrane to release ILVs to extracellular space, which are called exosomes (Vlassov et al., 2012; Pant et al., 2012; Ludwig et al., 2012). The biogenesis of exosome was recently found to require the syndecans, heparan sulphate, ESCRTs, and the interaction between syntenin (the cytoplasmic adaptor for the syndecan heparan sulphate proteoglycans) and ALIX, demonstrating exosome indeed originates from MVBs (Hurley et al., 2012; Baietti et al., 2012).

The exosome has been shown to play important roles in retrovirus dissemination due to its ability to reprogramme the recipient cells (Vlassov et al., 2012; Pant et al., 2012; Wurdinger et al., 2012). Many studies even suggest that retroviruses are exosomes at the fundamental level and propose the “Trojan exosome hypothesis” for the hijacking of exosome for virus egress and spreading (Booth et al., 2006; Izquierdo-Useros et al., 2010: Wurdinger et al., 2012). It is reasonable because the biogenesis of exosome and virus budding are convergent at MVB. However, there are still many debates and uncertainties about it. For example, the budding of HIV-1 was reported to be independent of exosomes in CD4(+) T lymphocytes (Park et al., 2010). The absence of CD45 from HIV-1 virions and exosomes was previously used to support the “Trojan exosome hypothesis”, but latter research showed that the CD45 is present only on exosomes but not virions (Coren et al., 2008). Thus, as a recent review pointed out (Wurdinger et al., 2012), it is more likely that the retroviruses have highly flexible ways to hijack host factors and can easily adapt to various types of cells or conditions, and the overlap of exosome pathway with the virus budding remains to be further studied.

HOST MODULATES MVB PATHWAY TO INHIBIT VIRUS BUDDING

ISGylation

ISGylation is a ubiquitination-like conjugation of ISG15, encoded by interferon-stimulated gene 15, and a dimeric homologue of ubiquitin (Skaug et al., 2010). ISGylation is a broad spectrum antiviral mechanism, which has its own activating enzyme (E1), conjugating enzyme (E2), ligase (E3) and deISGylation enzyme, and can modify viral or host proteins to block virus infection and replication (Harty et al., 2009; Zhang et al., 2011; Tang et al., 2010)

Recent studies (Okumura et al., 2008; Malakhova et al., 2008) showed that ISGylation can inhibit the interaction of the PTAP motif of HIV-1 Gag with TSG101 by ISGylating NEDD4 to inhibit UBL activity, and thus inhibit virus budding (Figure 2). Other studies (Pincetic et al., 2010; Kuang et al., 2011) revealed that all ESCRT III subunits (CHMP2A, CHMP3, CHMP4B, CHMP6) and CHMP5 could be ISGylated to block virus budding (Figure 2). ISGylation of CHMP5 inhibited the interactions of CHMP5-LIP5 and LIP5-VPS4, blocked membrane recruitment of LIP5 and VPS4, resulting to the failure of virus budding. Similarly, ISGylated CHMP2A lost its binding to LIP5 or VPS4, and ISGylated CHMP4B or CHMP6 could not bind VPS4. However, ISGylation did not affect CHMP3 binding to LIP5 or VPS4, nor CHMP1B binding to VPS4, the significance of which is not clear. When knocking out CHMP5, ISGylation could not inhibit the interaction of LIP5 and VPS4, nor their membrane locations. The inhibitory effect on virus budding was also lost, and the ISGylation of CHMP2A and CHMP6 was disrupted, indicating an essential role of CHMP5 for ISGylation of these proteins and the inhibition on virus budding (Kuang et al., 2011). Because ESCRT III and VPS4 are required for budding of almost all enveloped viruses, the ISGylation of ESCRT III and related proteins is considered to be a general antiviral mechanism and a novel function for ISG15.

Phosphorylation of VPS37C by TBK1

TBK1 is a Ser/Thr kinase that regulates NFΚB and interferon (IFN) pathways and is implicated in innate

immunity, cell proliferation and oncogenesis (Clément et al., 2008; Shen et al., 2011). A recent study revealed an IFN-independent antiviral role for TBK1, as the phosphorylation of VPS37C (ESCRT I subunit) by TBK1 can inhibit PTAP motif-dependent virus budding (Figure 2) (Da et al., 2011).

As reported by Da et al., TBK1 could bind and phosphorylate VPS37C but not other isoforms of VPS37 or other MVB proteins. It could inhibit the PTAP- but not PPxY or YPxL motif- dependent virus budding, and have no effect on the interaction of TSG101 with VPS28, the replication of viral genome, or the ubiquitination of Gag or assembly of virions. Overexpressed TBK1 could be co-eluted with TSG101, VPS28, VPS37C in gel filtration, indicating TBK1 may form a complex with them despite the fact that the involvement of MVB12 was not tested. TBK1 knockout did not affect the morphology of MVB and the degradation of EGFR, suggesting the MVB pathway may not be affected, although the effect of overexpressed TBK1 should also be checked, as well as MVB sorting of more proteins.

Nevertheless, HIV-1 budding could be prompted by TBK1 knockout and be inhibited by overexpressing TBK1 in the absence of the IFN-1 and Signal Transduction And Transcription1 (STAT1) pathway. Amazingly, the virus budding could be significantly stimulated by overexpressing a kinase mutant K38A, a much stronger effect than not overexpressed (background), indicating that K38A may facilitate virus budding via unknown mechanisms (Da et al., 2011). Unfortunately, whether the interaction of viral PTAP-TSG101 is inhibited by phosphorylation of VPS37C or TBK1 was not demonstrated.

Since more HIV-1 virions were observed to bud in MVB rather than at the cell surface, it is possible that TBK1 may relocate viral Gag proteins to late endosomes to induce budding into MVB (Da et al., 2011). Together, it is clear that phosphorylation of VPS37C is a novel, specific and IFN-independent antiviral role of TBK1, although there are still many uncertainties about molecular mechanisms of this regulation.

Phosphorylation of MVB12

MVB12A and MVB12B are also regulated by phosphorylation (Figure 2) to inhibit virus budding (Morita et al., 2007; Tsunematsu et al., 2010). Overexpression or knockout of MVB12 significantly inhibits virion assembly, Gag maturation and virus budding. Blocking phosphorylation of MVB12 does not disrupt the formation of ESCRT I, but causes much less inhibitory effect on virus budding, indicating MVB12 phosphorylation negatively regulates virus budding. In addition, phosphorylation of MVB12 regulates its ubiquitination and the MVB pathway (Tsunematsu et al., 2010). EGF can induce the phosphorylation of MVB12A, and increase its binding to and the MVB degradation of EGFR. The ubiquitination and degradation of MVB12B were up-regulated by EGF, but were inhibited by mutating phosphorylation sites, and the stabilized mutants were functional for EGFR degradation (Tsunematsu et al., 2010). Taken together, it seems likely the phosphorylation of MVB12 may act similarly as that of VPS37C to regulate ESCRT 1 and inhibit virus budding. However, the detailed molecular mechanism remains to be explored, as well as the identity of the kinase responsible for the phosphorylation of MVB12.

CONCLUSIONS AND PERSPECTIVES

Recent research has demonstrated that the MVB pathway is a newly discovered battlefield for the competition and gameplay between virus and host. Enveloped viruses use modular LDMs to hijack host MVB machinery for budding at the cell surface. The host cells modulate MVB proteins by ISGylation and phosphorylation to block virus budding. The result of this competition may decide whether virus infection can spread in the host system or the host can prevent virus dissemination.

Currently, available antiviral drugs are mainly inhibitors of protease, integrase or reverse transcriptase, all of which can induce drug resistance in viruses. A study showed that small inhibitors of the host-virus TSG101-PTAP interaction could specifically block virus budding (Liu et al., 2011). In addition, TSG101 was found to be exposed on the outside layer of plasma membranes of virus infected cells, and could be targeted by monoclonal antibodies to prompt the clearing of these cells (Diaz et al., 2010). These exciting studies will definitely stimulate research on the interaction of virus and host in the MVB pathway, and prompt development of new antiviral therapeutics.

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