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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., 2001). The NH_2 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

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Abbreviations used in this paper: CPY, carboxy peptidase Y; MVB, multivesicular body; Ub, ubiquitin; UEV, Ub E2 variant; UIM, Ub interaction motif.

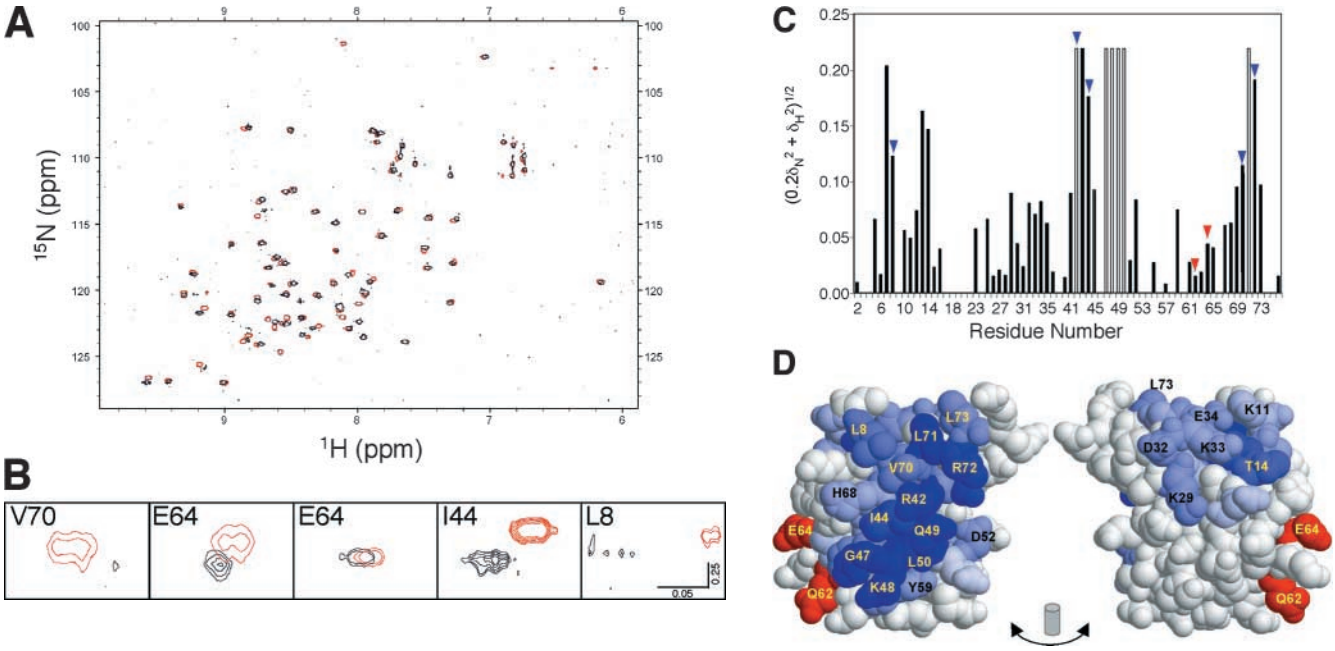

Figure 1. **NMR analysis of the Vps27 binding site on Ub.** (A) The ^{15}N ^{1}H NMR spectra of ^{15}N-labeled Ub in the presence (black) and absence (red) of 100 μM GST-Vps27$_{1–351}$. (B) Enhanced view of the chemical shift spectra for the indicated residues. Scale bars indicate ppm for ^{15}N (y axis) and ^{1}H (x axis). (C) Chemical shift differences for each Ub residue are plotted using the formula $(0.2\delta N^2 + \delta H^2)^{1/2}$, where δ = the chemical shift difference in the presence and absence of GST-Vps27. Blue arrows indicate Ub residues affected by Vps27 binding that were mutated. Red arrows indicate Ub residues affected by Tsg101 binding that were mutated. (D) Structural model of Ub showing the proposed binding site for Vps27 (blue) and two residues of the Tsg101 binding site Q_{62} E_{64} (red). Shading of the Vps27 binding surface is weighted to the magnitude of chemical shift difference upon binding.

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 ^{15}N-labeled Ub that interacts with Vps27 by [^{1}H,^{15}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 (Shekhtman 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 Ub-binding surface for the Tsg101 UEV domain (Pornillos 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 Ub-like (Ubl) domains that bind UIM motifs, thus providing ideal candidate targets to specifically alter the Vps23 binding site without perturbing Vps27 binding (Shekhtman 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 $K_{63}R$ mutations (R3ΔGG) 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-

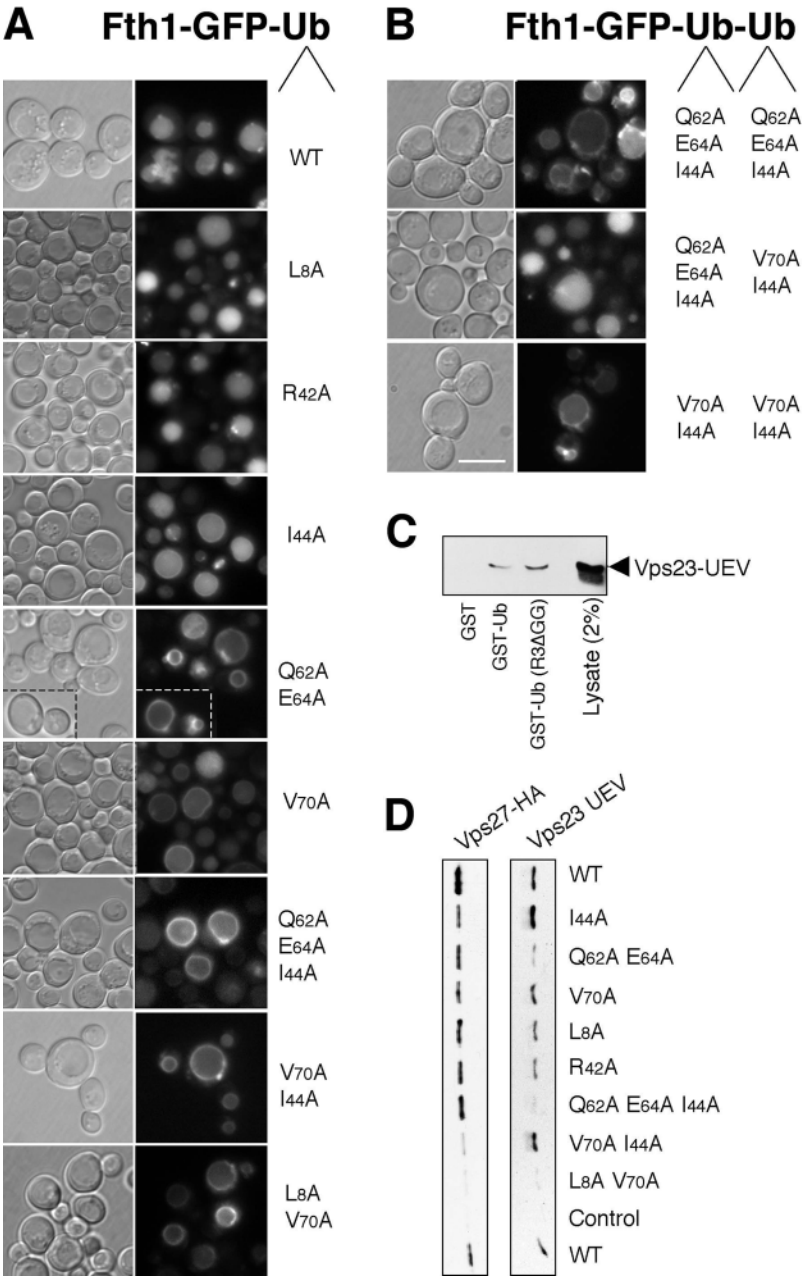

Figure 2. **Binding and sorting by mutant Ubs.** Mutations were placed in Ub also containing $K_{29}R$, $K_{48}R$, and $K_{63}R$ mutations and deleted of the COOH-terminal two glycine residues (R3ΔGG). (A) Mutant Ubs were fused in frame to an Fth1-GFP reporter protein and expressed in *pep4* cells. GFP fluorescence images and differential interference contrast (DIC) images that identify the yeast vacuole are shown. Dashed lines indicate composite figure. (B) The indicated combinations of mutant Ubs were incorporated into a tandem array at the COOH terminus of Fth1-GFP. Mutations in the first and second positions are indicated, and the GFP fluorescence and DIC images of their localization are shown. (C) Binding the Vps23-UEV domain to GST-Ub. Vps23 (1–161) flanked by a V5 epitope (Vps23-UEV-V5) was expressed from the T7 promoter. Cleared lysate was incubated in GSH agarose beads with either GST, GST-Ub, or GST-Ub (R3ΔGG). Beads were washed and immunoblotted with anti-V5 antibody along with a 2% equivalent of the lysate. (D) Binding of the Vps27 and Vps23-UEV to a panel of GST-Ub mutants. GSH agarose beads bound with GST-Ub (WT) or the indicated mutants were incubated with either lysate from *vps23* mutant yeast expressing Vps27-HA or bacterial lysate expressing Vps23 (1–161)-UEV-V5 domain. Beads were washed and immunoblotted with anti-HA or anti-V5 antibodies, respectively. All Ub mutants including the "wild-type" GST-Ub construct contained the R3ΔGG mutations. As a control, a truncated version of GST-Ub (Ub 1–35) is shown. Bar, 5 μm.

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 (R3ΔGG) (Fig. 2 C).

Alanine substitution of L_8 or R_{42} had no effect on Vps27 binding, and only modest effects on binding were observed by $I_{44}A$ or $V_{70}A$ mutations (Fig. 2 D). Thus, despite the large chemical shift differences in these residues upon Vps27 binding, no single mutation completely ablated Vps27 binding. This is likely due to the presence of other contacts provided by the Vps27-Hse1 complex. We next combined mutations with the $I_{44}A$ 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 $I_{44}A$ $V_{70}A$ and L_8A $V_{70}A$ markedly inhibited Vps27 binding. For Vps23-UEV binding, the L_8A, $R_{42}A$, $I_{44}A$, and $V_{70}A$ mutations had little effect on Vps23-UEV binding. In contrast, binding was significantly reduced by a $Q_{62}A$ $E_{64}A$ double mutation. A complete loss of Vps23-UEV binding was observed when $Q_{62}A$ $E_{64}A$ double mutation was combined with an $I_{44}A$ mutation. Surprisingly, we found that L_8A $V_{70}A$ also caused loss of Vps23 binding. Importantly, the Vps23-UEV domain bound well to the $I_{44}A$ $V_{70}A$ mutant, while Vps27 bound well to the $Q_{62}A$ $E_{64}A$ $I_{44}A$ triple mutant, thus defining Ub alleles that differentially bind Vps23 versus Vps27.

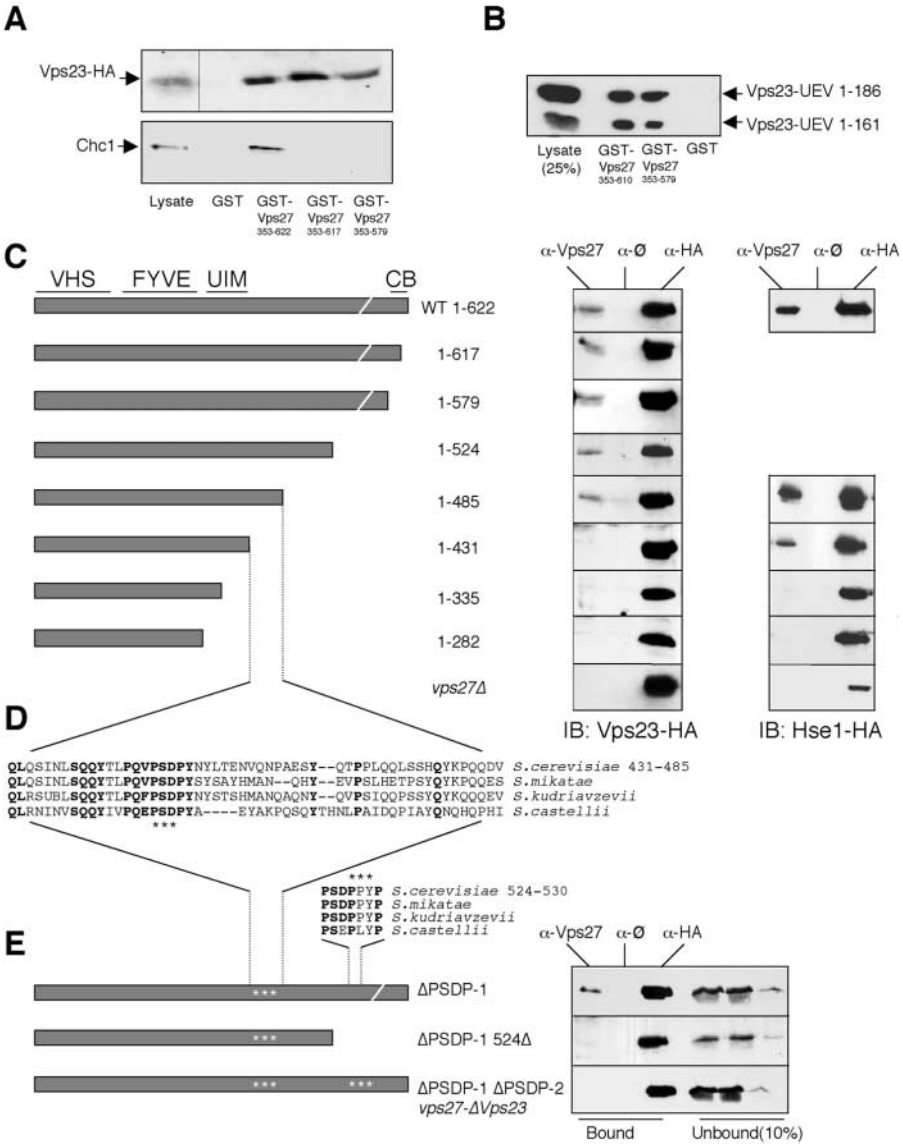

Figure 3. **Vps27 binds Vps23 directly via two PSDP motifs.** (A) Yeast lysate containing full-length Vps23-HA was incubated with GSH-agarose bound with GST, GST-Vps27(353–622), GST-Vps27 (353–617), or GST-Vps27(353–579). Beads were washed and analyzed by immunoblotting with anti-HA antibodies or anti-clathrin antibodies together with a 10% equivalent of starting lysate. (B) Similar to A, except that bacterial lysate containing Vps23 (1–161)-UEV-V5 and Vps23 (1–186)-UEV-V5 domains was incubated with GST and GST-Vps27 fusion proteins. (C, left) Schematic of Vps27 together with the indicated NH_2-terminal VHS (Vps27, Hgs, STAM) domain, the PI3P binding FYVE domain, the region containing two UIM domains, and the clathrin box (CB). The length of the indicated Vps27 truncation mutants is graphed below. (C, right) Immunoprecipitation experiments using the indicated Vps27 truncation mutants. Yeast lysates from cells with various *vps27* alleles also expressing full-length Vps23-HA or Hse1-HA were immunoprecipitated with preimmune (α-∅), anti-Vps27 NH_2 terminus (α-Vps27), or HA epitope (α-HA) antibodies. Beads were washed and analyzed by immunoblotting with monoclonal α-HA antibodies. (D) The Vps27 sequence between residues 431 and 485 is aligned with the same region from the Vps27 orthologues of other budding yeast. Identical residues are in bold. Residues within a PSDP motif selected for alanine substitution to make the *vps27-ΔPSDP-1* ($AAA_{447–449}$) allele are indicated (***). A second conserved PSDP motif is found between residues 524 and 530. Residues selected for alanine substitution to make the *vps27ΔPSDP-2* allele ($AAA_{525–527}$) are indicated (***). (E, left) Schematic of *vps27* alleles containing a ΔPSDP-1 mutation alone or in combination with truncation at residue 524 or the ΔPSDP-2 mutation, which comprises the *vps27-Δvps23* allele. (E, right) Cells expressing Vps23-HA and the indicated *vps27* alleles were immunoprecipitated and immunoblotted for Vps23-HA as in C.

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 *pep4* mutant strain (Fig. 2 A). Consistent with the Ub binding data, the L_8A or $R_{42}A$ mutations had no effect on sorting Fth1-GFP-Ub to the vacuole lumen. $I_{44}A$ had a modest effect consistent with previous results (Urbanowski and Piper, 2001), while $V_{70}A$ also had a slight effect. Ub mutations that greatly inhibited Vps27 binding ($I_{44}A$ $V_{70}A$ and L_8A $V_{70}A$) were defective in sorting Fth1-GFP to the vacuole interior. Likewise, the $Q_{62}A$ $E_{64}A$ mutant was defective in MVB sorting, and the defect was accentuated by the addition of $I_{44}A$. 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.

We next used a series of reporter proteins with two tandem Ub molecules to show that the presence of a Vps27-binding–defective Ub mutant could complement a Vps23-binding–defective Ub mutant in a tandem array (Fig. 2 B). This was not simply due to the presence of multiple but defective Ub-sorting motifs, as an Fth1-GFP-Ub-Ub reporter containing either two $I_{44}A$ $V_{70}A$ mutant Ubs or $I_{44}A$ $Q_{62}A$

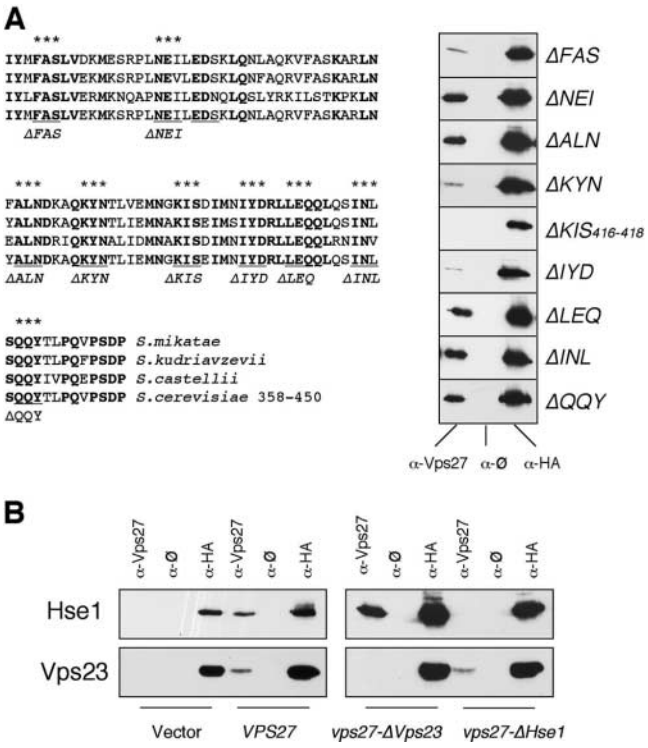

Figure 4. **dependent binding sites for Vps23 and Hse1.** (A) The Vps27 sequence (residues 358–450) aligned with Vps27 orthologues of other budding yeast. Identical residues are in bold. Residues selected for triple alanine substitution are indicated (***). Lysates from cells expressing Hse1-HA and the indicated *vps27* alleles were immunoprecipitated and immunoblotted for Hse1-HA. The *ΔKIS* allele (residues KIS416–418AAA) was renamed *vps27-ΔHse1*. (B) Lysates from *vps27Δ* cells with vector, *VPS27* wild type, or the *vps27-Δvps23* or *vps27-ΔHse1* alleles in combination with Vps23-HA or Hse1-HA were immunoprecipitated with anti-Vps27, control (∅), or anti-HA polyclonal antibodies.

$E_{64}A$ 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 physical interaction of the Hgs-STAM complex with another Ub-binding protein, Eps15 (Komada and Kitamura, 2001; Bache et al., 2003). Eps15 is important for the clathrin-dependent internalization of Ub-cargo such as EGFR (Confalonieri et al., 2000). Perhaps Ub-cargo is passed from one Ub-sorting receptor to another to effect its final delivery into the MVB degradative pathway. One prediction from this model is that Vps27 may also directly associate with Vps23. As an initial test, we found that high levels of HA-tagged Vps23 expressed in yeast lysates could bind to various fragments of Vps27 expressed as GST fusion proteins (Fig. 3 A). The Vps27 COOH terminus also bound clathrin via a clathrin box motif (EL**LI**E**L**) located at the extreme COOH terminus. Deletion of the clathrin box did not affect binding of Vps23. Interestingly, a truncated version of Vps27 lacking the clathrin box motif was normal for vacuolar protease sorting and Ste3-GFP localization to the vacuole lumen and only showed a defect in the clearance of Ste3 from the cell surface (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305007/DC1). The GST-Vps27 COOH-terminal fusion protein also bound high levels of recombinant Vps23-UEV (1–161) and Vps23-UEV (1–186) domains, demonstrating direct interaction (Fig. 3 B). Because the mammalian Vps23 homologue Tsg101 binds to PTAP motifs within viral *gag* proteins via its UEV domain, we suspected that a similar mechanism might explain the association of Vps23 with Vps27. A PTVP motif spans residues 581–584 in the COOH terminus of Vps27. However, a GST-Vps27 truncation containing residues 1–579 bound both Vps23-HA from yeast lysates as well as recombinant Vps23-UEV domain (Fig. 3, A and B). Truncation at residue 579 or beyond resulted in a class E phenotype (Fig. S1). 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 NH_2-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 yeast 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 Vps23. Mutation of the PSDP-1 (447–450) motif did not block Vps23 binding. However, additional truncation or alanine substitution of a second conserved PSDP motif (PSDP-2) resulted in loss of Vps23 coimmunoprecipitation (Fig. 3 E). Accordingly, the allele containing alanine substitutions in both PSDP motifs was renamed *vps27-ΔVps23*. This result was confirmed with recombinant protein in vitro (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200305007/DC1). The Vps27-ΔVps23 protein could associate with Hse1, indicating that it did not undergo global misfolding (Fig. 4 B). A similar truncation and alanine scanning mutagenesis approach was undertaken to interrupt the Hse1 binding site on Vps27 (Fig. 3 C and Fig. 4 A). The resulting *vps27-ΔHse1* 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 *vps27-ΔVps23* cells expressing the Fth1-GFP-Ub-Ub ($Q_{62}A$ $E_{64}A$ $I_{44}A$: $V_{70}A$ $I_{44}A$) reporter protein. If coordination of the Vps27-Hse1 complex with ESCRT-I were 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-*

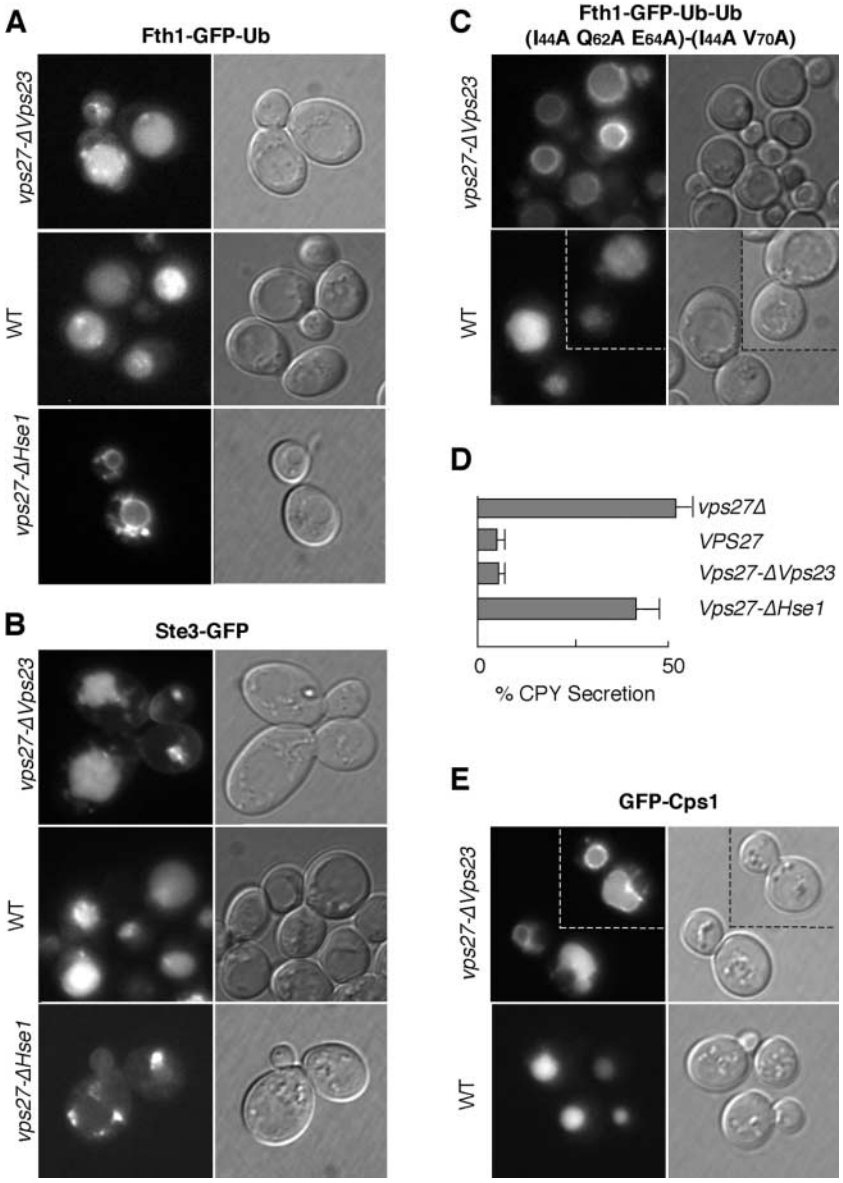

Figure 5. **Defects associated with loss of Vps27-Vps23 and Vps27-Hse1 association.** Cells (*vps27Δ::Kanr*) were transformed with low copy plasmid carrying the *vps27-ΔVps23* or *vps27-ΔHse1* alleles or wild-type *VPS27* along with plasmid expressing either (A) Fth1-GFP-Ub(Q_{62}A E_{64}A I_{44}A)-Ub(I_{44}A V_{70}A), (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 *vps27-ΔVps23* 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, *vps27-ΔVps23* 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 α 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 ^{15}N-labeling medium was from Spectra Stable Isotopes. ^{15}N-Ub was mixed with 7 mg of GST-Vps27 fusion protein (Vps27 residues 1–351 in pGEX-3x) or GST alone in 40 mM

$NaPO_4$, pH 7.2, containing 10% D_2O 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 $K_{29}R$, $K_{48}R$, and $K_{63}R$ substitutions and deletion of $G_{75}G_{76}$ (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/SmaI 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 SGSGTSGTR 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 *vps27* alanine substitution mutants were made by creating a mutant PCR fragment encoding bp 1–1800 and subcloning relevant fragments into a CEN-based *VPS27* plasmid (p1863). The Vps23-HA plasmid consisted of a centromeric plasmid expressing Vps23 flanked with a 3xHA tag expressed under the *VPS23* promoter. GFP-CPS1 plasmid was used as previously described (Katzmann et al., 2001), except carried in a *TRP1* low copy plasmid.

Binding studies

GST fusion protein (0.5 mg) bound to 100 μl of GSH-agarose was used to assess binding to Ub or the Vps27 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. Immune complexes 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 a 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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