ESCRT-0 Assembles as a Heterotetrameric Complex on Membranes and Binds Multiple Ubiquitinylated Cargoes Simultaneously*

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The ESCRT machinery consists of multiple protein complexes that collectively participate in the biogenesis of multivesicular endosomes (MVEs). The ESCRT-0 complex is composed of two subunits, Hrs and STAM, both of which can engage ubiquitinylated substrates destined for lysosomal degradation. Here, we conduct a comprehensive analysis of ESCRT-0:ubiquitin interactions using isothermal titration calorimetry and define the affinity of each ubiquitin-binding domain (UBD) within the intact ESCRT-0 complex. Our data demonstrate that ubiquitin binding is non-cooperative between the ESCRT-0 UBDs. Additionally, our findings show that the affinity of the Hrs double ubiquitin interacting motif (DUIM) for ubiquitin is more than 2-fold greater than that of UBDs found in STAM, suggesting that Hrs functions as the major ubiquitin-binding protein in ESCRT-0. In vivo, Hrs and STAM localize to endosomal membranes. To study recombinant ESCRT-0 assembly on lipid bilayers, we used atomic force microscopy. Our data show that ESCRT-0 forms mostly heterodimers and heterotetramers of Hrs and STAM when analyzed in the presence of membranes. Consistent with these findings, hydrodynamic analysis of endogenous ESCRT-0 indicates that it exists largely as a heterotetrameric complex of its two subunits. Based on these data, we present a revised model for ESCRT-0 function in cargo recruitment and concentration at the endosome.

Many plasma membrane proteins, including hormone receptors, must be internalized and degraded to appropriately regulate their activities (1). Defects in receptor turnover have been shown to result in constitutive cell signaling that can lead to disease (2). The down-regulation of cell surface proteins can be initiated by their ubiquitinylation, which allows for their recognition by members of a group of proteins collectively referred to as the ESCRT (endosomal sorting complex required for transport) machinery (3). Ubiquitinylation of membrane proteins occurs at both the plasma membrane and at early endosomes, which ultimately leads to their incorporation into multivesicular endosomes (MVEs) and degradation within acidified lysosomal compartments (4, 5). A subset of the ESCRT machinery selectively binds the ubiquitin modification on membrane proteins destined for degradation using a variety of ubiquitin-binding domains (UBDs; 6–8). The relative contributions of each UBD remain poorly defined, as do the overall functions of individual proteins within the ESCRT complexes.

The ESCRT machinery consists of five multi-subunit complexes, three of which have been shown to contain UBDs: ESCRT-0, ESCRT-I, and ESCRT-II (9). In metazoans, ESCRT-0 is currently the only ESCRT complex known to contain more than one ubiquitin binding site (10, 11). ESCRT-0 is composed of two proteins, Hrs and STAM, which form a 1:1 complex in solution (12). Hrs contains two well-characterized ubiquitin binding sites, both of which reside within its double ubiquitin interacting motif (DUIM) (11). STAM also contains two ubiquitin-binding sites, a Vps27/Hrs/STAM (VHS) domain and a ubiquitin interacting motif (UIM) (10). Thus, ESCRT-0 is believed to be capable of binding at least 4 ubiquitin moieties simultaneously. The binding affinities of individual UBDs in ESCRT-0 have been determined. In each case, indirect thermodynamic measurements were obtained using surface plasmon resonance (SPR), and most studies only used isolated domains from Hrs and STAM for their analyses (11–13). Therefore, the relative contributions of each UBD within the intact ESCRT-0 complex remain undefined.

In cells, both Hrs and STAM localize predominantly to endosomal membranes (5). Their association with endosomes is mediated by an interaction between the Hrs FYVE (Fab1/YOTB/Vacl/EAA1) domain and phosphatidylinositol 3-phosphate (PI3P), which is enriched there. In cells depleted of PI3P, neither Hrs nor STAM efficiently localizes to endosomes (14). Additionally, the stability of STAM depends on the presence of Hrs, suggesting that Hrs and STAM are constitutively bound together in vivo (15, 16). Although studies indicate that recombinant Hrs and STAM bind to one another in a 1:1 complex in solution (12), it remains unclear whether their association with membranes may affect ESCRT-0 assembly in vivo. Since the complex contains multiple UBDs that are each capable of interacting with a ubiquitinylated cargo, determining the stoichiometry of these interactions will be important to understanding the assembly and function of the ESCRT-0 complex.

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³The abbreviations used are: ESCRT, endosomal sorting complex required for transport; DUIM, double ubiquitin interacting motif; ILV, intralumenal vesicle; ITC, isothermal titration calorimetry; MVE, multivesicular endosome; SPR, surface plasmon resonance; UBD, ubiquitin binding domain; UIM, ubiquitin interacting motif; VHS, Vps27/Hrs/STAM.
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In this study, we conduct a comprehensive analysis of ESCR-T-0:ubiquitin interactions using isothermal titration calorimetry (ITC) and define the binding affinity of each UBD within the intact ESCR-T-0 complex. We further show that recombinant Hrs and STAM can form a heterotetrameric complex on membranes in vitro. Consistent with these findings, we demonstrate that endogenous ESCR-T-0 also assembles as a 2:2 complex of Hrs and STAM. Together, our data suggest that ESCR-T-0 can associate simultaneously with as many as 8 unique ubiquitin modifications in vivo, which may enhance cargo clustering during MVE biogenesis.

EXPERIMENTAL PROCEDURES

Recombinant Protein Purification—Hrs and STAM were amplified from a Caenorhabditis elegans cDNA library and cloned into the pRSETa vector that was modified for polycistronic expression. A polyhistidine tag was appended onto the amino terminus of Hrs to enable purification using nickel affinity chromatography. Protein expression was conducted in BL21 (DE3) cells induced with 0.1 mM IPTG for 3 h at 25 °C. Cells were harvested and resuspended in lysis buffer (50 mM Na phosphate (pH 8), 300 mM NaCl, 10 mM imidazole, 0.15% Tween-20, 5 mM β-mercaptoethanol) prior to sonication. Clarified lysates were incubated with 1 ml of Ni-NTA-agarose (Qiagen) for 1 h. The resin was washed with 200 ml of lysis buffer containing 0.10% Tween-20 and protease inhibitors. Proteins were eluted into elution buffer (50 mM Hepes (pH 7.6), 100 mM KCl, 1 mM EDTA, and 250 mM imidazole) and applied to a Superose 6 gel filtration column (GE Healthcare Life Sciences) equilibrated in elution buffer lacking imidazole. For ITC experiments, peak fractions were pooled and dialyzed overnight in 50 mM Hepes (pH 7.6), 100 mM KCl, and 1 mM EDTA.

Worm Strains and Preparation of Extracts—To prepare C. elegans embryo extracts, gravid adult hermaphrodites were grown in liquid culture, harvested, and treated with a 1:2 solution of 5 N sodium hydroxide and 6% sodium hypochlorite. Embryos isolated following this procedure were frozen in lysis buffer (50 mM Na phosphate (pH 7.6), 100 mM KCl, 1 mM EDTA, and 250 mM imidazole) and applied to a Superose 6 gel filtration column (GE Healthcare Life Sciences) equilibrated in elution buffer lacking imidazole. For ITC experiments, peak fractions were pooled and dialyzed overnight in 50 mM Hepes (pH 7.6), 100 mM KCl, and 1 mM EDTA.

Antibody Production and Site-directed Mutagenesis—C. elegans Hrs and STAM antibodies were raised in rabbits by immunization (Covance) with polyhistidine tagged forms of full length Hrs (C07G1.5) or STAM (C34G6.7) produced in Escherichia coli. For VPS-25 antibodies, a purified complex of ESCR-T-11 was used as the antigen. All antibodies were affinity purified from serum by binding to columns of the same antigens. Antibodies directed against EEA-1 have been described previously (19). Point mutations were generated using an overlap extension PCR technique and confirmed by gene sequencing.

Hydrodynamic Studies—Glycerol gradients (10–30%) were poured using a Gradient Master and centrifuged for 8 h following the application of a sample (100 µl) to the top of the gradient (4 ml). Fractions (200 µl) were collected by hand and analyzed via SDS-PAGE or Western blot. Gel filtration chromatography was performed using a Superose 6 gel filtration column, and 1 ml samples were collected for analysis. Standards with known sedimentation values and Stokes radii were processed similarly to generate standard curves, from which the hydrodynamic properties of samples were derived. To calculate the native molecular weight of proteins or protein complexes, the following equation was used: M = 6πηN/(1−η), where M is the native molecular weight, η is the viscosity of the medium, N is Avogadro’s number, a is the Stokes radius, s is the sedimentation value, ν is the partial specific volume, and ρ is the density of the medium (20).

Immunofluorescence and Atomic Force Microscopy—For immunofluorescence studies, embryos were fixed in cold methanol and stained using directly labeled polyclonal rabbit antibodies at a concentration of 1 µg/ml. Images were acquired on a swept field confocal microscope (Nikon Ti-E) equipped with a Roper CoolSnap HQ2 CCD camera using a Nikon 60×, 1.4NA Planapo oil objective lens. Acquisition parameters were controlled by Nikon Elements software. For each sample, 11 Z sections at 0.2 µm steps were acquired and used to generate a maximum intensity projection (2-µm thick). For studies using atomic force microscopy (AFM), liposomes containing phosphatidylcholine (PC, 54%), phosphatidylethanolamine (PE, 30%), phosphatidyserine (PS, 15%), and PI3P (1%) were adsorbed onto freshly cleaved mica in the presence or absence of recombinant ESCR-T-0. Imaging was performed using a Veeco Digital Instruments Multimode instrument controlled by a Nanoscope IIIa controller at room temperature. AFM images were plane-fitted to remove tilt, and each scan line was fitted to a first order equation. Particles were identified, and their dimensions were measured manually using the section tool. Molecular volumes were calculated based on the height and radius of each particle.

Isothermal Titration Calorimetry—All ITC experiments were performed on a Microcal ITC 200 calorimeter. Binding isotherms were recorded following 1-µl injections of ubiquitin (8 mM) into a cell containing recombinant ESCR-T-0 (~20 µM). Each experiment consisted of 38 injections performed at 2.5 min intervals. Background (injections of ubiquitin into a cell containing buffer alone) was subtracted from the binding isotherms and data were analyzed using a nonlinear least square program (Origin, MicroCal). Curve fitting with a one-site model yielded the binding constant (Kd), free energy change, enthalpy change, and entropy change, and dissociation constants were calculated as 1/Kd.
**ESCR-T-0 Assembly on Membranes and Interactions with Ubiquitin**

**RESULTS**

_C. elegans ESCR-T-0 Forms a 1:1 Complex of Hrs and STAM in Vitro—_The _C. elegans_ genome encodes single homologs of the human ESCR-T subunits, which we refer to as Hrs (C07G1.5) and STAM (C34G6.7). To determine whether Hrs and STAM form a complex in _C. elegans_, we generated affinity-purified antibodies directed against each protein and conducted a series of immunoprecipitation experiments. We found that both Hrs and STAM were recovered regardless of which antibodies were used, indicating that the proteins associate with one another (Fig. 1A). In contrast, neither component of ESCR-T-0 was recovered in an immunoprecipitation for the ESCR-T-II subunit Vps-25, suggesting that the interaction between Hrs and STAM is specific.

To determine whether any additional proteins constitutively associate with Hrs and STAM in _C. elegans_ extracts, we separated anti-STAM immunoprecipitates by SDS-PAGE and silver-stained the gel. Under a variety of salt concentrations ranging from 200–500 mM KCl, we observed bands corresponding to Hrs and STAM (Fig. 1B and data not shown). However, we failed to detect any additional proteins at similar levels of recovery. We therefore conclude that the ESCR-T-0 complex is composed of only Hrs and STAM in _C. elegans_ and that their association is resistant to disruption by relatively high concentrations of salt.

Previous reports have demonstrated that recombinant human ESCR-T-0 is composed of a 1:1 heterodimer of Hrs and STAM (12). Compared with globular standards, human ESCR-T-0 exhibits an aberrant migration profile during gel filtration chromatography that is consistent with its adopting an elongated conformation. We set out to determine whether the _C. elegans_ ESCR-T-0 complex assembles similarly to its human counterpart. _C. elegans_ Hrs and STAM were co-expressed using a polycistronic expression system in _E. coli_. A polyhistidine tag was appended onto the amino terminus of Hrs to enable purification using nickel affinity chromatography. Consistent with our finding that endogenous Hrs and STAM interact, we found that the recombinant proteins co-purify from nickel resin, even though Hrs alone harbors an affinity tag (Fig. 1C).

The hydrodynamic properties of the purified complex were analyzed by gel filtration chromatography and sedimentation through a glycerol gradient. In both cases, Hrs and STAM co-migrated as a single complex with a Stokes radius of ~7.05 nm and a sedimentation value of ~4.7 S (Fig. 1, C and D). By combining these values, we obtained an accurate measure of their native molecular mass (~139 kDa), which was similar to that of the predicted molecular weight of a 1:1 heterodimer of Hrs and STAM (134 kDa).

During ESCR-T-0 purification, we found two major contaminants consistently co-purified with Hrs and STAM following nickel affinity purification, gel filtration chromatography, and density gradient fractionation. Using mass spectrometry, both were identified as bacterial heat shock proteins (data not shown). However, based on the calculated native molecular weight of ESCR-T-0, the heat shock proteins did not appear to be directly bound to Hrs nor STAM. Instead, they were simply found in the same fractions during hydrodynamic analysis. Consistent with this idea, we found that the heat shock proteins fractionate similarly to ESCR-T-0 during gel filtration chromatography even when they are co-purified with other polyhistidine-tagged proteins (supplemental Fig. S1). We conclude that recombinant _C. elegans_ Hrs and STAM bind directly to one another with a 1:1 stoichiometry in solution, and similar to their human counterparts, form a complex with an elongated shape.

**Point Mutations in the UBDs of Hrs and STAM Do Not Affect ESCR-T-0 Assembly—_Mutations within the UBDs of Hrs and STAM Do Not Affect ESCR-T-0 Assembly—_Mutations within the UBDs of Hrs and STAM that inhibit their ability to associate with ubiquitin have been defined (11, 13, 21). To determine whether these point mutations affect the conformation of ESCR-T-0, we introduced them both individually and simultaneously into the ESCR-T-0 polycistronic expression construct. Specifically, mutations...
were made in the DUIM of Hrs (A263Q and A265Q), the UIM of STAM (A181Q and S185A), and the VHS domain of STAM (W29A) (Fig. 2A). In each case, the mutant complexes appeared stable and exhibited a similar Stokes radius to that of wild-type ESCRT-0 (Fig. 2, B and C). Additionally, deletion of the entire STAM VHS domain failed to disrupt ESCRT-0 assembly, consistent with the established role of the coiled-coil domains within Hrs and STAM mediating their association (Fig. 2B; 12, 22, 23). Our data indicate that mutations in ESCRT-0 that inhibit UBD:ubiquitin interactions do not significantly affect the conformation nor the assembly of the ESCRT-0 complex in solution.

Ubiquitin Binding Is Non-cooperative between ESCRT-0 UBDs—In nearly all cases reported, the affinities of UBDs identified within ESCRT subunits have been determined using indirect measurements of protein association, such as surface plasmon resonance (SPR) (11–13). Although valuable, SPR is susceptible to several artifacts, including mass transport effects, surface concentration, and problems caused by the immobilization of one binding partner onto a chip (24). In addition, the affinities of each UBD in the context of full-length ESCRT complexes have yet to be determined, prohibiting a direct comparison of their individual ubiquitin binding properties. Taking advantage of our ability to purify micromolar quantities of ESCRT-0 from bacteria, we used isothermal titration calorimetry (ITC) to determine the binding affinity between ESCRT-0 and ubiquitin. ITC offers several advantages over other binding studies (25). First, this technique uses an equilibrium solution, in which the proteins studied need not be labeled or adhered to a surface. Secondly, ITC is not affected by protein size or the optical properties of the sample. Finally, ITC can uniquely determine binding affinity, enthalpy, and entropy in a single experiment (26).

We titrated a concentrated solution of ubiquitin into a calorimetry cell containing ESCRT-0. Upon each titration, heat was released, and a binding curve was generated, from which we
determined the binding affinity between ESCRT-0 and ubiquitin (307 ± 14.5 μM) (Fig. 3A). To dissect the contributions of the individual UBDs within ESCRT-0, we examined the panel of mutants described above. Mutations that inactivate both STAM UBDs caused ESCRT-0 to bind ubiquitin more avidly (127 ± 3.5 μM), indicating that the Hrs DUIM binds ubiquitin more tightly than either of the STAM UBDs (Fig. 3B). Consistent with this idea, we found that the STAM UIM and VHS domains bound ubiquitin with affinities of 278 ± 20 μM and 549 ± 22 μM, respectively (Fig. 3, C and D; Table 1). An ESCRT-0 complex mutated at all four UBDs failed to exhibit detectable binding to ubiquitin (Table 1; supplemental Fig. S2, A and B). These data indicate that the overall binding affinity of ESCRT-0 for ubiquitin is an average of the affinities between each UBD and ubiquitin, demonstrating that individual ubiquitin molecules do not bind cooperatively within the complex.

**TABLE 1**

<table>
<thead>
<tr>
<th>ESCRT-0 complex</th>
<th>$K_d$ (μM)</th>
<th>Site(s) that bind Ub</th>
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<tbody>
<tr>
<td>STAM; Hrs</td>
<td>307 ± 14.5</td>
<td>VHS, UIM, DUIM (Hrs and STAM)</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;VHS,UIM&lt;/sup&gt;; Hrs&lt;sup&gt;DUIM&lt;/sup&gt;</td>
<td>No binding</td>
<td>None</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;VHS,UIM&lt;/sup&gt;; Hrs</td>
<td>127 ± 3.46</td>
<td>DUIM (Hrs)</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;VHS&lt;/sup&gt;; Hrs&lt;sup&gt;DUIM&lt;/sup&gt;</td>
<td>278 ± 20.1</td>
<td>UIM (STAM)</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;UIM&lt;/sup&gt;; Hrs&lt;sup&gt;DUIM&lt;/sup&gt;</td>
<td>485 ± 38.6</td>
<td>VHS and UIM (STAM)</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;UIM&lt;/sup&gt;; Hrs&lt;sup&gt;DUIM&lt;/sup&gt;</td>
<td>549 ± 22.0</td>
<td>VHS (STAM)</td>
</tr>
<tr>
<td>STAM&lt;sup&gt;V296A,UIM&lt;/sup&gt;; Hrs&lt;sup&gt;DUIM&lt;/sup&gt;</td>
<td>No binding</td>
<td>None</td>
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Furthermore, our data indicate that additional ubiquitin binding sites within ESCRT-0 are unlikely to exist, and that a heterodimer of Hrs and STAM can bind at most a total of 4 ubiquitin molecules.

**ESCRT-0 Undergoes Oligomerization When Bound to Membranes**—The ESCRT-0 complex functions at the endosomal membrane during cargo sorting in the MVE pathway. Using affinity-purified antibodies directed against Hrs and STAM, we confirmed that both proteins largely co-localize with the early endosomal marker EEA-1 in *C. elegans* embryos (Fig. 4A and data not shown). To study the assembly of ESCRT-0 on membranes where it functions *in vivo*, we used atomic force microscopy (AFM). Recombinant ESCRT-0, consisting of a 1:1 complex of Hrs and STAM, was incubated with unilamellar liposomes, which were adsorbed onto a mica surface to form a supported lipid bilayer. AFM demonstrated that individual particles of ESCRT-0 were distributed evenly throughout the bilayer (Fig. 4B). Upon closer examination of the particles, we

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**FIGURE 4. ESCRT-0 can undergo oligomerization on lipid bilayers.** A, control embryo fixed at the one-cell stage was stained with Cy2-labeled α-EEA-1 (top right) and Cy3-labeled α-Hrs antibodies (top left) (n = 12). Images are maximum intensity projections of 3D confocal datasets acquired over 2 μm. Bar, 10 μm. A color overlay of a higher magnification view of a portion of the embryo is shown below. Hrs staining is shown in red, and EEA-1 staining is shown in green. Bar, 2 μm. B, representative AFM images of bilayers composed of phosphatidylcholine (54%), phosphatidyethanolamine (30%), phosphatidylserine (15%), and phosphatidylinositol 3-phosphate (1%) assembled in the presence of ESCRT-0 (150 nM). A shade-height scale bar is shown on the right. Bar, 200 nm. C, an 8-fold magnification of the boxed region in B. Bar, 25 nm. D, analysis of the height distributions along the color-coded lines shown in C. A blue line was drawn over a particle with an approximate volume of 150 nm³, and a red line was drawn over a particle with an approximate volume of 300 nm³. E, frequency distribution of molecular volumes for the ESCRT-0 complex bound to the bilayer surface. Total number of particles analyzed is indicated. Two major peaks are observed, which are highlighted by color-coded arrowheads.
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FIGURE 5. ESCRT-0 assembles as a heterotetramer of Hrs and STAM. A and B: Western blots of wild-type (N2) or stamΔ (ok406) C. elegans embryo extracts prepared in the presence of 100 mKCl and fractionated over a Superose 6 gel filtration column (B) or a 10–30% glycerol gradient (C). Stokes radii and sedimentation values were calculated for Hrs based on comparison with the elution profiles of characterized standards (n = 2 each). C. Western blots of extracts prepared from wild-type and stam(ok406) mutant animals (n = 2 each). Serial dilutions of extracts prepared from control animals were loaded to quantify levels of each protein in the absence of STAM.

found that their volumes varied significantly (Fig. 4, C–E). Analysis of 278 particles in multiple experiments revealed two major peaks in the volume distribution, the first between 100–150 nm$^3$ and a second that was approximately twice as large (between 250–300 nm$^3$) (Fig. 4E). Based upon amino acid composition, a 1:1 heterodimer of Hrs and STAM is predicted to have a molecular volume of 162.3 nm$^3$, which is similar to that of the first peak. By extrapolation, the second peak likely corresponds to a heterotetramer of Hrs and STAM, which forms when ESCRT-0 associates with membranes. In addition, we observed that a significant percentage of the remaining particles exhibited even larger volumes, potentially corresponding to oligomers of Hrs and STAM (Fig. 4E). These data suggest that ESCRT-0 can oligomerize when bound to membranes.

ESCRT-0 Is a Heterotetramer of Hrs and STAM in Vivo—To determine whether ESCRT-0 is composed of multiple copies of Hrs and STAM in vivo, we performed a series of hydrodynamic studies on the native complex. Extracts generated from whole animals were separated by gel filtration chromatography, which provides information regarding the size and shape of a molecule. Similar to the recombinant ESCRT-0 complex, native Hrs and STAM each exhibited a Stokes radius of ~7.5 nm, indicating that ESCRT-0 also adopts an elongated conformation in vivo (Fig. 5A, top). To determine the sedimentation value of endogenous ESCRT-0, C. elegans extract was separated on a glycerol gradient and fractions were immunoblotted using Hrs antibodies. These studies demonstrated that ESCRT-0 exhibits a sedimentation value of 9.9 S, significantly larger than that observed of the recombinant complex (Fig. 5B, top; compare with Fig. 1D). Moreover, based on these findings, Hrs and STAM assemble into a ~310 kDa complex in vivo, similar to the size predicted for a 2:2 heterotetramer of Hrs and STAM.

To further substantiate our finding that Hrs and STAM form a 2:2 complex in vivo, we examined mutant animals harboring a deletion in STAM (ok406). Although animals lacking STAM exhibit a high level of embryonic lethality, ~10% are viable and grow to adulthood. In the absence of STAM, Hrs protein levels were reduced ~2-fold, suggesting that the stability of Hrs is partially dependent on its association with STAM (Fig. 5C). However, based upon gel filtration chromatography, Hrs retains an elongated conformation, exhibiting a Stokes radius of 6.5 nm, regardless of whether STAM is present (Fig. 5A, bottom). When stamΔ mutant extract was separated on a glycerol gradient, we found that Hrs exhibited a reduced S-value (7.1 S) as compared with control extract (Fig. 5B, bottom). By combining the Stokes radius and sedimentation value of Hrs in the absence of STAM, we found that Hrs assembles into a ~200 kDa complex. The difference in molecular weight between intact ESCRT-0 and ESCRT-0 lacking STAM was equivalent to two molecules of STAM, consistent with our finding that native ESCRT-0 is composed of a 2:2 heterotetrameric complex of Hrs and STAM. Additionally, these data strongly suggest that Hrs can self-associate to form a ~200 kDa dimer in vivo without STAM. Taken together, our findings indicate that Hrs can bind to both itself and STAM to mediate the formation of a heterotetrameric complex.

To characterize the assembly of endogenous ESCRT-0 further, we also treated wild-type C. elegans extracts with varying concentrations of salt (200–500 mM) and conducted a series of hydrodynamic studies. As demonstrated earlier, we failed to detect any changes in the composition of immunoprecipitated ESCRT-0 under these different conditions (see Fig. 1B). However, in the presence of 300 or 500 mM KCl, we found that the sedimentation value of endogenous ESCRT-0 dramatically decreased and became similar to that of recombinant ESCRT-0 purified in solution (Fig. 6A). In contrast, gel filtration analysis indicated that ESCRT-0 retained its elongated configuration, exhibiting a Stokes radius of 7.2 nm under these conditions (Fig. 6B). These data strongly suggest that elevated salt concentrations are sufficient to disrupt the heterotetrameric assembly of Hrs and STAM, but fail to perturb the conformation of the 1:1 ESCRT-0 complex. Taken together with our results using AFM, we speculate that formation of the ESCRT-0 heterotetramer likely requires an initial association with membranes, which may facilitate homotypic interactions between Hrs, leading to ESCRT-0 oligomerization.

DISCUSSION

Distinct from other components of the ESCRT machinery, ESCRT-0, -I, and -II have been shown to form heteropolymorphic complexes that assemble in solution (9). Each complex possesses a defined role in the early stages of MVE biogenesis, but
with affinities ranging from 190–450 nM. The DUIM of human Hrs has been shown to bind ubiquitin. However, this approach has led to contradictory findings. In a previous study, analysis of the Hrs VHS domain revealed extremely weak binding to ubiquitin ($K_d \approx 14$ mM), while another study demonstrated that binding to ubiquitin was not detectable to an identical region (12, 37). Such variation may be a consequence of the methodology used. We took advantage of an alternative approach that measures affinity between two proteins in solution. Moreover, we conducted our studies using isolated salt concentrations.

The interaction profiles of characterized standards.

**FIGURE 6. Assembly of the ESCRT-0 heterotetramer is sensitive to elevated salt concentrations.** A, Western blots of wild-type (N2) *C. elegans* embryo extracts prepared in the presence of 200–500 mM KCl and fractionated over a 10–30% glycerol gradient. B, Western blot of a wild-type (N2) *C. elegans* embryo extract prepared in the presence of 500 mM KCl and fractionated over a Superose 6 gel filtration column. The Stokes radius and sedimentation values were calculated for Hrs based on comparison with the elution profiles of characterized standards.

comparatively little is known about the relative contributions of individual subunits within the complexes. Here, we focused on ESCRT-0 and identified unique properties of its two components, Hrs and STAM. Using a thermodynamic approach, we defined the affinities of each UBD within intact ESCRT-0, demonstrating that the Hrs DUIM exhibits at least a 2-fold stronger affinity for ubiquitin as compared with UBDs found in STAM. These data suggest that Hrs is the major ubiquitin binding protein in ESCRT-0, and that STAM likely plays an accessory role in binding ubiquitylated cargoes. Additionally, we provide surprising new evidence that ESCRT-0 is composed of a 2:2 complex of Hrs and STAM in vivo, and association with membranes appears to play an important role in forming the heterotetrameric complex. Together, our studies suggest a new model for the role of ESCRT-0 in endocytic protein trafficking to the lysosome.

**Hrs and STAM Contribute to the Binding of Ubiquitylated Cargoes in Distinct Fashions**—Several studies have documented the affinities between ESCRT-0 UBDs and ubiquitin. In most cases, SPR has been used, and domains alone were analyzed. However, this approach has led to contradictory findings. The DUIM of human Hrs has been shown to bind ubiquitin with affinities ranging from 190–450 μM (11, 27), and studies of the STAM UIM have been similarly inconsistent (13, 27). In one case, analysis of the Hrs VHS domain revealed extremely weak binding to ubiquitin ($K_d = 1.4$ mM), while another study demonstrated that binding to ubiquitin was not detectable to an identical region (12, 37). Such variation may be a consequence of the methodology used. We took advantage of an alternative approach that measures affinity between two proteins in solution at equilibrium. Moreover, we conducted our studies using the intact ESCRT-0 complex instead of individual domains that may exhibit atypical properties in isolation. Based on our findings, we propose that Hrs plays a more significant role than STAM in binding mono- and poly-ubiquitylated substrates destined for lysosomal degradation, given its greater affinity for ubiquitin. In contrast, the weaker affinity between the STAM UBDs and ubiquitin suggests that STAM functions as an accessory protein, which may play a critical role in the binding of some poly-ubiquitylated substrates during their down-regulation (27). Consistent with this idea, loss of STAM function is typically better tolerated than loss of Hrs (28, 29). In *C. elegans* for example, deletion of Hrs is lethal, while animals lacking STAM are viable.

Our findings also establish that recombinant ESCRT-0 can bind up to 4 ubiquitin molecules simultaneously, and no additional UBDs await characterization in this complex. In contrast, additional UBDs may be present in other metazoan ESCRT complexes. Based on recent evidence that yeast ESCRT-1 harbors an addition UBD in its Mvb12p subunit (30), it is possible that additional UBDs await characterization in other ESCRT complexes. Analysis of the interactions between intact ESCRT-1 or -II and ubiquitin will be necessary to ultimately resolve this issue.

**ESCRT-0 Undergoes Oligomerization When Bound to Membranes**—Our findings indicate that recombinant *C. elegans* ESCRT-0, like human ESCRT-0, forms a 1:1 complex of Hrs and STAM in solution. However, the majority of ESCRT-0 localizes on endosomal membranes in vivo. Using AFM, we found that recombinant ESCRT-0 can undergo oligomerization when bound to membranes, with equal fractions of the complex assembling as 1:1 heterodimers and 2:2 heterotrimers. Although 2 bacterial heat shock proteins were present during our purification of ESCRT-0, neither contaminant bound to membranes *in vitro* (supplemental Fig. S3), strongly suggesting that Hrs and STAM were the only proteins associated with the supported lipid bilayer during our AFM studies.

To confirm that ESCRT-0 can assemble as a heterotetramer of Hrs and STAM, we examined the endogenous complex and found its native molecular mass is $\sim 310$ kDa, consistent with the formation of a 2:2 complex. However, previous studies indicate that both Hrs and STAM can associate with several other proteins, potentially accounting for the unexpectedly large mass of the complex. STAM has been shown to interact with multiple deubiquitinating enzymes and a ubiquitin ligase, which may help to regulate the sorting efficiency of cargoes into MVEs (31, 32). Hrs has been implicated in the recruitment of clathrin to MVEs, which likely functions to retain cargoes within microdomains of early endosomes (14, 33). In human cells, Hrs also interacts with Eps15b, an endosomally localized isoform of Eps15 that lacks EH domains and regulates receptor trafficking (34). In each of these cases, it remains unclear whether the interactions are transient or stable. Gel filtration profiles of the native and recombinant forms of human Hrs and STAM have been shown to be similar, suggesting that endogenous ESCRT-0 is composed of Hrs and STAM only (12). Our gel filtration studies using *C. elegans* Hrs and STAM isoforms confirmed these results. However, endogenous ESCRT-0 exhibited a significantly larger sedimentation value as compared with the recombinant complex, indicating that ESCRT-0 is not a 1:1 complex of Hrs and STAM in vivo. Instead, based on our data, we propose that ESCRT-0 contains two copies of each subunit. Consistent with this idea, we found that ESCRT-0 isolated from *stamΔ* mutant animals exhibited a loss of mass equivalent to two molecules of STAM. Additionally, these data indicate that in the absence of STAM, Hrs exists a dimer that is not.
capable of maintaining sufficient ESCRT-0 function for *C. elegans* viability. This finding is supported by previous work showing that the Hrs FYVE domain can undergo dimerization, leading to a higher affinity for PI3P in the endosomal membrane (35, 36).

Another study has recently suggested that recombinant Hrs purified in the absence of STAM may form hexamers, again supporting the idea that Hrs can self-associate (37). We failed to observe the formation of Hrs hexamers in extracts from *stam*Δ animals. However, our AFM studies suggest that ESCRT-0 can oligomerize on lipid bilayers. Homotypic binding of Hrs may facilitate the formation of endosomal microdomains that have been observed previously in human cells (33). These regions of the endosome membrane would likely exhibit enhanced ESCRT-I, -II, and -III assembly, ultimately driving MVE formation. Further studies are necessary to define the precise site(s) within Hrs that mediate self-association, to determine whether microdomain formation on endosomes requires Hrs multimerization.

**A Revised Model for Cargo Sorting during MVE Biogenesis**—Several models have been proposed to describe how the ESCRT machinery recruits ubiquitinylated cargoes for deposition into lumenal vesicles within the MVE (3). In the “conveyor belt model,” cargoes are passed from the ESCRT-0 complex sequentially to ESCRT-I and then ESCRT-II (3). Although each complex contains at least one subunit that can bind to ubiquitin, the low affinity interaction between ubiquitin and the UEV domain of the ESCRT-I subunit Tsg101 (510 μM) does not support the idea of cargo movement from ESCRT-0 (38). Studies in yeast suggest that UBDs within ESCRT-0, -I, and -II may cooperate to sort ubiquitinylated cargoes efficiently, potentially assembling into a “supercomplex” of ESCRT machinery (30, 39). However, it remains to be shown whether the early acting ESCRT complexes co-assemble into a larger complex on endosomal membranes. Based on our data, we propose an alternate model, in which ESCRT-0 functions as the primary, multivalent ubiquitin-binding factor during cargo transport into the MVE (Fig. 7). By undergoing oligomerization on the endosomal membrane, ESCRT-0 brings together several UBDs, with affinities as high as 127 μM, which are likely sufficient for cargo sequestration and concentration. Subsequently, ESCRT-I and ESCRT-II function in vesicle formation, while ESCRT-III is responsible for vesicle scission (40). Additional work will be required to fully dissect this pathway and determine exactly how cargoes associated with ESCRT-0 are released into lumenal vesicles for eventual degradation within the lysosome lumen.

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**REFERENCES**

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Heat shock proteins isolated during the purification of another polyhistidine-tagged protein exhibit a gel filtration elution profile similar to ESCRT-0. Polyhistidine-tagged VPS-32 was purified using nickel affinity chromatography. A Coomassie stained gel of the fractions isolated after separation on a Superose 6 gel filtration column is shown. Two bacterial heat shock proteins are found in the same fractions in which ESCRT-0 normally elutes during its purification.

Figure S2. Mutations in four UBDs within ESCRT-0 abolish binding to ubiquitin. (A and B) The binding kinetics between ubiquitin and mutant forms of ESCRT-0 were determined using isothermal titration calorimetry. The top panels correspond to the raw heat released upon each injection of ubiquitin (8 mM). The bottom panels show the binding isotherms that were generated directly from the raw heat data and fitted using the equivalent non-interacting sites model. The domain(s) deleted or mutated are indicated in superscripted text. The minor heat signals generated with each injection of ubiquitin are likely the result of non-specific binding between ubiquitin and ESCRT-0, since they do not saturate following the addition of high concentrations of ubiquitin over time.

Figure S3. Bacterial heat shock proteins that co-purify with ESCRT-0 do not interact with membranes. A co-flotation assay was used to determine whether the two bacterial heat shock proteins that co-purify with ESCRT-0 interact with membranes composed of phosphatidylcholine (52%), phosphatidylethanolamine (30%), phosphatidyserine (15%) and phosphatidylinositol 3-phosphate (3%). Neither protein is recovered in the membrane-associated fraction following flotation. The total amount of protein used in the assay is shown on the left, and lanes 1 and 2 show the total recovery following two flotation reactions done in parallel.

SUPPLEMENTAL REFERENCE

Figure S2

(A) Time (min)

STAM\textsuperscript{W29A,UIM}, Hrs\textsuperscript{DUIM}

µcal/sec

kcal/mole of injectant (Ub)

Molar Ratio (Ub/ESCRT-0)

(B) Time (min)

STAM\textsuperscript{AVHS,UIM}, Hrs\textsuperscript{DUIM}

µcal/sec

kcal/mole of injectant (Ub)

Molar Ratio (Ub/ESCRT-0)