Interaction of AMSH with ESCRT-III and Deubiquitination of Endosomal Cargo*

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The “class E” vacuolar protein sorting (VPS) pathway mediates sorting of ubiquitinated cargo into the forming vesicles of the multivesicular bodies (MVB), and it is essential for down-regulation of signaling by growth factors and budding of enveloped viruses such as Ebola and HIV-1. Work in yeast has identified DOA4 as a gene that is recruited by the class E machinery to remove ubiquitin from the endosomal cargo before it is incorporated into MVB vesicles, but the identity of the mammalian counterpart is unclear. Here we report the interaction of AMSH (associated molecule with the SH3 domain of STAM), an endosomal deubiquitinating enzyme, with the endosomal sorting complex required for transport (ESCRT-III) subunits CHMP1A, CHMP1B, CHMP2A, and CHMP3. We also show that a catalytically inactive AMSH inhibits retroviral budding in a dominant-negative manner and induces the accumulation of ubiquitinated forms of an endosomal cargo, namely murine leukemia virus Gag. Finally, VPS4 and AMSH compete for binding to the C-terminal regions of CHMP1A and CHMP1B, revealing a coordinated interaction with ESCRT-III. Taken together, these results are consistent with a role of AMSH in the deubiquitination of the endosomal cargo preceding lysosomal degradation.

Ubiquitin plays an essential role in the trafficking of membrane proteins into the degradative lysosomal pathway. Covalent attachment of ubiquitin to certain activated cell surface receptors serves as a sorting signal for entry into the endocytic vesicles at the plasma membrane (1). Subsequently to this internalization, the class E vacuolar protein sorting (VPS)2 pathway directs the ubiquitinated cargo into the forming vesicles of the late endosomal compartment, termed multivesicular bodies (MVB) (2, 3). In the last step, the MVB fuses with the lysosomal membrane and the cargo is delivered to the lumen of the lysosome for degradation.

Work in yeast has identified 18 class E VPS proteins that are required for sorting into the lumen of MVBs (2, 4, 5). Importantly, at least one human homolog for every class E protein has been identified and recent studies show that most of the protein-protein interactions between the class E proteins are conserved from yeasts to humans (6–8). A subset of class E proteins form one of three endosomal sorting complexes required for transport (ESCRT-I, -II, and -III) (9–11), and current models propose that these complexes are sequentially recruited by the endosomal cargo, forming a membrane-associated lattice that functions in vesicle invagination during MVB biogenesis. ESCRT-III is believed to contain the core sorting machinery of the class E pathway and it is composed of two functional subcomplexes, a membrane-proximal complex that interacts with the endosomal membrane and a peripheral subcomplex that seems to recruit accessory proteins (9).

After completion of cargo sorting and vesicle formation, ESCRT-III recruits the AAA ATPase Vps4 through direct protein-protein interactions, mediating the disassembly of the ESCRT machinery for recycling into the cytoplasm thus allowing further rounds of MVB sorting (12, 13). Work in yeast has identified Doa4 as an additional enzymatic activity that recycles ubiquitin through its removal from ubiquitin-tagged cargo prior to vacuolar degradation (14). Recruitment of Doa4 by the class E machinery to the endosomes is still controversial since both ESCRT-III and Bro1 have been shown to interact with Doa4 (14–16). The identity of the mammalian ubiquitin hydrolase that removes ubiquitin from MVB cargoes is still unclear.

A number of recent studies have provided an exciting connection between the class E VPS pathway and budding of enveloped viruses that include deadly pathogens like HIV-1 and Ebola virus (17). Notably, vesicle budding into the MVB and virion formation at the plasma membrane are topologically identical processes both involving a vesicle budding away from the cytoplasm. Most of the enveloped viruses encode at least one of the known late budding domains (L-domains) whose mutation induces an accumulation of immature virions at the plasma membrane (18). Viral L-domains are encoded by PT/SAP, PPXY, LYPXL, or FPV sequence motifs that facilitate viral egress by recruiting the class E VPS machinery via the interaction with alternative adaptor proteins (17). Intriguingly, several observations suggest that ubiquitin plays a general role in viral budding. It is known that proteasome inhibitors block retroviral release (19–22) and a catalytically active HECT ubiqui-

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2 The abbreviations used are: VPS, vacuolar protein sorting; AMSH, associated molecule with the SH3 domain of STAM; CHMP, charged MVB protein; DUB, deubiquitinating enzyme; EGFR, EGF receptor; ESCRT, endosomal sorting complex required for transport; HIV, human immunodeficiency virus; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; L-domain, late budding domain; MLV, murine leukemia virus; MVB, multivesicular body; siRNA, small interfering RNA; STAM, signal-transducing adaptor protein; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; dsBM, deletion of the STAM binding motif.
uitin ligase is required for PPHX-mediated viral budding (23). Previous work has also shown that viral L-domains influence the ubiquitination status of retroviral Gag proteins, although this activity does not necessarily correlate with their ability to facilitate budding. The presence of either PTAP or LYPXL motifs results in decreased ubiquitination levels of Gag (24, 25), suggesting that an ubiquitin hydrolase activity is recruited by these motifs. In contrast, it has been reported that PPHX-type L-domains increase Gag ubiquitination, probably as a consequence of the interaction with HECT ubiquitin ligases (22, 24).

In this study, we describe the interaction of AMSH (associated molecule with the SH3 domain of STAM), an endosome-associated ubiquitin isopeptidase (26), with several components of the peripheral subunit of the human ESCRT-III, namely CHMP1A, CHMP1B, CHMP2A, and CHMP3. Retroviral budding is used as a model to characterize the function of AMSH, finding that a catalytically inactive AMSH inhibits viral budding, while knockdown experiments suggest that AMSH is not essential for viral budding. This apparent paradox is explained, at least in part, by the mutually excluding interaction of AMSH and VPS4 with the C-terminal region of CHMP1A and CHMP1B, thus revealing a coordinated interaction of AMSH and VPS4 with ESCRT-III.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All the yeast two-hybrid plasmids, mammalian class E VPS factors, murine leukemia virus (MLV) Gag-GST, HA-tagged form of ubiquitin, Myc-VPS4, and HA-VPS4 expression plasmids have been described elsewhere (6, 24). AMSH and AMSH-LP were amplified by PCR from the Mammalian Gene Collection clones DNA (IMAGE 6170296 and 3898533, respectively) using primers directed to the 5' and 3' ends of the coding sequence and containing NotI sites to insert the PCR product into pGBK7T7 (Clontech) and pVP16 (27) for yeast two-hybrid assays and into pCR3.1/YFP and pCAGGS/GST for protein expression experiments. The AMSH D348A, AMSH, VPS4, and CHMP1A and -1B deletions derivatives were generated by PCR.

Yeast Two-hybrid Assay—Yeast Y190 cells were transformed with 1 μg of each of the pGBK7T7 and pVP16 constructs and transformants were selected on medium lacking tryptophan and leucine for 3 days at 30 °C. Interactions were determined by β-galactosidase activity in yeast extracts as previously described (28).

Coprecipitation Assays—293T cells were transfected with GST and YFP expression vectors (1 μg each) using polyethylenimine (Polysciences, Inc.) (29). 36 h later, cells were harvested and lysed in 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, and a protease inhibitor mixture (complete mini-EDTA-free, Roche Applied Science). Clarified lysates were incubated with glutathione-Sepharose beads (Amersham Biosciences) for 3 h at 4°C and washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 0.1% Triton X-100). The bead-bound proteins were eluted by boiling in 100 μl of sodium dodecyl sulfate sample buffer and analyzed by Western blotting with α-GFP monoclonal antibody.

Microscopy—293T cells were seeded onto glass coverslips and transfected with YFP-AMSH expression plasmid along with CFP-CHMP1B, CFP-HRS, or CFP unfused vectors. Cells were fixed with 4% paraformaldehyde 24 h after transfection, and images were taken with a TCS SP2 AOBS confocal microscope (Leica; HCX PL APO CS 63.0 × 1.4 oil objective).

Infectivity Assays—For MLV infectivity experiments, plasmids encoding the MLV proviruses pNCS (MLV), and pNCS P6PY (MLV/hPTAP) were used (30). 293T cells (24 well plates) were transfected with 125 ng of YFP derivatives, 300 ng of MLV proviral plasmid, 100 ng of a vesicular stomatitis virus-G envelope expression plasmid and 300 ng of pM5SCV/Tat. Similarly, for HIV infectivity assays, cells were transfected with 125 ng of the YFP fusions and 300 ng of pNL/HXB. Indicator HeLa-TZM-bl cells (CD4+, CXCR4+, CCR5+, HIV-1 LTR-LacZ) (31) were infected with 100 μl of filtered supernatant, harvested from 293T cells 36 h after transfection. Finally, 48 h after infection, β-galactosidase activities in cell lysates were measured using the chemiluminescent detection reagent Galacto-Star (Applied Biosystems).

Culture supernatants, collected 48 h after transfection, were clarified by low speed centrifugation, and particles present in 250 μl were obtained by centrifugation through a 20% sucrose cushion at 14,000 rpm for 2 h. Viral protein content in cell and particle lysates was analyzed by Western blotting with α-Gag antibodies.

EGF Receptor Degradation Assay—HeLa cells were transfected with c-Cbl expression plasmid and YFP control, YFP-AMSH, or YFP-AMSH D348A fusion proteins. The next day, cells were serum-starved for 5 h in Dulbecco’s modified Eagle’s medium containing 0.1% fatty acid-free bovine serum albumin (Sigma) and stimulated with 50 ng/ml EGF (Sigma) for 0, 1, 2, or 3 h. One hour prior to addition of EGF cells were treated with 5 μg/ml cycloheximide (Sigma). At the required time point, cells were harvested and resuspended in 100 μl of sodium dodecyl sulfate sample buffer. Endogenous EGF receptor was analyzed by Western blotting with α-EGFR antibody.

RNA Interference—To assay inhibition of viral production by siRNA-mediated depletion of cellular AMSH, 293T cells were initially transfected with 50 pmol of siRNA using Lipofectamine 2000 (Invitrogen) and split the next day. 48 h after initial transfection, cells were cotransfected with another 50 pmol of siRNA and the MLV or HIV proviral plasmids as described in the infectivity assays.

The target sequences for the AMSH-specific, Tsg101-specific and luciferase control siRNAs were UUAACAAU-CUGCUUGUCUUU, CCUCAGUCUUUCUGGUC and CUGCCUGCGAGAUUC, respectively.

MLV Gag Ubiquitination Assays—MLV Gag-GST fusion protein and HA-tagged form of ubiquitin were expressed by transfection of 293T cells. The cells were harvested 48 h post-transfection and lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 5 mM N-ethylmaleimide, and a protease inhibitor mixture (Roche Applied Science). MLV Gag-GST and ubiquitinated conjugates were precipitated with glutathione-agarose beads and analyzed by Western blotting with α-Gag or α-HA antibodies.
Western Blot Analysis—Cell extracts, as well as virion lysates, were separated on 10 or 12% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were sequentially probed with monoclonal antibodies α-HIV-1 p24 (183-H12–5C), α-GFP (Roche Applied Science), α-HA (HA.11, Covance) or α-TSG101 (4A10, Abcam), rabbit polyclonal antibodies α-AMSH (gift of S. Urbe, Physiological Laboratory, University of Liverpool), α-EGFR (Cell Signaling) or α-Hsp90 (Santa Cruz Biotechnology) or goat polyclonal α-MLV p30 (gift of M. Malin, Department of Infectious Diseases, King’s College London School of Medicine) and with a peroxidase-conjugated antibody against mouse (AP308, Chemicon), goat (AP106P, Chemicon), or rabbit (Pierce) and developed using chemiluminescent substrate reagents (Pierce).

RESULTS

Interaction of AMSH with the Class E VPS Pathway—Based on the assumption that the class E VPS pathway is highly conserved across divergent species, we searched for deubiquitinating enzymes (DUBs) interacting with ESCRT-III components using a global map of protein/protein interactions generated by yeast two-hybrid in Drosophila (32). The orthologue of AMSH (CG2224) was found to interact with CG8055 and CG9779, the orthologues of CHMP3 and CHMP4. Based on these results, we decided to study the human AMSH and the closely related protein AMSH-LP, which shares a 73% of amino acid similarity with AMSH. Interestingly, AMSH has been recently described as an endosome associated ubiquitin isopeptidase (26), and it has also been shown to be associated with STAM (signal transducing adaptor molecule) (33), an endosomal component of the HRS complex. Interactions between these DUBs and the different components of the human class E pathway were tested by yeast two-hybrid assays (Fig. 1A), and positive interactions between AMSH and the ESCRT-III subunits CHMP1A, CHMP1B, CHMP2A, and CHMP3 were observed. Importantly, none of these ESCRT-III proteins interacts with AMSH-LP, suggesting a highly specific interaction of AMSH and the peripheral subunit of ESCRT-III.

Mapping studies by yeast two-hybrid analysis determined that the N-terminal region of AMSH (residues 1–191) is necessary and sufficient for interaction with ESCRT-III (Fig. 1B). Importantly, the region containing the STAM binding motif (residues 229–239) is dispensable for this interaction. This result was confirmed by GST pulldown, showing that the deletion of the STAM binding motif (dSBM) in AMSH (34) had no effect in the interaction with CHMP1B (Fig. 1D), also excluding STAM as a bridging factor between AMSH and ESCRT-III.

The positive interactions obtained by yeast two-hybrid were confirmed by a GST coprecipitation assay (Fig. 1, C and D). Unexpectedly, a catalytically inactive form of AMSH (D348A) showed an increased binding to GST-CHMP1A and GST-CHMP2A as compared with wild type AMSH. This effect might indicate that AMSH dissociates from ESCRT-III after the endosomal cargo is deubiquitinated and could also contribute to the more pronounced endosomal localization exhibited by AMSH D348A (26).

Colocalization studies by confocal microscopy revealed that the YFP-AMSH fusion protein exhibits a diffuse cellular distribution (Fig. 1E), but the coexpression of CFP-CHMP1B resulted in a dramatic relocation of YFP-AMSH to CFP-CHMP1B-containing intracellular vesicles. Importantly, the expression of CFP-HRS, which also presents a marked vesicular localization, did not induce a change in the localization of YFP-AMSH and only a marginal colocalization between both proteins was observed.

A Catalytically Inactive AMSH Inhibits Viral Budding and Degradation of Endosomal Cargo—There is an increasing body of evidence showing that retroviruses utilize the class E VPS pathway to facilitate viral egress, thus making retroviral budding a useful tool to study the ESCRT machinery. In this model, the viral protein Gag is the equivalent of the late endosomal cargo and different L-domains recruit the ESCRT proteins through the interaction with different cellular adaptor proteins. It is widely accepted that PTAP motifs recruit the ESCRT-I complex through the interaction with TSG101 (28, 35, 36), whereas PPXY motifs utilize a subset of HECT ubiquitin ligases to facilitate viral budding (23, 37–39). A third type of L-domain containing the LYPX motif recruits the ESCRT machinery by interacting with AIP1/ALIX (6, 7), the human homologue of Bro1.

To study the role of AMSH in viral budding we used previously described MLV, which encode either the natural PPXY L-domain (MLV) or a PTAP motif derived from HIV-1 (MLV-hPTAP) (30, 40). The overexpression of YFP-AMSH resulted in a modest inhibition of budding of both MLV and MLV/hPTAP. In contrast, the expression of YFP-AMSH D348A resulted in a strong inhibition of virion release by MLV and MLV/hPTAP (Fig. 2A). This inhibition of PPXY and PTAP L-domains suggest that AMSH D348A inhibits the core class E machinery. Importantly, an accumulation of ubiquitinated forms of MLV Gag was observed in the presence of YFP-AMSH D348A and similar results were obtained with MLV Gag/hPTAP (Fig. 2B), suggesting that AMSH is recruited by an endosomal cargo, namely MLV Gag.

Recent work by McCullough et al. (26) describes an increased association of AMSH D348A with STAM as compared with wt AMSH, suggesting that STAM might play a role in AMSH D348A inhibition of viral budding. This hypothesis was studied by testing the inhibitory activity of AMSH mutants that contain a deletion of the STAM binding motif (dSBM), finding that the D348A mutation exhibits a similar phenotype both in the wt and dSBM contexts (Fig. 2A), thus indicating that the dominant-negative properties of AMSH D348A are independent of the interaction with STAM and therefore might be linked to its binding to ESCRT-III.

The inhibition of the class E VPS pathway by AMSH D348A was confirmed by studying the degradation of the EGF receptor upon activation by EGF. These experiments were performed in the absence of protein synthesis by adding cycloheximide 1 h before and during the activation with EGF. As described previously (41), a slower degradation of the EGF receptor was observed upon overexpression of HRS as compared with the controls (Fig. 2C). In agreement with the results obtained in the viral budding assays, the expression of AMSH D348A resulted in a block of EGFR degradation (Fig. 2C), whereas no significant
FIGURE 1. AMSH binds to the ESCRT-III components CHMP1A, CHMP1B, CHMP2A, and CHMP3 in a STAM1-independent way. A and B, full-length AMSH and AMSH-LP or AMSH deletions fused to the VP16 activation domain were tested by yeast two-hybrid with the mammalian components of ESCRT-I, -II, and -III fused to the GAL4 DNA binding domain. Protein interactions were determined by the levels of β-galactosidase activity and are expressed as absorbance units (A\textsubscript{540} nm). Error bars indicate the standard deviations from the mean of triplicate measurements. C, co-precipitation assays to validate the interactions found by yeast two-hybrid experiments. 293T cells were cotransfected with plasmids encoding YFP-AMSH, YFP-AMSHD348A, or YFP-AMSH-LP and the different ESCRT-III subunits fused to GST. Cell lysates were subjected to precipitation with glutathione-Sepharose beads as described under "Experimental Procedures," and the bound fractions were analyzed by immunoblotting with an α-GFP monoclonal antibody. D, a form of AMSH lacking the STAM binding motif is able to bind to CHMP1B but not to STAM1. 293T cells were cotransfected with plasmids encoding GST-AMSH (WT), GST-AMSHdSBM (dSBM), or pCAGGS/GST alone (GST) and CHMP1B or STAM1 fused to YFP. Cell lysates were subjected to precipitation with glutathione-Sepharose beads as before and analyzed by Western blot with α-GFP monoclonal antibody. E, YFP-AMSH localization in 293T cells in the presence of either CFP-CHMP1B or CFP-HRS. Cells were examined by confocal microscopy, and each set of three panels shows images acquired using YFP (left) and CFP (middle) settings to generate overlaid images (right).
effects in this assay were observed by overexpression of wild type AMSH.

AMSH Is Not Essential for Viral Budding—To characterize in more detail the role of AMSH in viral budding, a knockdown approach by siRNA was followed. As shown in Fig. 3, A and B, specific depletion of AMSH induced a 2–3-fold increase in infectious viral release by MLV, MLV/hPTAP, or HIV-1. Depletion of TSG101 was included for control purposes (Fig. 3B), resulting in a 20-fold reduction of infectious HIV-1 production as described previously (35). Overall, these results show that AMSH is not essential for viral budding and suggest that the dominant-negative effect exhibited by AMSH D348A might be due to a competition or interference with essential components of the ESCRT machinery that are recruited by L-domains. This notion is also consistent with the increased L-domain activity observed in the absence of endogenous AMSH.

The C-terminal Region of CHMP1A and CHMP1B Is Important for Binding to AMSH and VPS4—A deletion analysis by yeast two-hybrid methods determined that the C-terminal region of CHMP1B (residues 61–196) is sufficient to interact with CHMP1A, AMSH, and VPS4 (Fig. 4A). These results are in agreement with recently published experiments showing that the MIT domain of VPS4 binds with the same affinity to both full-length and residues 65–196 of CHMP1B (42). Conversely, a deletion of 12 amino acids at the C terminus of CHMP1B resulted in loss of binding to AMSH and VPS4, while the interaction with CHMP1A was not affected by this deletion (Fig. 4B). CHMP1A and CHMP1B amino acid sequences are very similar, and we reasoned that the C-terminal region of CHMP1A might have similar properties as the observed with CHMP1B. In fact, a CHMP1A construct lacking 11 amino acids at the C terminus (1–185) was no longer able to interact with AMSH or VPS4, while the interaction with CHMP1B was observed at similar levels as full length CHMP1A (Fig. 4B).

The previous results suggested that the C-terminal region of CHMP1A and CHMP1B could be important for ESCRT-III complex functioning, and to investigate this concept we followed a dominant-negative approach overexpressing the truncated forms of CHMP1A and CHMP1B. As previously published and shown in Fig. 4B (6), the overexpression of a YFP-CHMP1B fusion inhibits MLV and HIV-1 release by 2–3-fold as determined by
Western blot and infectivity assays. Importantly, YFP-CHMP1B 1–184 exhibited an enhanced dominant-negative activity on MLV and HIV-1 viral release. The fact that this effect is observed in viruses that encode different types of L-domain suggests that the core ESCRT machinery is inhibited by the truncated CHMP1B. Similar experiments with CHMP1A showed a reduced dominant-negative effect of CHMP1A 1–185 on MLV budding as compared with the analogous deletion in CHMP1B (Fig. 4B). In contrast, CHMP1A 1–185 did not induce a significant inhibition of HIV-1 budding, suggesting that different viruses might use differentially the ESCRT-III complex, although this needs further investigation. In summary, these data indicate that the C-terminal region of CHMP1A and CHMP1B is required for ESCRT-III function, presumably by interacting with VPS4 and AMSH.

Mutually Exclusive Binding of AMSH and VPS4 to ESCRT-III—Based on the previous observations, we hypothesized that AMSH and VPS4 might bind to overlapping regions in CHMP1A and -1B and therefore compete with each other for binding to the ESCRT-III complex. In this model, the increased association of AMSH D348A with the ESCRT-III complex would allow a more effective competition with VPS4 thus explaining the dominant-negative activity exhibited by this mutant. To test this hypothesis, pulldown experiments were performed, in which a GST-AMSH construct was cotransfected with YFP-CHMP1B in 293T cells, in the presence or absence of overexpressed VPS4. As shown in Fig. 5A, the overexpression of VPS4 resulted in a decreased binding of YFP-CHMP1B to GST-AMSH, and a similar result was obtained with GST-AMSH D348A. Further deletion analysis showed that the MIT domain of VPS4 is necessary and sufficient to compete with AMSH for binding to CHMP1B. However, a reduced competition was observed by the isolated MIT domain as compared with full-length VPS4. This result might be a consequence of the oligomeric structure of VPS4 as opposed to a monomeric MIT domain (42, 43).

An important control to determine the specificity of the competition assay showed that the binding of YFP-STAM1 to GST-AMSH is not inhibited by the overexpression of VPS4. This result is coherent with the fact that STAM1 and CHMP1B interact with different regions in AMSH.

Last, the model in which AMSH D348A inhibits L-domain function by preventing the VPS4/ESCRT-III interaction was functionally tested by determining the dominant-negative activity by YFP-AMSH D348A in the presence of increased levels of VPS4. As shown in Fig. 5C, overexpression of VPS4 alleviated in part the inhibitory activity of YFP-AMSH D348A as
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Figure 4. The C-terminal regions of CHMP1A and CHMP1B interact with VPS4 and AMSH and are important for virus production. A, yeast two-hybrid mapping of the regions in CHMP1A and CHMP1B important for AMSH and VPS4 interaction. Full-length AMSH, VPS4, CHMP1A, or CHMP1B fused to the VP16 activation domain were coexpressed with full-length CHMP1A and CHMP1B (FL) or their truncated forms at the C terminus (1–185 and 1–184) fused to the GAL4 DNA binding domain and β-galactosidase activity was determined. Error bars indicate the standard deviations from the mean of triplicate measurements. B, CHMP1A and -1B deletions inhibit virus release. YFP fused to full-length CHMP1A and -1B (FL), to their C-terminal deletions (1–185 and 1–184), or unfused (Control) was cotransfected with MLV or HIV-1 proviruses. Infectious virus release was measured as described in the legends to Figs. 2 and 3. Error bars indicate the standard deviation from the mean of triplicate measurements. The inhibition of viral particles release was analyzed in cell lysates and extracellular virions by Western blot with α-MLV Gag antibodies. The bottom right panel shows the expression levels of YFP fusions as analyzed by Western blot with α-GFP antibody (WBα).

Figure 5. AMSH and VPS4 compete for CHMP1B. A, lysates prepared from 293T cells transfected with pairs of GST-AMSH (WT), GST-AMSHD348A (D348A), or empty pCAGGS/GST (None) and YFP-CHMP1B or YFP-STAM1 in the presence of HA-VPS4 (+ VPS4), HA-MIT domain of VPS4 (+ MIT), HA-VPS4dMIT (+ VPS4dMIT), or HA empty vector (Control) were precipitated with glutathione-Sepharose beads and analyzed by Western blot with α-GFP monoclonal antibody as before. B, analysis of HA fusion protein expression levels in cell lysates with α-HA monoclonal antibody. C, rescue of MLV release induced by overexpression of YFP-AMSHD348A in the presence of VPS4. 293T cells were transfected with sets of MLV proviral DNA, Myc-VPS4 (+ VPS4), or empty (control) expression vectors and YFP-AMSH, YFP-AMSHD348A, or unfused (control) plasmids and infectious virus release was measured as described above. Determined by infectious virus release. In contrast, augmenting the level of VPS4 did not increase infectious virus release in the presence of YFP-AMSH or unfused YFP. Attempts to obtain a complete rescue of the inhibitory effect induced by AMSH-D348A by expressing higher levels of VPS4 failed (data not shown). However, a general inhibition of viral budding in these conditions was observed, possibly precluding a full rescue of MLV budding. It is also possible that the wt and catalytically inactive AMSH inhibit the ESCRT machinery by additional mechanisms.

Discussion

This study describes the interaction of AMSH, an endosomal deubiquitinating enzyme, with several components of the peripheral subunit of ESCRT-III complex, namely CHMP1A, -1B, -2B, and -3. The relevance of the ESCRT-III/AMSH binding is highlighted by an evolutionary conserved interaction described in a genome-wide screen in Drosophila.
(32). Additional evidence suggesting that AMSH and the ESCRT machinery are functionally related is given by work in Schizosaccharomyces pombe (44) showing that the orthologues of AMSH and other class E proteins are suppressor genes in cells that are mutated in the homologue of Fab1p, a kinase that regulates vacuole morphology through its product PtdIns(3,5)P2 and it is essential for protein sorting in the MVB (45).

Deubiquitination of the endosomal cargo precedes vacuolar/lysosomal degradation (46, 47). Intuitively, a spatial and temporal regulation of the MVB-associated DUB would be important to avoid the deubiquitination of the endosomal cargo prior to its ubiquitin-dependent commitment into the forming luminal vesicle of an MVB. The interaction of AMSH with several components of ESCRT-III suggests that AMSH might mediate the deubiquitination of the class E cargo at a late stage in the sorting process. In agreement with this role, we observe that AMSH D348A induces an increased ubiquitination of an endosomal cargo, namely MLV Gag. A role of UBPY in the deubiquitination of endosomal cargo in transit to the MVB has also been proposed (48), but there are important differences with AMSH. The activity of UBPY seems to be dependent of the interaction with STAM/HRS (48), whereas the dominant-negative activity of AMSH and its interaction with ESCRT-III are independent of STAM1 binding. Interestingly, AMSH has been shown to process K63-linked polyubiquitin (26, 48), which regulates endocytosis and lysosomal sorting (49) whereas UBPY processes K48-linked polyubiquitin, a signal for proteasomal degradation.

The current models support a late role of VPS4 in the class E pathway by disassembling ESCRT-III from the endosomal membranes after the sorting process has been completed. This study shows the mutually exclusive interaction of AMSH and VPS4 with the peripheral subunit of ESCRT-III, suggesting that the C-terminal region of CHMP1A and CHMP1B is important for ESCRT-III function by coordinating the interaction with VPS4 and AMSH. In this context, it seems likely that the recruitment of VPS4 might terminate the deubiquitination of the endosomal cargo by occupying the AMSH binding site in ESCRT-III. Intriguingly, Vps4 and Doa4 interact with Vps32 in yeasts (15), suggesting that a similar mechanism might regulate DOA4 activity. The interaction of AMSH with CHMP2A and CHMP3 indicates that additional regulatory mechanisms might modulate the interaction of AMSH with ESCRT-III.

A catalytically inactive AMSH inhibits viral budding and degradation of endosomal cargo, while there is no apparent inhibition of the ESCRT function when AMSH is depleted from cells. We explain this phenomenon by the increased binding of AMSH D348A to some of the ESCRT-III subunits, allowing a more efficient competition with VPS4 for binding to the CHMP proteins. This result is reminiscent of a “substrate trap” effect and we speculate that decreasing the affinity of AMSH for ESCRT-III upon deubiquitination of the endosomal cargo would allow VPS4 to interact more efficiently with ESCRT-III and mediate the disassembly of the class E machinery.

The main function of DOA4 in the class E pathway seems to be the removal of ubiquitin from the endosomal cargo to main-
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