Hrs and STAM Function Synergistically to Bind Ubiquitin-Modified Cargoes In Vitro

Hirohide Takahashi,1 Jonathan R. Mayers,2 Lei Wang,2 J. Michael Edwardson,1 and Anjon Audhya2,*

1Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, United Kingdom; and 2Department of Biomolecular Chemistry, University of Wisconsin-Madison School of Medicine and Public Health, Madison, Wisconsin

ABSTRACT The turnover of integral membrane proteins requires a specialized transport pathway mediated by components of the endosomal sorting complex required for transport (ESCRT) machinery. In most cases, entry into this pathway requires that cargoes undergo ubiquitin-modification, thereby facilitating their sequestration on endosomal membranes by specific, ubiquitin-binding ESCRT subunits. However, requirements underlying initial cargo recognition of mono-ubiquitinated cargos remain poorly defined. In this study, we determine the capability of each ESCRT complex that harbors a ubiquitin-binding domain to bind a reconstituted integral membrane cargo (VAMP2), which has been covalently linked to mono-ubiquitin. We demonstrate that ESCRT-0, but not ESCRT-I or ESCRT-II, is able to associate stably with the mono-ubiquitinated cargo within a lipid bilayer. Moreover, we show that the ubiquitin-binding domains in both Hrs and STAM must be intact to enable cargo binding. These results indicate that the two subunits of ESCRT-0 function together to bind and sequester cargoes for downstream sorting into intralumenal vesicles.

INTRODUCTION

The ESCRT machinery is composed of five multisubunit complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and the Vps4 complex) and several accessory proteins (1–3). A majority of these factors are conserved among eukaryotic species, suggesting that they play key roles in regulating cellular function (4). Consistent with this idea, ESCRT components have been implicated in a number of membrane scission events, including intralumenal vesicle (ILV) biogenesis on endosomes, cytokinetic abscission to separate daughter cells after division, and the budding of many retroviruses from the plasma membrane (5–7). In each case, adaptors recruit ESCRT complexes that ultimately bend and/or cleave the lipid bilayer. For example, in mammalian cells, Cep55 recruits the ESCRT-I complex to the site of abscission, whereas viral Gag isoforms bring ESCRT-I to sites of virus budding on the plasma membrane (8,9). Accumulation of ESCRT-I and other downstream complexes on endosomal membranes depends on ESCRT-0, which assembles as a heterotetramer of two unique subunits, Hrs and STAM, on lipid bilayers (10–12). In addition to its role as an adaptor for ESCRT-mediated ILV formation, ESCRT-0 also plays an important role in promoting ubiquitin-dependent cargo sorting by virtue of its ability to bind directly to ubiquitin (13–15). Both ESCRT-I and ESCRT-II also harbor domains that associate with ubiquitin, raising the possibility that multiple complexes in the ESCRT machinery participate in cargo recognition before deposition into ILVs (1–3). However, the individual roles of these ESCRT complexes in promoting cargo sequestration remain ill-defined.

The ubiquitin-interacting domains found within subunits of the early-acting ESCRT complexes (ESCRT-0, ESCRT-I, and ESCRT-II) have been demonstrated to bind ubiquitin weakly in solution, with affinities ranging from ~100 to 600 μM (2). The membrane-associated heterotetrameric ESCRT-0 complex harbors at least eight ubiquitin-binding surfaces, all of which can engage ubiquitin simultaneously (12). In contrast, ESCRT-I contains no more than two domains capable of interacting with ubiquitin (depending on the presence of mammalian UBAP1 in the complex), and ESCRT-II contains only a single ubiquitin-binding surface (16–19). Although all early-acting ESCRT complexes exhibit the ability to bind to endosomally enriched phosphatidylinositol 3-phosphate (PI3P), their affinities vary significantly. At steady state, only ESCRT-0 accumulates stably on endosomes, whereas ESCRT-I and ESCRT-II exhibit a more transient association (2). Current evidence suggests that ESCRT-0 recruits ESCRT-I, which in turn brings ESCRT-II onto the membrane. These data suggest that a supercomplex of the early-acting ESCRT machinery may form to sequester cargoes (13). However, assembly of such a higher-order oligomer is unlikely to be stable, as the distributions of each complex have been shown to differ in vivo (10).

Recently, the ESCRT-0 complex, but not ESCRT-I, was shown to accumulate at sites of clathrin-mediated endocytosis at the plasma membrane, through its ability to interact with a set of clathrin adaptors (20). In the absence of this pool of ESCRT-0, ubiquitin-modified cargoes continue to be
internalized normally, but exhibit a substantial delay in endosomal trafficking. These findings highlight the possibility that initial recognition and/or sequestration of cargoes by ESCRT-0 in clathrin-coated pits accelerates their downstream sorting into ILVs. Similarly, ubiquitin-modified biosynthetic cargoes leaving the trans-Golgi network encounter a pool of ESCRT-0 on early endosomes, which may result in their local concentration before endosome maturation and the accompanying recruitment of downstream ESCRT complexes (1). In both of these models, cargo sequestration depends on ESCRT-0, whereas other ESCRT components function to promote membrane bending and scission (21).

To directly test the ability of early-acting ESCRT complexes to bind cargo within a lipid bilayer, we developed a system to measure the distribution of a mono-ubiquitin-conjugated substrate at nanometer-scale resolution using atomic force microscopy (AFM). Importantly, in contrast to earlier studies that used modified lipid headgroups to tether ubiquitin to the surface of a bilayer (22), we took advantage of a reconstituted integral membrane, mono-ubiquitin-modified protein (VAMP2). We demonstrate that ESCRT-0, but not ESCRT-I or ESCRT-II, is capable of binding this model cargo stably, in a ubiquitin-dependent manner. Moreover, we show that ubiquitin-binding domains in both subunits of ESCRT-0 are critical for this effect, indicating that Hrs and STAM function synergistically in this process. Our data define the minimal number of lipid binding modules that must function simultaneously to restrict cargo diffusion within a physiologically relevant lipid bilayer.

MATERIALS AND METHODS

Recombinant protein purification

Cloning and polyclintranscript co-expression of wildtype and mutant C. elegans ESCRT-0, ESCRT-I, and ESCRT-II was conducted as described previously (12,23,24). Briefly, to enable purification of each ESCRT complex, a polyhistidine tag was appended onto the amino-terminus of a single subunit to enable capture using nickel affinity chromatography. All complexes were purified further using size exclusion chromatography. Purification of murine VAMP2 or an amino-terminal translational fusion between ubiquitin and VAMP2 was conducted as described previously (25). Briefly, bacterial pellets were lysed by sonication and supplemented with ~1% Triton X-100 before binding to nickel affinity resin. Proteins were washed and eluted in the presence of ~1% n-octylglycide, to maintain solubility. Purifications of glutathione S-transferase (GST) and GST fusions to mono- and di-ubiquitin were carried out using glutathione agarose beads. The immobilized GST proteins were subjected to extensive washing using 20 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Tween 20, 1 mM PMSF, and 1 mM DTT before a 30-min incubation with purified ESCRT-0, ESCRT-I, or ESCRT-II. Subsequent to the binding reaction, beads were washed five times using 20 mM Tris (pH 7.5) and 150 mM NaCl and then eluted using 50 mM Tris (pH 7.5), 10 mM glutathione, and 150 mM NaCl. Antibodies directed against Hrs, STAM, Mvb12, and Vps22 have been described previously (12,20,23).

Reconstitution of VAMP2 into liposomes

Phosphatidycholine (PC), phosphatidylethanolamine (PE), and phosphatidyserine (PS) were obtained as chloroform solutions from Avanti Polar Lipids (Alabaster, AL). Phosphatidylinositol 3-phosphate (PI3P) was obtained as a solution in chloroform/methanol/water (1:2:0.8) from Echelon Biosciences (Salt Lake City, UT). A lipid mixture of composition 54% PC, 30% PE, 15% PS, and 1% PI3P was produced, and the solvents were evaporated in a stream of nitrogen gas. Dried lipid (400 µg) was resuspended in 200 µL of 2% CHAPS in HEPES-buffered saline (HBS; 100 mM NaCl, 20 mM HEPES, pH 7.5), containing 1 µg of protein (VAMP2 or ubiquitin-conjugated VAMP2). The mixture was then dialyzed against 1 L of HBS for 3 days at 4°C.

Atomic force microscopy

Liposomes (either with or without integrated protein) were mixed with individual ESCRT complexes (final concentration of 150 nM in each case) and incubated for 30 min at 37°C. The suspension was then adsorbed onto freshly cleaved mica for 3 min at room temperature. Imaging was performed in tapping mode under fluid (HBS) using a Bruker Multimode instrument (Santa Barbara, CA), controlled by a Nanoscope IIIa controller, at room temperature. Cantilevers (MikroMasch HQ:NSC18/AL BS, Innovative Solutions Bulgaria, Sofia, Bulgaria) were tuned to between 10% to 20% below the peak of the resonance frequency, generally found between 25 and 35 kHz in fluid conditions (12,24). During the approach, the cantilever was set at 0.9 of free amplitude. During imaging, the set point value was kept as high as possible so that the applied force was minimized. Images were captured at a scan rate of 2 Hz (unless otherwise noted), and with 512 scan lines per area. At least four cantilevers were used to generate each volume distribution. Data analysis was performed using commercially available software (NanoScope III software; Digital Instruments, Santa Barbara, CA). Free-standing particles were identified. Particle heights and diameters were measured manually by the Nanoscope software and used to calculate the molecular volume of each particle using the following equation:

$$V_m = \left(\frac{\pi h}{6}\right)\left(3r^2 + h^2\right),$$

where $h$ is the particle height and $r$ the radius (25). This equation assumes that the adsorbed particles adopt the form of a spherical cap. Asymmetric particles, and particles at the bilayer edges, were excluded from the volume analysis. More than 200 particles, from two to three independent experiments, were analyzed for each condition.

To check that there were no systematic errors in measurement of $h$ and $r$, which could skew the determination of volumes, we constructed scatter plots of $h$ against particle diameter for the various particles imaged. These are shown in Supporting Material. In all cases there was no strong correlation between $h$ and diameter, excluding the possibility of systematic errors.

RESULTS

An AFM-based assay to study the distribution of an integral membrane, ubiquitin-modified cargo

The ESCRT machinery mediates the ubiquitin-dependent turnover of integral membrane cargoes in the endolysosomal system. Previous work indicates that the translation fusion of monoubiquitin to a transmembrane protein results in its recognition by the ESCRT machinery and degradation within the lysosome lumen (17). We sought to capitalize on this finding and develop a model cargo that could be reproducibly reconstituted into a lipid bilayer and visualized without the use of conjugated dyes or other tags, which might influence associations with binding partners. AFM affords label-free imaging of proteins and protein complex...
assembly on membranes at nanometer scale resolution. To generate bilayers that mimic the surface of early endosomes (26), we adsorbed liposomes composed of 54% PC, 30% PE, 15% PS, and 1% PI3P onto mica. Imaging of protein-free bilayers by AFM revealed a relatively homogenous surface, with occasional gaps that exposed the underlying mica (Fig. 1 A), as observed previously (12,24). Importantly, the supported membrane exhibited a highly uniform thickness of ~4 nm, consistent with the formation of a single lipid bilayer.

Previous studies indicate that transmembrane proteins in the soluble NSF attachment protein receptor (SNARE) family are amenable to recombinant expression and reconstitution into synthetic liposomes (27). We purified a recombinant form of murine VAMP2 and used detergent (1% n-octylglucoside) to maintain its solubility in solution. By dialyzing away the detergent in the presence of phospholipids, we were able to achieve spontaneous insertion of VAMP2 into membranes. To verify incorporation, we conducted a co-flotation assay, in which reconstituted vesicles were mixed with Accudenz density medium and overlaid with decreasing concentrations of Accudenz. After centrifugation, vesicles floated to the top of the gradient and were collected for analysis by SDS-PAGE. We found that VAMP2 efficiently floated with vesicles in this assay, indicating that VAMP2 was membrane-embedded (Fig. 1 B). Fractions from elsewhere in the gradient were devoid of protein (Fig. 1 B).

Next, VAMP2-containing liposomes were adsorbed onto freshly cleaved mica for imaging. Numerous free-standing particles were observed in the VAMP2-containing bilayers (Fig. 1 C). In addition, VAMP2 induced the formation of small pores in the bilayer (Fig. 1 C, arrows). These pores had raised edges, suggesting that they were lined with protein, unlike the larger gaps in the bilayer (Fig. 1 C, asterisks), which were also observed in the absence of VAMP2 (Fig. 1 A). A zoomed image of a section of bilayer containing both a protein-lined pore and a free-standing particle is shown in Fig. 1 D. Our subsequent analysis focused on the free-standing particles only. These particles exhibited a random distribution and had a peak molecular volume of 50 to 75 nm³ (Fig. 1 E). Given that the expected molecular volume of VAMP2, based on its amino acid composition, is 16 nm³, we estimate that a typical particle contains three or four VAMP2 molecules.

We should point out caveats associated with the measurement of molecular volumes by AFM. For instance, it is well known that the geometry of the scanning AFM probe introduces a tendency to overestimate particle radii, a convolution that becomes especially significant when imaging under fluid, because fluid-imaging tips are blunter than air-imaging tips. In addition, volume measurements of particles bound to lipid bilayers do not take into account any penetration of the bilayer by the protein, or any squashing of the protein or the lipid by the tip. For this reason, we regard the measured volumes as estimates rather than precise values. Nevertheless, the reasonably close correspondence seen in this study between the measured and expected volumes gives us confidence that our interpretation of the imaging data is correct.

We translationally fused ubiquitin to the amino-terminus of VAMP2 and purified the recombinant protein identically to the wildtype form. Importantly, the efficiency of reconstituting ubiquitin-modified VAMP2 (Ub-VAMP2) into liposomes was similar with that of unmodified VAMP2 (Fig. 1 B). The protein:lipid ratio of the liposomes was ~1:400 (w/w). Following assembly of supported lipid bilayers, we found that Ub-VAMP2 continued to distribute randomly,
and we again observed pores in the bilayer (Fig. 1 F; arrows). The only difference detected between Ub-VAMP2 and wildtype VAMP2 was an increase in its molecular volume to 75 to 125 nm$^3$ (Fig. 1 G). The addition of a ubiquitin moiety should increase the molecular volume of a VAMP2 molecule to 27 nm$^3$. Hence, a typical particle again would contain three or four Ub-VAMP2 molecules. Together, these findings indicated that Ub-VAMP2 met all of our criteria for determining whether components of the ESCRT machinery can influence cargo distribution within a membrane. Additionally, parallel analysis of unmodified VAMP2 provided an ideal control for all studies.

**Ubiquitin-binding domains in both Hrs and STAM are required for cargo binding**

Previous work suggests that the ESCRT-0 complex acts upstream of other ESCRT components during the sorting of ubiquitin-modified cargoes (2). ESCRT-0 is composed of two subunits, Hrs and STAM, both of which contain at least two ubiquitin-binding domains with affinities ranging from ~130 to 550 μM (12). Our goal was to determine whether these low-affinity ubiquitin-binding domains could facilitate the stable recruitment of an integral membrane protein in a ubiquitin-dependent manner. We first confirmed that recombinant ESCRT-0, assembled using *C. elegans* Hrs and STAM, bound to supported lipid bilayers composed of 54% PC, 30% PE, 15% PS, and 1% PI3P and in the absence of VAMP2. Consistent with our previous work (12), we found that individual particles of ESCRT-0 were distributed evenly across the bilayer (Fig. 2 A). Analysis of their volumes highlighted two major peaks, at 125 to 150 nm$^3$ and 225 to 275 nm$^3$, as indicated by the arrows in Fig. 2 B). The expected volume of a heterodimer of Hrs and STAM, based on the amino acid compositions of the two proteins, is 162 nm$^3$. We conclude, therefore, that the two volume peaks correspond to heterodimers and heterotetramers of Hrs and STAM. We next examined mutant ESCRT-0 complexes, which harbor point mutations that impair the ability of either Hrs or STAM (or both) to associate with ubiquitin (12). Mutation of the DUIM (double ubiquitin interacting motif) within Hrs did not affect particle size or particle volume distribution as compared with wildtype ESCRT-0 (Fig. 2 C and D). Similarly, point mutations in the Vps27/Hrs/STAM (VHS) (W29A) and UIM domains of STAM failed to affect the assembly of ESCRT-0 on lipid bilayers, either alone or in combination with mutations in the Hrs DUIM domain (Fig. 2 E–H). Importantly, these data highlight that both wildtype and mutant ESCRT-0 complexes exhibit similar volume distributions, which are distinct from those calculated for VAMP2 and Ub-VAMP2.

To assess whether ESCRT-0 could interact with an integral membrane cargo in bilayers, we compared the volume distributions for ESCRT-0 and VAMP2 imaged together with the distributions for the proteins imaged separately.
volume distribution obtained for wildtype ESCRT-0 alone, we found that co-incubation of ESCRT-0 and VAMP2 reduced the frequency of particles exhibiting a volume between 100 to 300 nm$^3$ (heterodimers and heterotetramers of Hrs and STAM), whereas the number of particles with a volume below 100 nm$^3$ (VAMP2 alone) increased (Fig. 3, A and B). We also examined mutant forms of ESCRT-0, which contain mutations within its ubiquitin binding domains, and obtained similar results (Fig. 3, C–H). Together, these data strongly suggest that ESCRT-0 does not bind to VAMP2.

In contrast to studies using unmodified VAMP2, we observed a striking redistribution of particle volumes when we co-incubated wildtype ESCRT-0 with Ub-VAMP2 (Fig. 4, A and B). In particular, we found that the frequency of particles exhibiting a volume larger than 300 nm$^3$ (larger than ESCRT-0 heterotetramers or Ub-VAMP2) increased dramatically as compared with wildtype ESCRT-0 alone. These data strongly suggest that ESCRT-0 binds directly to Ub-VAMP2 in lipid bilayers to form stable complexes.

To determine whether the association between ESCRT-0 and Ub-VAMP2 was dependent on the presence of its multiple ubiquitin binding domains, we took advantage of ESCRT-0 complexes harboring point mutations in the Hrs DUIM domain, the STAM VHS domain, and the STAM UIM domain. The DUIM domain of Hrs exhibits the strongest affinity for ubiquitin (~130 μM) (12). In contrast to the wildtype complex, ESCRT-0 harboring point mutations in the DUIM domain of Hrs failed to promote the formation of enlarged particles when mixed with Ub-VAMP2 (Fig. 4, C and D). These data indicate that the capture of monoubiquitin-modified cargoes by ESCRT-0 requires the DUIM domain of Hrs. We also tested the impact of mutations in the VHS and UIM domains of STAM, which exhibit affinities of ~280 and 550 μM for monoubiquitin, respectively (12). Interestingly, the mutant complex was not able to bind stably to Ub-VAMP2, as reflected by the absence of particles larger than 300 nm$^3$ (Fig. 4, E and F). As expected, based on these data, mutant ESCRT-0 harboring mutations in all ubiquitin-binding sites also failed to bind to Ub-VAMP2 (Fig. 4, G and H). Taken together, these data indicate that ubiquitin binding sites in Hrs and STAM function together to facilitate stable, ubiquitin-dependent cargo binding.

**ESCRT-I and ESCRT-II bind to immobilized ubiquitin but are unable to stably associate with an integral membrane, ubiquitin-modified substrate**

In addition to regulating ubiquitin-dependent trafficking to the lysosome, ESCRT-0 also recruits the ESCRT-I complex to endosomal membranes. ESCRT-I is composed of four distinct subunits, which co-assemble with a 1:1:1:1 stoichiometry (2). The combined molecular mass of the complex is 126 kDa, giving an expected molecular volume of 153 nm$^3$. In all organisms, including *C. elegans*, at least one subunit of the complex harbors a ubiquitin-binding domain (Tsg101). Although ESCRT-I binds only weakly to membranes (2), we were able to visualize its accumulation by AFM, even in the absence of ESCRT-0 (Fig. 5 A). We found that ESCRT-I exhibited a peak molecular volume of ~150 to 200 nm$^3$ when imaged on a membrane surface (Fig. 5 B). These data are consistent with the formation of a 1:1:1:1 heterotetramer and indicate that ESCRT-I does not undergo further oligomerization when bound to a lipid bilayer. Upon addition of ESCRT-I to bilayers containing VAMP2, we observed a downward shift in the particle volume distribution, indicating that VAMP2 did not bind to ESCRT-I.
A similar downward redistribution of particles was observed when ESCRT-I was incubated with Ub-V AMP2 (Fig. 5, E and F). To confirm that the intact ESCRT-I complex can bind to ubiquitin, we immobilized GST alone or a mixture of mono- and di-ubiquitin fused to GST on glutathione agarose beads, and incubated them with purified ESCRT complexes (Fig. 6 A). We found that ESCRT-I exhibited preferential binding to beads harboring GST-tagged ubiquitin as compared with GST alone, as observed for ESCRT-0 components (Fig. 6 B). Together, these data strongly suggest that ESCRT-I is incapable of binding stably to an integral membrane cargo modified only by mono-ubiquitin.

The ESCRT-II complex acts downstream of ESCRT-I and harbors three distinct subunits, Vps22, Vps25, and Vps36, which assemble with a 1:2:1 stoichiometry in solution. The combined molecular mass of this complex is 116 kDa, giving an expected molecular volume of 140 nm³. The Vps36 subunit harbors a single ubiquitin interacting motif within its amino terminus. Using a GST pull-down assay, we confirmed that intact *C. elegans* ESCRT-II binds directly to ubiquitin (Fig. 6 B). Furthermore, we found that ESCRT-II assembles on lipid bilayers using AFM, exhibiting a peak molecular volume of ~200 nm³ in the presence of 54% PC, 30% PE, 15% PS, and 1% PI3P, consistent with our previous work (24; see also Fig. 7, A and B). The measured volume is somewhat higher than the expected volume; nevertheless, because of the caveats associated with volume measurement by AFM, we suggest that the volume peak represents a single ESCRT-II complex. In contrast to our results with ESCRT-0, we found that neither the addition of VAMP2 or Ub-VAMP2 resulted in an upward shift in particle

![Figure 4](image_url) ESCRT-0 associates stably with ubiquitin-conjugated VAMP2. (A, C, E, and G) Representative AFM images of bilayers assembled in the presence of Ub-V AMP2 and either (A) wildtype ESCRT-0, (C) ESCRT-0 harboring point mutations in the DUIM domain of Hrs, (E) ESCRT-0 harboring two-point mutations in the VHS and UIM domains of STAM, or (G) ESCRT-0 harboring point mutations in all ubiquitin-binding domains. A shade-height scale bar is shown. Scale bar = 200 nm. (B, D, F, and H) The frequency distributions of molecular volumes for particles observed by AFM are shown. Each graph indicates the form of ESCRT-0 visualized together with Ub-VAMP2.

![Figure 5](image_url) ESCRT-I does not associate stably with VAMP2 or ubiquitin-conjugated VAMP2. (A, C, and E) Representative AFM images of bilayers assembled in the presence of either (A) ESCRT-I alone, (C) ESCRT-I together with VAMP2, or (E) ESCRT-I together with Ub-VAMP2. A shade-height scale bar is shown. Scale bar = 200 nm. (B, D, and F) The frequency distributions of molecular volumes for particles observed by AFM are shown. Each graph indicates the proteins present in the experiment. In panel B, a representative Coomassie stained gel of recombinant ESCRT-I is also shown. The molecular mass markers are indicated by dashes on the right. From top to bottom, the markers have masses of 50, 37, 25, and 20, and 10 kDa. The ESCRT-I components, Tsg101, Mvb12, Vps37, and Vps28, are highlighted.
These data indicate that ESCRT-II is not able to bind stably to integral membrane proteins, irrespective of the presence of ubiquitin. Together, our findings highlight the specific ability of ESCRT-0 to bind and sequester integral membrane proteins in a manner dependent on the presence of ubiquitin.

**DISCUSSION**

Modification of many transmembrane proteins by ubiquitin conjugation serves as a sorting signal for transport into the lumen of multivesicular endosomes. Unlike proteasomal targeting, which typically utilizes polyubiquitin chains, conjugation of one or a few ubiquitin moieties is sufficient for entry into ILVs (28). In mammalian cells, the E3 ubiquitin ligase c-Cbl modifies several cell surface receptors, including activated epidermal growth factor receptor, and mutations in c-Cbl that impair its ability to ubiquitin-modify substrates have been linked to oncogenic transformation (29). Similarly, numerous mutations that perturb transport through the endocytic system are also associated with cancer, potentially by affecting receptor degradation (30). Together, these data underscore a need to better understand the mechanisms that regulate ubiquitin-dependent receptor sorting.

Mechanisms by which the ESCRT machinery handles ubiquitin-modified cargoes have long been debated. In one widely cited model, ESCRT-0 delivers substrates directly to ESCRT-I and/or ESCRT-II (31). However, structural and thermodynamic studies fail to support this hand-off scenario (32). Additionally, our data do not support the possibility that ESCRT-0 transfers cargoes to other complexes in the pathway, as neither ESCRT-I nor ESCRT-II was capable of stably binding a mono-ubiquitin-modified...
substrate. Alternatively, ESCRT-0 may act as a central nucleating factor, sequestering ubiquitin-modified cargoes within subdomains on endosomal membranes, and subsequently aiding the recruitment of downstream ESCRT complexes (12,32). In this model, eventual release of ESCRT-0 from the site of vesicle formation would require coordination with the internalization of cargoes into ILVs, which may involve ubiquitin-binding domains in ESCRT-I and/or ESCRT-II to restrict cargo diffusion. Further studies are necessary to validate such a model.

Although our studies indicate that ESCRT-II forms individual heterotetrameric complexes on membranes, previous work suggests that the lipid environment can influence ESCRT complex assembly. Specifically, the presence of 25 mol % cholesterol was shown to promote ESCRT-II clustering in vitro, even in the absence of its effector ESCRT-0 (33). On average, the presence of cholesterol caused ~50 ESCRT-II complexes to coalesce on a model membrane, and these clustered complexes were capable of binding to ubiquitin. This finding is consistent with our data that numerous low-affinity ubiquitin-binding domains must be present to stably recruit and sequester ubiquitin-modified cargoes. However, it remains unclear whether such a large number of ESCRT-II complexes assemble simultaneously on the endosome in vivo. Based on biochemical data and localization studies, the majority of ESCRT-II is found in the cytoplasm at steady state, making it an unlikely candidate to cluster cargoes before their deposition into ILVs (34). By contrast, ESCRT-0 binds to membranes stably, and our data indicate that its multiple ubiquitin-binding domains can bind cargo efficiently, unlike other ESCRT complexes. Together, our findings strongly suggest that ESCRT-0 acts upstream of ESCRT-I and ESCRT-II to bind ubiquitin-modified cargoes during multivesicular endosome biogenesis.

SUPPORTING MATERIAL

Seven figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)01185-0.

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