Vps27-Hse1 and ESCRT-I complexes cooperate to increase efficiency of sorting ubiquitinated proteins at the endosome

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Ubiquitin (Ub) attachment to cell surface proteins causes their lysosomal degradation by incorporating them into lumenal membranes of multivesicular bodies (MVBs). Two yeast endosomal protein complexes have been proposed as Ub-sorting “receptors,” the Vps27-Hse1 complex and the ESCRT-I complex. We used NMR spectroscopy and mutagenesis studies to map the Ub-binding surface for Vps27 and Vps23. Mutations in Ub that ablate only Vps27 binding or Vps23 binding blocked the ability of Ub to serve as an MVB sorting signal, supporting the idea that both the Vps27-Hse1 and ESCRT-I complexes interact with ubiquitinated cargo. Vps27 also bound Vps23 directly via two PSDP motifs present within the Vps27 COOH terminus. Loss of Vps27-Vps23 association led to less efficient sorting into the endosomal lumen. However, sorting of vacuolar proteases or the overall biogenesis of the MVB were not grossly affected. In contrast, disrupting interaction between Vps27 and Hse1 caused severe defects in carboxy peptidase Y sorting and MVB formation. These results indicate that both Ub-sorting complexes are coupled for efficient recognition of ubiquitinated cargo.

Introduction

Degradation of cell surface membrane proteins is mediated by their incorporation into lumenal membranes of the late endosome (or multivesicular body [MVB]), which are ultimately delivered to lysosomes. Attachment of ubiquitin (Ub) to a variety of membrane proteins is both necessary and sufficient to guide proteins into this degradative pathway (Piper and Luzio, 2001). The group of “class E” Vps proteins and the PI 3-kinase Vps34p are required for the generation of MVB lumenal membranes and protein sorting into those membranes (Piper and Luzio, 2001; Katzmann et al., 2002). Some of these proteins also catalyze the topologically related process of gag-mediated retrovirus budding (Perez and Nolan, 2001; Carter, 2002).

Two class E Vps protein complexes have been proposed as endosomal Ub-sorting receptors. Vps23p, and its mammalian counterpart Tsg101, is a component of the ESCRT-I complex (Babst et al., 2000; Garrus et al., 2001; Katzmann et al., 2002). The NH2 termini of Vps23 and Tsg101 contain a Ub E2 variant (UEV) domain, and the Tsg101 UEV domain binds directly to Ub (Pornillos et al., 2002). The Tsg101 UEV domain also binds to a PTAP motif within a region of viral gag proteins required for budding, providing a mechanism for recruiting other class E Vps components (Perez and Nolan, 2001). A complex of two other class E Vps proteins, Vps27-Hse1, as well as their mammalian equivalent, Hgs-STAM1/2, also binds Ub via Ub interaction motifs (UIMs) (Bilodeau et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Both the yeast and mammalian complexes localize to early endosomes, and EM studies have pinpointed Hgs to clathrin-rich subdomains where MVB formation ensues (Komada and Kitamura, 2001; Sachse et al., 2002). Mutations within the UIM domains of both Vps27 and Hse1 prevent sorting of ubiquitinated proteins to the MVB interior while other functions provided by the Vps27-Hse1 complex remain intact (Bilodeau et al., 2002).

Although there are two potential Ub-sorting receptors on endosomes, it remains unclear whether each recognizes ubiquitinated cargo (Ub-cargo) in vivo or whether Ub-co}
binding serves another purpose. Vps23 can associate with Ub-cargo, but overexpression of other class E Vps proteins (ESCRT-II complex) obviates a requirement for Vps23 in MVB sorting (Babst et al., 2002). Also, while mutation of Vps23 in the UEV domain blocks MVB sorting and vacuolar protease sorting (Katzmann et al., 2001), the dual nature of the Tsg101 UEV domain (i.e., binding Ub and PTAP) may indicate another role of the Vps23-UEV domain. Likewise, while a specific function for sorting Ub-cargo can be assigned to the UIM domains of the Vps27-Hse1 complex (Bilodeau et al., 2002), UIM domains may also direct ubiquitination of the UIM-containing proteins themselves for a yet unknown function (Polo et al., 2002).

We examined the contribution of Vps27 and Vps23 in the recognition and sorting of Ub-cargo into the MVB interior by identifying two sets of Ub mutations: one that binds Vps27-Hse1 but not Vps23-UEV and vice versa. MVB sorting of Ub-conjugated reporter proteins is blocked by either set of mutations. We also find that Vps27 binds the Vps23 UEV domain by PTAP-related motifs, providing a mechanism for the cooperation of these two Ub-sorting complexes.

**Results and discussion**

We showed that a fragment of Vps27 (1–351) containing both UIM domains bound Ub when isolated from yeast lysates (Bilodeau et al., 2002). A GST fusion of this fragment was used to map the surface on Ub that interacts with Vps27 by $[^{15}N]^{-N}$-HSQC NMR. Many differences in the HSQC spectrum of Ub were observed in the presence of GST-Vps27 (1–351) but not with GST alone; these included changes in chemical shifts and peak broadening or disappearance (Fig. 1). The Ub surface residues most affected by Vps27 binding were L$_8$, T$_{14}$, R$_{42}$, I$_{44}$, G$_{47}$, K$_{48}$, Q$_{49}$, V$_{70}$, and R$_{72}$. Many of these residues are also affected upon binding peptides containing the UIM domains from the Rpn10 proteasome subunit or from Hgs (Shekhman and Cowburn, 2002; Walters et al., 2002). The UIMs of Rpn10 also interact with the Ub-like (Ubl) domains of PLIC-2, HR23a, and Parkin (Walters et al., 2002; Sakata et al., 2003). These combined data indicate a core group of residues, L$_8$, R$_{42}$, I$_{44}$, and V$_{70}$, that likely define a common binding interface.

A similar NMR approach was used to map the Ubl-binding surface for the Tsg101 UEV domain (Formillos et al., 2002). This surface includes I$_{44}$, K$_{48}$, Q$_{62}$, and E$_{64}$. The Q$_{62}$ and E$_{64}$ residues are far from the Vps27 binding site (Fig. 1). Furthermore, these residues are not conserved in other Ubl domains that bind UIM motifs, thus providing ideal candidate targets to specifically alter the Vps23 binding site without perturbing Vps27 binding (Shekhman and Cowburn, 2002; Walters et al., 2002).

Mutations in either the putative Vps27 or Vps23 binding site on Ub were made to assess their contribution to the MVB sorting signal. We placed our mutations within a Ub that lacked the two COOH-terminal glycine residues and contained K$_{29}$R, K$_{48}$R, and K$_{68}$R mutations (R3/H9004, GG) to ensure that polyubiquitination or formation of Ub adducts would not occur in vivo. Because Vps27 forms a complex with the UIM-containing protein Hse1, we performed bind-
ing analyses with yeast lysates containing both proteins to ensure that we assessed binding of the whole complex. This was done using yeast lacking VPS23 as previously described (Bilodeau et al., 2002). Analysis of Vps23 binding was performed using a recombinant Vps23 UEV domain. Despite key differences in the residues of TSG101 important for Ub binding and the aligned residues of Vps23 (Pornillos et al., 2002), the Vps23 UEV domain specifically bound GST-Ub and GST-Ub (R3/H9004GG) (Fig. 2 C).

Alanine substitution of L8 or R42 had no effect on Vps27 binding, and only modest effects on binding were observed by I44A or V70A mutations (Fig. 2 D). This is likely due to the presence of other contacts provided by the Vps27-Hse1 complex. We next combined mutations with the I44A mutation, reasoning that since this residue lies within the binding surface of Ub for both Vps27 and Tsg101 UEV domain, we could destabilize binding enough to reveal a role for residues that constitute a specific binding site for Vps27 or Vps23. We found that double mutations I44A/V70A and L8A/V70A markedly inhibited Vps27 binding. For Vps23-UEV binding, the L8A, R42A, I44A, and V70A mutations had little effect on Vps23-UEV binding. In contrast, binding was significantly reduced by a Q62A/E64A double mutation. A complete loss of Vps23-UEV binding was observed when Q62A/E64A double mutation was combined with an I44A mutation. Surprisingly, we found that L8A/V70A also caused loss of Vps23 binding. Importantly, the Vps23-UEV domain bound well to the L8A/V70A mutant, while Vps27 bound well to the Q62A/E64A/I44A triple mutant, thus defining Ub alleles that differentially bind Vps23 versus Vps27.
We then assessed how well these mutant forms of Ub could direct MVB sorting by fusing them to the COOH terminus of an Fth1-GFP reporter protein, which otherwise localizes to the limiting membrane of the vacuole (Urbanowski and Piper, 2001). To prevent degradation of intravacuolar vesicles, we used a \textit{pep4} mutant strain (Fig. 2 A). Consistent with the Ub binding data, the L8A or R42A mutations had no effect on sorting Fth1-GFP-Ub to the vacuole lumen. I44A had a modest effect consistent with previous results (Urbanowski and Piper, 2001), while V70A also had a slight effect. Ub mutations that greatly inhibited Vps27 binding (I44A V70A and L8A V70A) were defective in sorting Fth1-GFP to the vacuole interior. Likewise, the Q62A I44A mutant was defective in MVB sorting, and the defect was accentuated by the addition of I44A. Thus, Ub mutations in either the Vps23- or Vps27-binding site blocked the ability of Ub to serve as an MVB sorting signal.

These data indicate that both the Vps27-Hse1 complex and the Vps23-containing ESCRT-I complex recognize Ub-cargo proteins to effect their sorting into intralumenal membranes. One caveat is that there may yet be another endosomal Ub-binding protein that fulfills this function. No candidates have so far been identified, and given the correlation between loss of binding and the loss of MVB sorting in the various Ub mutants, these data support the idea that direct recognition of Ub-cargo by Vps27 and Vps23 is necessary for efficient MVB sorting.
E64A mutant Ubs was still defective in sorting to the vacuole interior. The ability of these Ub alleles to complement indicated that the mutations specifically affected Vps27 or Vps23 binding.

The observation that Vps27-Hse1 and ESCRT-I recognize Ub-cargo but not necessarily the same Ub moiety suggests that Ub-cargo could interact sequentially with each Ub-receptor component for final incorporation into the MVB lumen. Tsg101 and UIM-containing proteins bind Ub with low affinity, which may allow for shuttling Ub conjugates between different sorting receptors (Pornillos et al., 2002). Indeed, the putative binding sites of Vps27 and TSG101 on Ub partly overlap, potentially prohibiting simultaneous binding. Such a model is attractive given the multisite nature of Ub-conjugates which may allow for shuttling Ub conjugates between different sorting receptors (Pornillos et al., 2002). Indeed, the putative binding sites of Vps27 and TSG101 on Ub partly overlap, potentially prohibiting simultaneous binding.

Figure 4. Dependent binding sites for Vps23 and Hse1. (A) The Vps27 sequence (residues 358–450) aligned with Vps27 orthologues of other budding yeast. Identical residues are in bold. Residues selected for triple alanine substitution are indicated (**). Lysates from cells expressing Hse1-HA and the indicated vps27 alleles were immunoprecipitated with Vps27 NH2-terminal antibodies when parent Vps27-Hse1 Vps23 protein bound high levels of recombinant Vps27-Hse1 (wild-type) and Vps27-Hse1 (KIS416–418AAA) was renamed vps27ΔKIS-1. (B) Lysates from vps27Δ cells with vector, VPS27 wild type, or the vps27Δ−vps23 or vps27Δ−Hse1 alleles in combination with Vps23-HA or Hse1-HA were immunoprecipitated with anti-Vps27, control ( ), or anti-HA polyclonal antibodies.

To map the Vps23 binding site on Vps27, we performed coimmunoprecipitation studies using Vps27 truncation mutants. Approximately 10% of Vps23 could be immunoprecipitated with anti-Vps27 NH2-terminal antibodies when full-length Vps27 was present or when truncated at residues 617, 579, 524, or 485. Further truncation analysis showed that residues 430–484 were required for Vps23 binding (Fig. 3 C). To determine what motifs serve as a binding interface between Vps23 and Vps27, we analyzed an alignment of Vps27 orthologues from several other budding yeasts because the evolutionary distance of these related species might be close enough to conserve protein interaction sites but distant enough to allow irrelevant residues to vary. We identified a proline-containing region (PSDP 447–450 [PSDP-1]) as a candidate interaction site for Vps27. Mutagenesis approach was undertaken to interrupt the Hse1 binding site on Vps27 (Fig. 3 C and Fig. 4 A). The resulting vps27Δ−Hse1 allele did not coimmunoprecipitate with Hse1 but did associate with Vps23 (Fig. 4 B). To assess the role of coupling between Vps27 and Vps23, we examined the phenotype of vps27Δ−Vps23 cells expressing the Fth1-GFP-Ub (Q62A/E64A/I44A; V70A/I44A) reporter protein. If coordination of the Vps27-Hse1 complex with ESCRT-I was required for the efficient sorting of cargo into the MVB, we reasoned that this reporter protein would not sort to the vacuolar lumen in cells where Vps27 did not associate with Vps23. Fig. 5 C shows that vps27−
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Figure 5. Defects associated with loss of Vps27-Vps23 and Vps27-Hse1 association. Cells (vps27Δ::Kanr) were transformed with low copy plasmid carrying the vps27ΔVps23 or vps27ΔHse1 alleles or wild-type VPS27 along with plasmid expressing either (A) Fth1-GFP-Ub(Q62A E64A I44A)-Ub(I44A V70A), (B) Fth1-GFP-Ub, or (C) Ste3-GFP. (D) Quantitation of CPY secretion from cells with the indicated vps27 allele (±SD). (E) Wild-type SEY6210 cells or isogenic vps27Δ cells with a stably integrated vps27ΔVps23 allele were analyzed for sorting of GFP-Cps1. Cells were viewed by fluorescence and differential interference contrast (DIC) microscopy. Dashed lines indicate composite figure.

ΔVps23 cells did not efficiently sort the Fth1-GFP-Ub-Ub reporter. We also examined the sorting of GFP-Cps1, a ubiquitinated MVB marker protein that is transported along the biosynthetic pathway to the vacuole interior (Katzmann et al., 2001). Unlike wild-type cells, vps27ΔVps23 cells were partly defective in delivery of GFP-Cps1 to the vacuole interior (Fig. 5 E; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200305007/DC1). However, the process of MVB formation was not grossly perturbed since MVB substrates, such as Ste3-GFP, accumulated in the vacuole lumen (Fig. 5 B) and carboxy peptidase Y (CPY) secretion was normal (Fig. 5 D). Thus, the binding of Vps27 to Vps23 served more to enhance the efficiency of sorting MVB cargo rather than serve an essential function in MVB formation. The linkage between Vps27 and Vps23 could provide a cooperative effect on Ub binding by Vps27 and Vps23. This may provide a better "chain-of-possession" amongst the Ub receptors to ensure processive sorting of Ub-cargo for degradation and prevent access of Ub-cargo to Ub peptidases that would interrupt the degradative process. In contrast, coupling between Vps27 and Hse1 was essential for both CPY sorting and MVB formation, consistent with previous data indicating that Vps27 is required for Hse1 localization (Bilodeau et al., 2002).

Materials and methods

Materials

Reagents, cell culture, microscopy, CPY secretion assays, immunoblotting, and recombinant protein production were previously described (Urbanowski and Piper, 2001; Bilodeau et al., 2002). The yeast strains used were MAT a SF838–9D pep4-3 as wild type and a vps27Δ::Kanr derivative (Urbanowski and Piper, 2001; Bilodeau et al., 2002). SEY6210 with VPS27 replaced by stable integration was used for analysis of GFP-Cps1 (Katzmann et al., 2001; Urbanowski and Piper, 2001). Monoclonal antibodies to clathrin were from S. Lemmon (Case Western Reserve University, Cleveland, OH).

NMR analysis

Recombinant Ub was expressed and purified as detailed previously (Sivaraman et al., 2001; Sundd et al., 2002). The 15N-labeling medium was from Spectra Stable Isotopes. 15N-Ub was mixed with 7 mg of GST-Vps27 fusion protein (Vps27 residues 1–351 in pGEX-3x) or GST alone in 40 mM
NaPO₄, pH 7.2, containing 10% D₂O at 25°C. Resonance assignments were used as previously described (Schneider et al., 1992).

**Plasmids**

Mutations in Ub used a derivative of p1717 that contained K₂₉R, K₄₈R, and K₆₃R substitutions and deletion of G₇₅G₇₆ (Urbanowski and Piper, 2001). This cassette contained the 3′ UT of PHO8 downstream of the Ub coding region. A BglII/NruI fragment encoding the various mutant Ub alleles was subcloned into the BglII/NruI site of Fth1-GFP-Ub plasmid p1717 to create GFP reporter proteins or into the BamHI/Smal site of pGEX-3X to create GST-Ub proteins. The Fth1-GFP-Ub-Ub plasmids were similar to the Fth1-GFP-Ub constructs except that the last Ub moiety was preceded by a linker encoding SCGTSCTGTR and an MluI site. The Vps23-UEV expression plasmids were made by subcloning a PCR fragment encoding 1-161 or 1-186 of Vps23 downstream of a GAL1 promoter and T7 promoter and upstream of a V5 epitope within pYES2.1 (Invitrogen). Expression of the Vps23-UEV was performed in BL21-DE3-RIL codon plus bacteria (Stratagene) with the addition of IPTG to induce production of T7 RNA polymerase. The Vps23-AL plasmid expressing Vps23 flanked with a 3xHA tag expressed under the Tac promoter and T7 promoter and upstream of the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain.

**Binding studies**

GST fusion protein (0.5 mg) bound to 100 µl of GSH-agarose was used to assess binding to Ub or the Vps23 COOH terminus. Yeast or bacterial lysates containing HA-tagged Vps27 and Hse1 (p1779 and p1808; Bilodeau et al., 2002) or the Vps23-UEV domain were added to the beads and incubated at 4°C for 2 h. Bacterial cells were lysed with a French Press, and yeast spheroplast lysates were produced with 600 mM sorbitol, 20 mM Hepes, 50 mM KCl, 100 mM potassium acetate, 1% TX-100 (lysis buffer). Beads were washed in lysis buffer and analyzed along with an equivalent of lysate by SDS-PAGE and immunoblotting with either monoclonal HA or V5 antibodies.

**Immunoprecipitations**

Cleared lysate from 100 OD of yeast spheroplasts resuspended in lysis buffer was divided and incubated with 2 µl of anti-Vps27, nonimmune, or anti-HA rabbit serum at 4°C for 2 h. Immunocomplexes were then isolated on fixed Staph-A beads, washed three times in lysis buffer, and analyzed by SDS-PAGE and immunoblotting.

**Online supplemental material**

The supplemental material (Figs. S1–S3) is available at http://www.jcb.org/cgi/content/full/jcb.200305007/DC1. The effect of deleting the clathrin binding region of Vps27 is shown in Fig. S1. Confirmation of Vps23 binding to two PSDP motifs in Vps27 is shown in Fig. S2. Quantitation of sorting defects is shown in Fig. S3.

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