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Vps27 recruits ESCRT machinery to endosomes during MVB sorting

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Down-regulation (degradation) of cell surface proteins within the lysosomal lumen depends on the function of the multivesicular body (MVB) sorting pathway. The function of this pathway requires the class E vacuolar protein sorting (Vps) proteins. Of the class E Vps proteins, both the ESCRT-I complex (composed of the class E proteins Vps23, 28, and 37) and Vps27 (mammalian hepatocyte receptor tyrosine kinase substrate, Hrs) have been shown to interact with ubiquitin, a signal for entry into the MVB pathway. We demonstrate that activation of the MVB sorting reaction is dictated largely through interactions between Vps27 and the endosomally enriched lipid species phosphatidylinositol 3-phosphate via the FYVE domain (Fab1, YGL023, Vps27, and EEA1) of Vps27. ESCRT-I then physically binds to Vps27 on endosomal membranes via a domain within the COOH terminus of Vps27. A peptide sequence in this domain, PTVP, is involved in the function of Vps27 in the MVB pathway, the efficient endosomal recruitment of ESCRT-I, and is related to a motif in HIV-1 Gag protein that is capable of interacting with Tsg101, the mammalian homologue of Vps23. We propose that compartmental specificity for the MVB sorting reaction is the result of interactions of Vps27 with phosphatidylinositol 3-phosphate and ubiquitin. Vps27 subsequently recruits/activates ESCRT-I on endosomes, thereby facilitating sorting of ubiquitinated MVB cargoes.

Introduction

The multivesicular body (MVB) sorting pathway is required for a number of key biological processes, including receptor down-regulation, cell growth control, developmental signaling, antigen presentation, and even the budding of certain viruses from host cells (for review see Katzmann et al., 2002). MVB formation occurs at a late endosomal compartment, when its limiting membrane invaginates and buds small vesicles into its lumen, giving rise to the characteristic morphology of numerous intraluminal vesicles within a larger membrane-enclosed endosome (Felder et al., 1990; Futter et al., 1996; Odorizzi et al., 1998). Cargoes destined for delivery to these intraluminal vesicles, such as internalized cell surface receptors, appear to be selected on the basis of both cis-acting sorting signals, such as ubiquitin (Ub), and trans-acting factors, such as the endosomal sorting complex required for transport (ESCRT) machinery (Katzmann et al., 2001; Babst et al., 2002a,b). Heterotypic fusion between an MVB and the vacuole/lysosome results in the delivery of the intraluminal vesicles (and their cargoes) into the lumen of this hydrolytic organelle where they are degraded. In contrast, certain RNA viruses, including HIV-1, utilize the cellular ESCRT machinery to facilitate their escape from host cells by redirecting ESCRT complexes to the cell surface where they appear to drive the budding and fission of the viral particles (for reviews see Perez and Nolan, 2001; Carter, 2002; Pornillos et al., 2002c). Topologically, MVB vesicle formation and viral budding are very similar, both involve budding membranes out of the cytoplasm. However, important questions remain concerning the mechanisms that regulate the processes of

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Abbreviations used in this paper: CPS, carboxypeptidase S; ESCRT, endosomal sorting complex required for transport; FYVE domain, Fab1, YGL023, Vps27, and EEA1; Hrs, hepatocyte receptor tyrosine kinase substrate; LDAO, lauryldimethylamineoxide; MVB, multivesicular body; NBD-PC, 7NBD-labeled phosphatidylcholine; NEM, *N*-ethylmaleimide; PI(3)P, phosphatidylinositol 3-phosphate; STAM, signal-transducing adaptor molecule; Ub, ubiquitin; UIM domain, Ub-interacting motif; VHS domain, Vps27, Hrs, and STAM; Vps, vacuolar protein sorting.

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membrane site selection, cargo recognition, and vesicle budding during the MVB sorting reaction.

We have previously defined ESCRT-I as a 350-kD protein complex that consists of the *Saccharomyces cerevisiae* class E vacuolar protein sorting (Vps) proteins Vps23, Vps28, and Vps37 (Katzmann et al., 2001). ESCRT-I is required for MVB sorting and interacts with ubiquitinated MVB cargoes. This interaction appears to occur via the Vps23 subunit of ESCRT-I, as a substitution in its UBC-like domain renders the complex incapable of Ub binding in vitro and confers an MVB sorting defect in vivo (Katzmann et al., 2001). Similarly, the mammalian homologue of Vps23, Tsg101, is part of a 350-kD complex that binds Ub and is required for the function of the MVB pathway (Babst et al., 2000; Bishop and Woodman, 2001; Garrus et al., 2001; Bishop et al., 2002; Pornillos et al., 2002b). However, ubiquitinated proteins exist throughout the cell, begging the question of how ESCRT-I is properly recruited and activated at its correct site of action, the endosome. The yeast class E protein Vps27 and its mammalian homologue, hepatocyte receptor tyrosine kinase substrate (Hrs), are also required for protein sorting in the MVB pathway. Each can bind Ub in vitro (Bilodeau et al., 2002; Bishop et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002), and possesses a FYVE domain (for Fab1, YGL023, Vps27, and EEA1), known to interact with the endosomally enriched lipid phosphatidylinositol 3-phosphate (PI(3)P; Burd and Emr, 1998; Misra and Hurley, 1999; Sankaran et al., 2001; Stahelin et al., 2002). Therefore, we sought to test the potential role for Vps27 in the regulation of the MVB sorting pathway.

Here, we examine the role for PI(3)P in the recruitment/activation of Vps27 and the role of Vps27 in the recruitment/activation of the ESCRT machinery. We report that endosomally localized PI(3)P is required to recruit Vps27 to the endosome and Vps27 appears to serve as a docking site for the ESCRT-I complex, thereby, initiating the MVB sorting reaction at the limiting membrane of the endosome. ESCRT-I physically interacts with membrane-bound Vps27 through a motif in the COOH-terminal portion of Vps27. Therefore, the Vps27 protein appears to direct the compartment-specific activation of MVB sorting and Vps27 function is regulated by specific interactions with both PI(3)P and ubiquitinated cargo at the late endosome.

Results

Vps27 and ESCRT-I bind Ub independently

Among the class E Vps proteins, both Vps27 and the Vps23-containing ESCRT-I complex have been shown to bind Ub and play a critical role in the function of the MVB sorting pathway (Katzmann et al., 2001; Bilodeau et al., 2002; Shih et al., 2002). To address the functional relationship between these Ub-interacting proteins in the context of the MVB sorting reaction, we initially analyzed the ability of each of these class E Vps proteins to bind Ub independently. This was accomplished by performing protein–protein interaction studies using either GST or Ub fused to GST as described previously (Katzmann et al., 2001; Shih et al., 2002). To address a requirement for Vps27 in ESCRT-I binding to Ub, immobilized GST or Ub-GST was incubated with cellular extracts prepared from wild-type cells, *vps27*Δ cells, or cells expressing a mutant form of Vps27 bearing substitutions in the Ub-interacting motifs (UIM domains; $Vps27^{S313D,\ S270D}$), previously shown to display dramatically reduced binding affinity for Ub in vitro (Shih et al., 2002). Subsequent to incubation with cell extracts, beads were washed and bound material was analyzed by Western blotting with anti-Vps23 antisera. As expected, the ESCRT-I subunit Vps23 bound to Ub-GST, but not GST, when lysate was prepared from wild-type cells (Fig. 1 A). Vps27 was not essential for this interaction, but appears to contribute to this binding, as deletion of *VPS27* resulted in a small decrease (less than twofold) in the amount of bound ESCRT-I, whereas the $vps27^{S313D,\ S270D}$ UIM mutation caused no obvious defect in binding. This in-

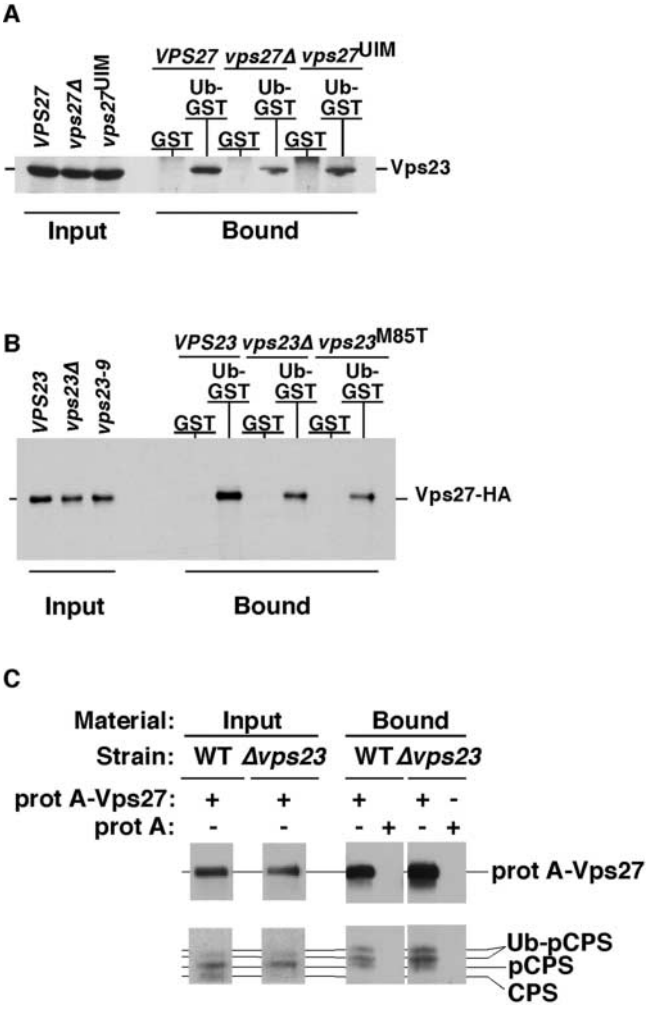

Figure 1. **ESCRT-I and Vps27 bind Ub independently of one another in vitro.** (A) Extracts were prepared from MBY52 cells expressing the indicated *VPS27* alleles and incubated with immobilized GST or Ub-GST (Ub-GST). ESCRT-I binding was visualized by Western blotting with anti-Vps23 antisera. (B) Extracts were prepared from DKY61 cells expressing the indicated *VPS23* alleles and incubated with immobilized GST or Ub-GST. Vps27-HA binding was visualized by Western blotting with anti-HA antisera. (C) Cellular membranes were isolated from DKY61, solubilized and protein A–Vps27 was purified under native conditions and bound material was probed by Western blotting with anti-Vps27 or anti-CPS antisera.

dicated that ESCRT-I binding to Ub did not require the Ub-binding function of Vps27, although Vps27 may contribute to the stability of this interaction.

Likewise, the ability of Vps27 to bind Ub independently of ESCRT-I function was addressed by preparing lysates from both *vps23*Δ and *vps23*^{M85T} mutant cells. The *vps23*^{M85T} mutation has been shown to prevent binding of ESCRT-I to Ub without affecting ESCRT-I complex formation (Katzmann et al., 2001). As expected from previous results (Bilodeau et al., 2002; Shih et al., 2002), Vps27 in lysates from wild-type cells was capable of binding to Ub in vitro (Fig. 1 B). Loss of ESCRT-I function, in the case of either *VPS23* deletion or the *vps23*^{M85T} point mutation, revealed a minor decrease (approximately twofold) in the amount of Vps27 recovered by this binding assay (Fig. 1 B). Similar to our previous results with ESCRT-I binding Ub independently of Vps27, these data indicated that Ub binding by Vps27 did not require ESCRT-I function, although this interaction may be stabilized by ESCRT-I.

We have previously shown that ESCRT-I and the MVB cargo ubiquitinated precursor carboxypeptidase S (Ub-pCPS) can be copurified from solubilized membranes (Katzmann et al., 2001). This assay was used to address whether Vps27 was also capable of interacting with this MVB cargo. Membranes were prepared and solubilized from wild-type or *vps23*Δ cells expressing a protein A–Vps27 chimera. Protein A-Vps27 was isolated from this fraction using IgG Sepharose and the bound material was analyzed by Western blotting (Fig. 1 C). It can be seen that Vps27 associated with Ub-pCPS from wild-type cells. Furthermore, this interaction appeared to be stabilized when MVB function was blocked in a *vps23*Δ strain. These results suggested that Vps27 also interacted with MVB cargo and that this interaction was not dependent on ESCRT-I expression. We were unable to perform the reciprocal experiment of ESCRT-I binding MVB cargo in a *vps27* mutant, as ESCRT-I is mislocalized to the cytoplasm under these conditions (see Results).

As both Vps27 and ESCRT-I interacted with Ub in vitro and MVB cargo in vivo, we wished to understand the mechanisms responsible for the selective subcellular localization of these proteins to the MVB sorting compartment. Our previous results suggested that a sequential interaction may occur between Vps27, ESCRT-I, and MVB cargo. Thus, the presence of a PI(3)P-specific binding domain within Vps27 was intriguing in that this could represent a mechanism to explain the specificity by which the MVB sorting machinery is initially recruited to the endosome to execute its function. Specificity for MVB sorting would, thereby, be determined not only by the presence of ubiquitinated MVB cargoes, but also by the presence of the endosomally enriched lipid PI(3)P.

Vps27 localization requires Vps34 function, PI(3)P, and the FYVE domain of Vps27

To examine the subcellular localization of Vps27 in vivo, we constructed a GFP-Vps27 chimera and expressed this in cells. We chose an approach that employed intact, viable cells because we have found that PI(3)P and other phosphoinositide species were rapidly degraded upon cell lysis (unpublished data). This GFP-Vps27 fusion protein was functional, as addressed by its ability to complement the sorting defects for the vacuolar hydrolysis carboxypeptidase Y and carboxypepidase S (CPS) observed in *vps27*Δ cells (unpublished data). In wild-type cells, the fusion protein localized to intracellular punctate structures (Figs. 2 B, left; Fig. 2 C, top left). Next, we examined whether these punctate structures represented endosomal membranes. To do so, we fused DsRed to the FYVE domain from mammalian EEA1 as a reporter for PI(3)P-enriched membranes and expressed this fusion protein in cells together with the GFP-Vps27 chimera. The FYVE domain of EEA1 has previously been shown to bind specifically to PI(3)P in vitro and direct endosomal localization in vivo (Burd and Emr, 1998; Gaullier et al., 1998). In wild-type cells expressing DsRed-FYVE, red fluorescence was observed on punctate structures adjacent to the vacuole, weakly on the vacuole limiting membrane, and in some cases within the vacuole itself, consistent with previous studies that used GFP-FYVE^{EEA1} and GST-FYVE^{EEA1} fusions (Burd and Emr, 1998; Gillooly et al., 2000; Fig. 2 B, middle; Fig. 2 C, top middle). In cells coexpressing GFP-Vps27 and DsRed-FYVE significant colocalization was observed between GFP-Vps27 and DsRed-FYVE (Fig. 2 B, right), which is consistent with Vps27 being recruited to PI(3)P-containing endosomal membranes.

To directly address a role for PI(3)P in the efficient endosomal recruitment of the FYVE domain-containing Vps27 protein, we examined the localization of GFP-Vps27 in *vps34*Δ mutant cells. *S. cerevisiae* has been shown to possess a single PI 3-kinase isoform, Vps34. It has been shown that inactivation of Vps34 by deletion of the *VPS34* gene results in a block in the synthesis of PI(3)P (Schu et al., 1993; Stack et al., 1993). Consistent with this, the DsRed-FYVE reporter redistributed to the cytoplasm in *vps34*Δ cells (Fig. 2 C, middle). Likewise, GFP-Vps27 displayed a highly diffuse cytoplasmic pattern in *vps34*Δ cells, although there were a few faint puncta visible (Fig. 2 C, middle). The role of the FYVE domain in endosomal recruitment was addressed by deletion of this domain from the GFP-Vps27 fusion protein. In wild-type cells, only a small fraction of mutant GFP-Vps27$^{\Delta FYVE}$ was recruited to PI(3)P-containing endosomal compartments labeled with DsRed-FYVE (Fig. 2 C, bottom). Similar results were obtained using a point mutation that renders the FYVE domain nonfunctional (unpublished data). Together, these results indicated that efficient recruitment of Vps27 to the endosomal membrane was mediated by interactions between PI(3)P and the FYVE domain of Vps27.

ESCRT-I endosomal localization requires Vps27 function

Next, we addressed the mechanism by which ESCRT-I is recruited to its site of action, as there are clearly numerous membrane sites within the cell that contain ubiquitinated proteins (e.g., endoplasmic reticulum, Golgi compartments, plasma membrane, and endosomes), indicating this cannot be the sole mechanism for its localization. To examine the subcellular localization of ESCRT-I, we used a previously described chromosomally integrated gene fusion between Vps23 and GFP (Katzmann et al., 2001). As expected, in wild-type cells, Vps23-GFP was observed on intracellular punctate structures as well as in the cytoplasm (Fig. 3 A). As efficient Vps27 localization to endosomal membranes re-

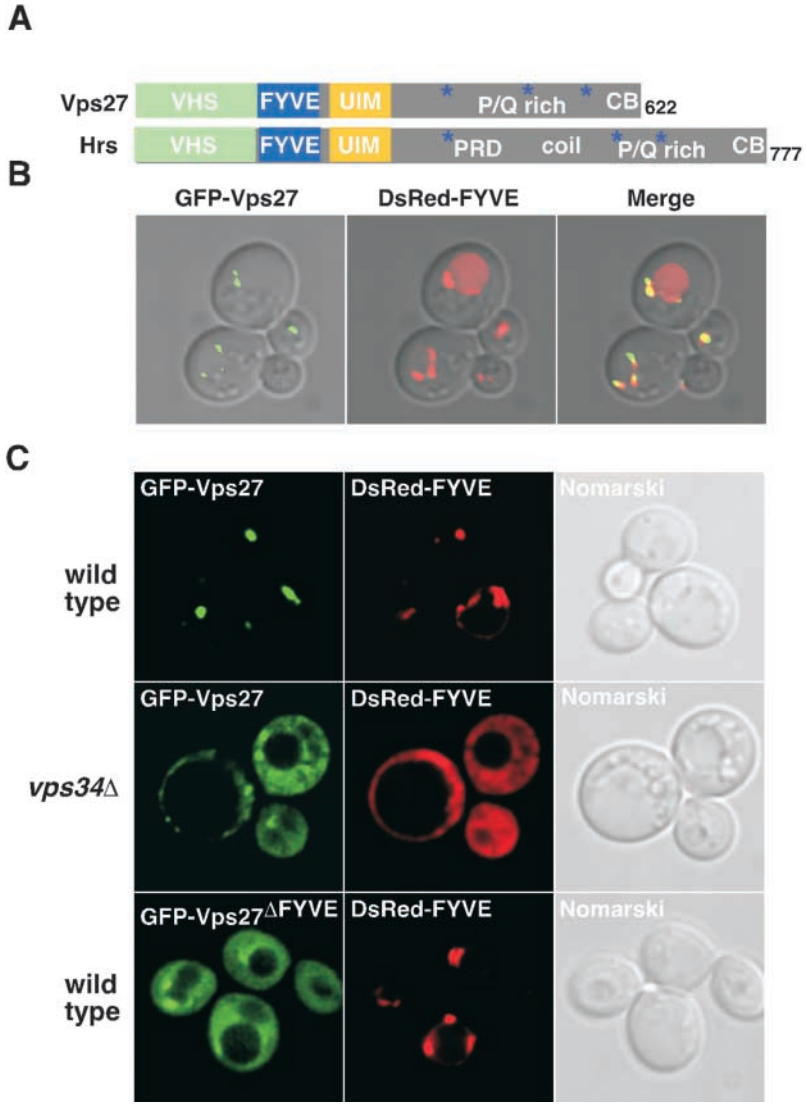

Figure 2. **The steady state localization of Vps27 on PI(3)P-containing endosomal membranes is dependent on Vps34 activity and the FYVE domain in Vps27.** (A) Schematic representations of Vps27 and its mammalian orthologue, Hrs. The VHS (light green), FYVE (blue), and UIM (yellow) domains are indicated. The proline/glutamine-rich region (P/Q rich), and clathrin-binding motifs (CB) are indicated in Vps27 and Hrs. The proline-rich (PRD) and coiled-coil domain (coil) in Hrs are also indicated. The asterisks indicate the relative positions of candidate PTAP-like motifs found in Vps27 ($PSDP^{447–450}$, $PSDP^{524–527}$ and $PTVP^{581–584}$) and Hrs ($PSAP^{348–351}$, $PSGP^{583–586}$ and $PSMP^{620–623}$). (B) Wild-type cells (SEY6210) coexpressing GFP-Vps27 and DsRed-$FYVE^{EEA1}$ fusions were observed by fluorescent and Nomarski microscopy using a DeltaVision deconvolution system. (C) Wild-type (top) or *vps34*Δ cells (PHY102; middle) coexpressing GFP-Vps27 and DsRed-$FYVE^{EEA1}$ fusions or wild-type cells coexpressing GFP-$Vps27^{\Delta FYVE}$ and DsRed-$FYVE^{EEA1}$ fusions (bottom) were observed by fluorescent and Nomarski microscopy.

quired the production of PI(3)P, we examined whether PI(3)P was also required for the recruitment of ESCRT-I. In *vps34*Δ cells, Vps23-GFP exhibited largely a cytoplasmic distribution with almost no intracellular puncta (Fig. 3 A). One interpretation of this result would be that the PI(3)P effector Vps27 is required for bridging the interaction between ESCRT-I and PI(3)P. To test this idea, Vps23-GFP localization was analyzed in *vps27*Δ cells. Vps23-GFP redistributed to a diffuse cytoplasmic pattern in *vps27*Δ cells (Fig. 3 A).

We have previously shown that in cells lacking the Vps4 AAA-ATPase, Vps23-GFP is localized almost entirely onto the endosomal membranes that accumulate in these mutant cells (Katzmann et al., 2001; Fig. 3 A). This was consistent with other data that indicated the Vps4 AAA-ATPase is responsible for dissociating ESCRT proteins from the endosomal membrane (Babst et al., 1998, 2002a,b). These studies suggested that Vps4 activity is required as one of the last steps in the MVB sorting reaction. To order the actions of Vps27, ESCRT-I, and Vps4, we addressed Vps23-GFP localization in a *vps4*Δ *vps27*Δ double mutant strain. Vps23-GFP displayed a highly diffused cytoplasmic distribution in *vps4*Δ *vps27*Δ cells, similar to the *vps27*Δ strain (Fig. 3 A), suggesting an early requirement for Vps27 function in the recruitment of ESCRT-I to endosomal membranes.

To address whether Vps27 binding to endosomes required ESCRT-I, the reciprocal experiment was performed by localizing GFP-Vps27 in *vps23*Δ mutant cells. As can be seen in Fig. 3 B, GFP-Vps27 localized to punctate, intracellular structures even in the absence of ESCRT-I. Together, these data indicated that Vps27 localization did not depend on ESCRT-I, whereas ESCRT-I localization was dependent on Vps27 function, even in the absence of Vps4 activity. These findings place Vps27 function upstream of ESCRT-I and indicate a role for Vps27 in the endosomal recruitment of ESCRT-I. Moreover, these data highlight the importance of the interaction between PI(3)P and the FYVE domain of Vps27 in determining the subcellular compartment where MVB sorting occurs.

ESCRT-I interacts with Vps27

To clarify the role of Vps27 in the recruitment of ESCRT-I to endosomal membranes, we examined whether Vps27 and ESCRT-I associate on membranes. Wild-type cells expressing functional Vps23–protein A (Babst et al., 2000) and

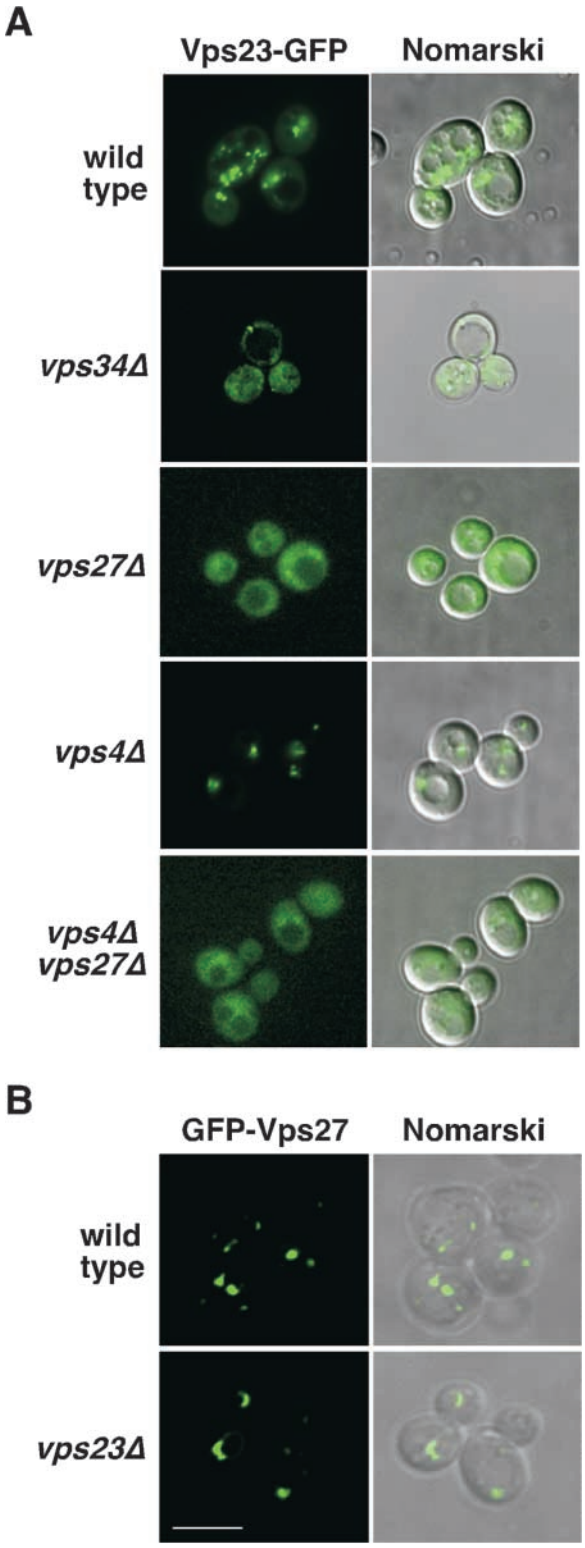

Figure 3. **Localization of ESCRT-I to endosomes is dependent on Vps34 activity and function of the PI(3)P-binding protein, Vps27.** (A) Localization of Vps23-GFP in wild-type (DKY54), *vps34*Δ (DKY82), *vps27*Δ (DKY78), *vps4*Δ (DKY55), or *vps4*Δ *vps27*Δ (DKY79) living cells by fluorescence and Normarski microscopy. (B) ESCRT-I is not essential for the recruitment of Vps27 to membranes. Wild-type (top) or *vps23*Δ cells (EEY6–2; bottom) expressing GFP-Vps27 were observed by fluorescence and Nomarski microscopy. Bar: (A and B) 5 μM.

Vps27-HA (Shih et al., 2002) fusion proteins were fractionated into soluble and membrane fractions. After solubilizing the membrane fraction, Vps23–protein A was isolated from both cell fractions under native conditions. Bound material was probed for the presence of Vps27-HA. The majority of Vps27 in cell lysates was present in the soluble fraction, likely due to PI(3)P hydrolysis upon cell lysis (Fig. 4 A, Input). Regardless, the vast majority of Vps27-HA copurified with Vps23–protein A in the membrane fraction, but not the soluble fraction (Fig. 4 A). This indicated that Vps27 and ESCRT-I specifically associate on membranes—consistent with the previous finding that soluble ESCRT-I did not contain Vps27 (Katzmann et al., 2001)—and suggested that this interaction did not occur after lysis.

In reciprocal experiments, this interaction was addressed by using the protein A–Vps27 construct using solubilized membrane fractions because our previous results indicated that Vps27 and Vps23 only associate on membranes. Both Vps23 and Vps28 copurified with protein A–Vps27 from the membrane fraction (Fig. 4 B). Next, we used this assay to examine whether the interaction between Vps27 and ESCRT-I was mediated by indirect binding to ubiquitinated MVB cargo. First, we examined whether substitutions in the UIM domains of Vps27 ($Vps27^{S313D, S270D}$) conferred defects upon Vps27/ESCRT-I association. Previous studies have shown that the UIM domains of Vps27 were necessary for the efficient binding of Vps27 to Ub in vitro (Bilodeau et al., 2002; Shih et al., 2002) However, the Vps27/ESCRT-I interaction was not disrupted by substitutions in the UIM domains of protein A–Vps27 (Fig. 4 B). In addition, previous work implicated the VHS domain (for Vps27, Hrs, and STAM) of Vps27 in the efficient binding of Vps27 to Ub in vitro (Bilodeau et al., 2002). We found that a mutant form of Vps27 lacking an intact VHS domain was competent for ESCRT-I association (Fig. 4 B). Thus, the interaction between Vps27 and ESCRT-I was not mediated by simultaneous binding to ubiquitinated MVB cargo, which is consistent with our finding that Vps27 and ESCRT-I bound Ub independently. Also, the interaction between Vps27 and ESCRT-I was specific, as a subunit of the downstream ESCRT-III complex, Snf7 (Babst et al., 2002a), was not present in the bound fraction (Fig. 4 B).

Identification of a domain in Vps27 that controls ESCRT-I recruitment and MVB sorting

These interaction studies revealed that association of Vps27 and ESCRT-I did not require Vps27 NH_2-terminal VHS and UIM domains, suggesting that COOH-terminal residues within Vps27 may be involved in this interaction. Accordingly, we constructed a series of deletions in the COOH-terminal domain of Vps27 and examined Vps23-GFP localization in *vps27*Δ mutant cells expressing various forms of Vps27 from a low copy plasmid. Truncation of Vps27 at residue 581 ($Vps27^{\Delta 581–622}$) conferred a defect in Vps23-GFP localization, as Vps23-GFP was no longer efficiently recruited to punctate compartments but was mainly diffuse throughout the cytoplasm in $vps27^{\Delta 580–622}$ mutant cells (Fig. 5, left column). In contrast, deletion of Vps27 residues 619–622 that comprise a candidate clathrin-binding

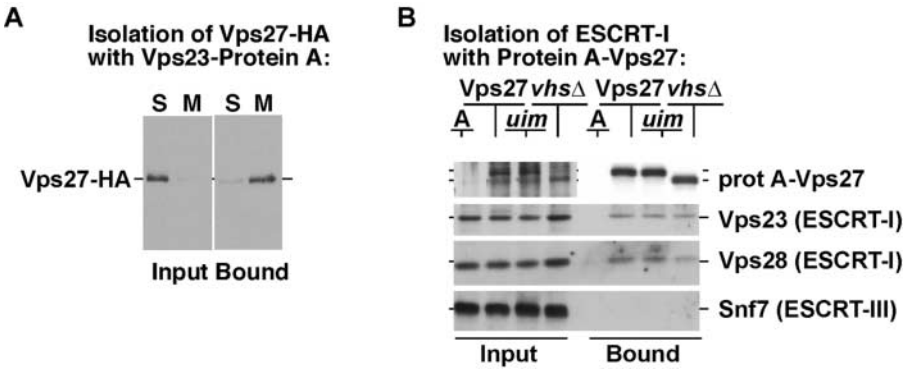

Figure 4. **Vps27 and ESCRT-I interact on membranes.** (A) Wild-type cells (TVY614) expressing Vps23–protein A and Vps27-HA were lysed and separated into low speed membrane pellet (M) and soluble fractions (S). Both fractions were adjusted to 0.5% LDAO, cleared, and Vps23–protein A was purified under native conditions. Isolated material was probed with anti-HA antibody to detect Vps27-HA. Input lanes show 20% of the total Vps27-HA present in the lysates. (B) MBY52 cells expressing protein A alone (A), protein A–Vps27 (Vps27), protein A–Vps27S313D, S270D (*uim*), or protein A–Vps27DVHS (*vhsΔ*) were lysed. Low speed membrane fractions were generated and solubilized with 0.5% LDAO and protein A–Vps27 was purified under native conditions. Bound material was Western blotted using antisera as indicated.

motif had no effect on Vps23-GFP localization (unpublished data), suggesting that residues 581 to 618 in Vps27 control ESCRT-I localization.

The HIV-1 encoded Gag protein has been shown to interact with the mammalian orthologue of Vps23 (Tsg101) and, thereby, recruits ESCRT-I to the plasma membrane where it functions in viral budding (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). Furthermore, structural analysis of the Tsg101 Ub E2 variant domain indicated that association of Gag with Tsg101 was mediated through a conserved motif (containing the amino acids PTAP) in the late domain of Gag (Pornillos et al., 2002a,b). Analysis of Vps27 revealed the presence of three PTAP-like motifs within Vps27 (Fig. 2 A), two PSDP motifs (residues 447–450 and 524–527), and a PTVP motif (residues 581–584). Because our initial findings implicated residues 581 to 618 in Vps27, we generated a mutant form of Vps27 bearing substitutions in the PTVP$^{581–584}$ motif. In cells expressing Vps27$^{P581G,\ T582S}$ alone, we observed that Vps23-GFP displayed an increased cytoplasmic distribution as compared with wild-type cells (Fig. 5, left column), suggesting a role for the PTVP motif in the efficient recruitment of ESCRT-I.

Because Vps27 has multiple PTAP-like motifs, we examined whether additional Vps27 residues contributed to ES-

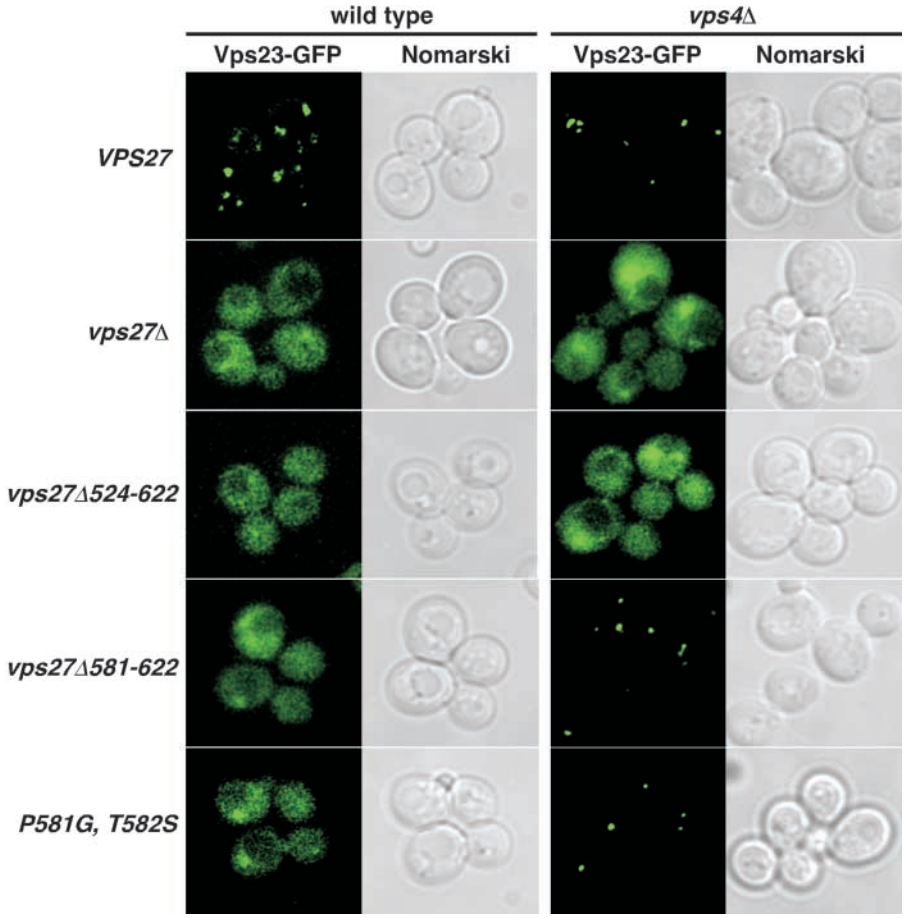

Figure 5. **Identification of a region in Vps27 involved in the efficient recruitment of ESCRT-I to endosomes.** Localization of Vps23-GFP in *vps27*Δ cells (MBY21; left column) or *vps4*Δ *vps27*Δ cells (DKY79; right column) expressing either wild-type or indicated mutant forms of Vps27 from a plasmid was observed by fluorescence and Normarski microscopy.

CRT-I interactions by performing experiments in a *vps4*Δ background that improved detection of Vps23-GFP on endosomal membranes. Interestingly, Vps23-GFP was still found on punctate structures in *vps27*$^{P581G,\ T582S}$ *vps4*Δ and *vps27*$^{\Delta 581–622}$ *vps4*Δ double mutant cells (Fig. 5, right column). Thus, although the *vps27*$^{P581G,\ T582S}$ mutation impaired recruitment of ESCRT-I to endosomes in otherwise wild-type cells, accumulation could be observed if Vps4 was not present to dissociate ESCRT-I from these compartments. This suggested that residues upstream of 581 contributed to ESCRT-I localization as well, possibly the PSDP motifs at residues 524–527 and 447–450. In agreement with this prediction, truncation of Vps27 at residue 524 (Vps27$^{\Delta 524–622}$) did confer a defect in Vps23-GFP localization even in the absence of Vps4 ATPase activity. In *vps27*$^{\Delta 524–622}$ *vps4*Δ double mutant cells, Vps23-GFP was no longer efficiently recruited to punctate compartments but was mainly diffuse throughout the cytoplasm (Fig. 5). Thus, these findings have demonstrated that a region of Vps27 within residues 524–618 was essential for ESCRT-I endosomal localization. However, these results did not exclude potential contributions from additional regions in Vps27, such as the PSDP$^{447–450}$ motif.

Next, we expressed mutant forms of Vps27 in *vps27*Δ cells and examined MVB sorting of a GFP-CPS fusion reporter (Odorizzi et al., 1998). As expected, in cells expressing wild-type Vps27, GFP-CPS was delivered to the lumen of the vacuole, resulting in GFP fluorescence localized to the vacuole lumen (Fig. 6). As shown previously, GFP-CPS was mislocalized to the outer, limiting membrane of the vacuole and aberrant endosomal structures in cells lacking Vps27 (Odorizzi et al., 2000; Shih et al., 2002; Fig. 6). Consistent with a defect in ESCRT-I recruitment, GFP-CPS was missorted to the limiting membrane of the vacuole and accumulated on prevacuolar endosomes in cells expressing Vps27$^{\Delta 524–622}$ alone (Fig. 6). Interestingly, truncation of Vps27 at residue 581 (Vps27$^{\Delta 581–622}$) conferred a defect in MVB sorting of GFP-CPS similar to that found in *vps27*Δ cells (Fig. 6), likely due to impaired endosomal recruitment of Vps23-GFP as observed in *vps27*$^{\Delta 581–622}$ mutant cells (Fig. 5). Likewise, in cells expressing Vps27$^{P581G,\ T582S}$, GFP-CPS was missorted to the outer, limiting membrane of the vacuole (Fig. 6), in further support of a role for this conserved PTVP motif in Vps27/ESCRT-I association. We also followed the localization of another MVB reporter, 7NBD-labeled phosphatidylcholine (NBD-PC), in *vps27* mutant cells. In *pep4*Δ cells expressing wild-type Vps27, NBD-PC was efficiently sorted into the MVB pathway and subsequently transported to the vacuole lumen as previously shown (Bilodeau et al., 2002). However, *pep4*Δ *vps27*$^{\Delta 524–622}$ and *pep4*Δ *vps27*$^{\Delta 581–622}$

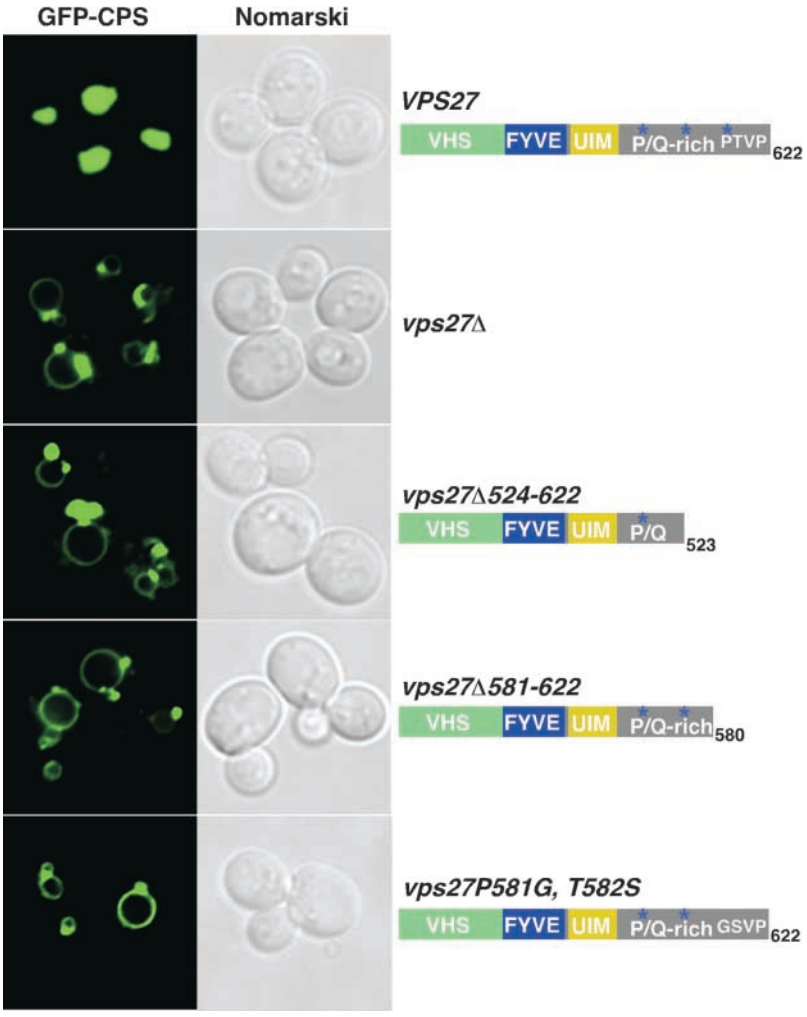

Figure 6. **Mutant forms of Vps27 defective in the efficient recruitment of ESCRT-I to endosomes display MVB sorting defects.** Localization of GFP-CPS (encoded by pGO45) in *vps27*Δ cells (MBY21) expressing either wild-type or the indicated mutant forms of Vps27 was observed by fluorescence and Normarski microscopy. Schematic representations of wild-type and mutant forms of Vps27 are shown. Truncations in the proline/glutamine-rich region (P/Q rich) and substitutions in the PTVP peptide motif are indicated. Asterisks indicate the presence of candidate PTAP-like motifs (PSDP$^{447–450}$, PSDP$^{524–527}$, and PTVP$^{581–584}$) in the various forms of Vps27.

mutant cells were impaired in the transport of NBD-PC to the vacuole lumen. Instead, NBD-PC accumulated in aberrant prevacuolar compartments (unpublished data), suggesting that Vps27/ESCRT-I association was necessary for the formation of MVB vesicles. Altogether, our results have identified a region in the COOH-terminal domain of Vps27 necessary for efficient recruitment of ESCRT-I to endosomes and implicated the PTVP motif in this process.

Discussion

Here, we examined the role for the yeast class E Vps proteins Vps27 and ESCRT-I (Vps23, Vps28, and Vps37) during MVB sorting. Both Vps27 and the ESCRT-I complex bound Ub and ubiquitinated MVB cargo. Efficient localization of Vps27 on endosomes required PI(3)P synthesis and an intact FYVE domain in Vps27. We found that Vps27 and ESCRT-I physically interact specifically on membranes. Our data indicated that Vps27 and ESCRT-I act sequentially during the initiation of the MVB sorting pathway. Recruitment of ESCRT-I to endosomal membranes required the function of Vps27 (via a COOH-terminal domain containing PTAP-like motifs), whereas localization of Vps27 did not require ESCRT-I function. Thus, MVB sorting is spatially and temporally controlled by the regulated and localized synthesis of PI(3)P at the endosome which then programs the ordered recruitment/activation of Vps27 followed by ESCRT-I. Vps27 and by extension its mammalian homologue, Hrs, therefore appear to function as key upstream activators of the MVB sorting pathway.

Recruitment of Vps27/Hrs to PI(3)P-containing endosomes

Numerous studies have implicated a critical role for phosphoinositides in membrane trafficking, such as PI(3)P in endosomal transport, which appears to exert its effects through effector proteins (Corvera et al., 1999; Odorizzi et al., 2000; Simonsen et al., 2001). Several lines of evidence suggest an important role for PI(3)P in the control of Vps27 in endosomal sorting. First, we observed extensive colocalization between GFP-Vps27 and the DsRed-based PI(3)P reporter used in this work (Fig. 2). Second, GFP-Vps27 was mislocalized throughout the cytoplasm in *vps34*Δ cells that are defective in PI(3)P synthesis. Third, in wild-type cells, mutant GFP-Vps27$^{\Delta FYVE}$ was not efficiently recruited to PI(3)P-containing endosomes. Together, these results indicated that Vps27 association with endosomal membranes was mediated by interactions between PI(3)P and the FYVE domain of Vps27. Finally, a previous study has demonstrated that substitutions in the FYVE domain of Vps27 impair its function, as assessed by effects upon the sorting and processing of the vacuolar hydolase carboxypeptidase Y (Piper et al., 1995). Although these findings highlight the importance of PI(3)P in activation/initiation of the MVB sorting pathway on late endosomes, they do not exclude additional roles for PI(3)P in endosomal sorting, such as the previously published roles for PI(3)P in endosome to vacuole transport (Sato et al., 2001) or in membrane recycling from the endosome to the Golgi compartments (Burda et al., 2002).

Although we have found that binding to PI(3)P was necessary for the efficient recruitment of Vps27 to endosomes, we cannot rule out the possibility that additional domains in Vps27 may stabilize membrane association via protein–protein interactions. Consistent with this idea, a small fraction of GFP-Vps27 could be observed in some punctate structures in *vps34*Δ cells. However, similar to our work on Vps27, previous studies have implicated the FYVE domain of Hrs in targeting to early endosomes. Earlier studies on Hrs indicated that endosomal localization of Hrs was disrupted in cells treated with wortmannin, an inhibitor of PI 3-kinase activity (Komada and Soriano, 1999; Raiborg et al., 2001a). Furthermore, a substitution in the FYVE domain has been shown to impair membrane targeting of Hrs. This particular study also identified additional coiled-coil domains in Hrs that mediate endosomal targeting in cooperation with PI(3)P binding (Raiborg et al., 2001b).

Interestingly, the coiled-coil domains in Hrs implicated in endosomal targeting have recently been shown to direct interactions with signal-transducing adaptor molecule (STAM) proteins (Bache et al., 2003). STAM proteins, which have several conserved domains including VHS, UIM, and SH3, were initially identified as proteins that interact with Eps15, a protein implicated in endocytic trafficking (Lohi and Lehto, 2001). Likewise, a previous study in yeast has indicated that Vps27 is present in a complex with a STAM-like protein, Hse1 (Bilodeau et al., 2002). Although the Vps27-Hse1 complex associated with Ub in vitro, Hse1 function was not necessary for the interaction of Vps27 with Ub in vitro or endosomal membranes in vivo (Bilodeau et al., 2002). We have found that deletion of *HSE1* did not confer a detectable defect in MVB sorting of CPS, or ESCRT-I membrane recruitment (unpublished data). Thus, additional protein–protein interactions may contribute to membrane recruitment of Vps27/Hrs as well. In support of this idea, one study has implicated another domain in Hrs consisting of a COOH-terminal proline/glutamine-rich region as essential for targeting to early endosomes (Hayakawa and Kitamura, 2000). We have identified an analogous domain in Vps27 as necessary for endosomal targeting of ESCRT-I (see Discussion). However, we have shown that the localization of GFP-Vps27 does not depend on ESCRT-I function (Fig. 3 B). Thus, potential interactions between ESCRT-I and this region are apparently not essential for endosomal localization of Vps27. Furthermore, we have GFP-tagged the mutant form of Vps27 (Vps27$^{\Delta 524–622}$) defective in ESCRT-I recruitment and observed that GFP-Vps27$^{\Delta 524–622}$ was present on endosomal membrane compartments (unpublished data). However, efficient membrane association may involve several interactions of Vps27/Hrs with lipid, ubiquitinated cargo, and ESCRT-I. Indeed, we have found that ESCRT-I may stabilize Vps27 binding to Ub (Fig. 1 B).

Recruitment/activation of ESCRT-I by Vp27/Hrs

Based on our data, we propose an ordered reaction in the mechanisms that initiate or activate MVB sorting (Fig. 7). First, Vps27 localization to endosomal membranes requires the production of PI(3)P, as demonstrated by increased cyto-

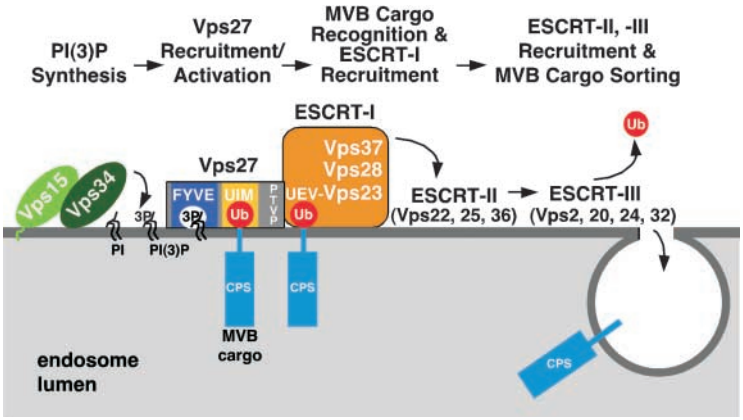

Figure 7. **Model for compartment specification of Ub-dependent sorting into MVB vesicles.** The Vps15/Vps34 complex synthesizes PI(3)P on endosomal membranes. The class E Vps protein Vps27 is targeted to PI(3)P-containing endosomal membranes via its FYVE domain (dark blue) where it can bind ubiquitinated MVB cargo via its UIM motifs (yellow). Vps27 subsequently recruits/activates ESCRT-I (orange) on endosomes. Ubiquitinated cargo (such as pCPS) is recognized by ESCRT-I (via the Ub E2 variant domain of Vps23), which initiates cargo entry into MVB vesicles. The action of a number of additional class E Vps proteins (ESCRT-II and ESCRT-III) is required for not only the function of this pathway but also for recruiting the deubiquitinating enzyme to remove Ub from cargo before its entry into invaginating vesicles. The concerted action of these proteins results in the sorting of cargo into the MVB pathway.

plasmic localization of GFP-Vps27 in *vps34*Δ cells. On endosomes, the PI(3)P effector Vps27 binds ubiquitinated MVB cargo and subsequently recruits and activates ESCRT-I. Activation of Vps27 on membranes may occur through a conformational change triggered by binding PI(3)P and Ub, thereby, exposing a binding site in Vps27 for ESCRT-I. In support of this model, we found that ESCRT-I physically interacts with Vps27 specifically on membranes but not in the cytoplasm (Fig. 4). Upon recruitment/activation by Vps27 on endosomes, ESCRT-I binds ubiquitinated MVB cargo as well. A possible role for the Vps27/ESCRT-I complex may be to facilitate the transfer of MVB cargo to the downstream ESCRT complexes. These later-acting ESCRTs are required for the continued sorting/concentration of MVB cargoes into nascent intraluminal vesicles, and also appear to coordinate the association of accessory factors responsible for such activities as Ub removal, ESCRT dissociation, and vesicle fission (Babst et al., 2002a,b). After assembly of downstream ESCRT machinery, Vps27 may be released to catalyze recruitment of additional ESCRT-I complexes.

Given that both Vps27 and ESCRT-I interacted with Ub in vitro and MVB cargo in vivo, we suggest that these proteins may interact with cargo in a sequential manner. Consistent with this, our results demonstrated that both Vps27 and ESCRT-I bound Ub independently of one another in vitro (Fig. 1, A and B). In addition, we found that Vps27 was able to associate with ubiquitinated precursor CPS in the absence of ESCRT-I function (Fig. 1 C). In such a model, binding of Vps27 to PI(3)P and ubiquitinated cargo might occur before recruitment of ESCRT-I. Several lines of evidence placed Vps27 upstream of ESCRT-I function and indicated a role for Vps27 in the membrane recruitment/activation of ESCRT-I. First, endosomal localization of Vps23 (ESCRT-I) was disrupted in the absence of Vps27 (Fig. 3 A). Second, Vps27 localization did not depend on the presence of Vps23 (Fig. 3 B). Third, in a companion paper, Bache and coworkers have demonstrated that Hrs and Tsg101 physically associate in vivo and that Hrs recruits Tsg101 to early endosomes (see Bache et al., 2003, in this issue).

We found that ESCRT-I physically interacts with Vps27 on membranes but not in the cytoplasm, consistent with a role for Vps27 in endosomal recruitment of ESCRT-I. COOH terminally truncated forms of Vps27 revealed a proline/glutamine-rich domain located within residues 524–622 as responsible for the endosomal localization of ESCRT-I (Fig. 5). This region of Vps27 contains two motifs ($PSDP^{524–527}$ and $PTVP^{581–584}$) that are similar to a peptide sequence within the HIV-1 Gag protein (PTAP) which is capable of interacting with Tsg101 (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). Analogous to the interaction of Gag and Tsg101, Vps27 may recruit/activate ESCRT-I on endosomal membranes via these motifs. Consistent with this idea, substitutions in the $PTVP^{581–584}$ motif of Vps27 resulted in increased cytoplasmic distribution of ESCRT-I in otherwise wild-type cells (Fig. 5) and a defect in MVB sorting (Fig. 6). Surprisingly, we did not observe MVB sorting defects in $vps27^{P524G}$ mutant cells, as GFP-CPS was properly localized within the vacuole lumen in these cells (unpublished data), suggesting that these motifs may not act in a simple, additive fashion. Accordingly, stabile association with ESCRT-I may involve multiple interactions with additional residues within this region of Vps27. In two companion studies, the Bache and Pornillos laboratories have identified a PSAP motif and a proline/glutamine-rich domain in Hrs that mediate interactions with Tsg101 (see Bache et al., 2003; and Pornillos et al., 2003 in this issue). Thus, multiple inputs within Vps27/Hrs appear to drive ESCRT-I recruitment/activation on endosomes.

We were unable to address whether ESCRT-I binding to MVB cargo occurs in a *vps27* mutant, as ESCRT-I is mislocalized to the cytoplasm in this context. This did not allow us to eliminate an alternative model to the sequential action of Vps27 and ESCRT-I in MVB sorting. UIM domain-containing proteins such as Eps15 and Hrs have been shown to be ubiquitinated in a manner that depends on their UIM domains, suggesting an auto-regulatory mechanism (Katz et al., 2002; Polo et al., 2002). An alternative possibility is that ubiquitination of Vps27/Hrs mediates association with ESCRT-I via Ub on Vps27/Hrs. Precedent for this alternative model exists in the case of Gag1, which is a PTAP motif-containing protein that is also ubiquitinated and this dramatically increases Tsg101's affinity for Gag (Pornillos et al., 2002b). However, we do not presently favor this model because ubiquitination of Eps15 and Hrs is mediated through the UIM domains of these proteins (Katz et al., 2002; Polo et al., 2002). Accordingly, the $Vps27^{UIM}$ mutant should not be ubiquitinated, nor would it interact with ESCRT-I (if Ub on Vps27 was a bridge for interaction). Importantly, Vps27

and ESCRT-I associate on membrane fractions from cells expressing Vps27^{UIM} and Vps27^{VHS} mutants (Fig. 4 B). Moreover, we have observed that Vps23-GFP is efficiently recruited to endosomal membranes in cells expressing Vps27^{UIM} and Vps27^{VHS} mutants (unpublished data). Regardless of this other possibility, our results clearly place Vps27 function upstream of ESCRT-I activity.

Our results suggest a model in which compartment specificity for the MVB sorting reaction is controlled by an interaction between Vps27 and PI(3)P, allowing ubiquitinated MVB cargoes to be selected at the appropriate location on late endosomes. Therefore, it is intriguing that sequences involved in Vps27 function and ESCRT-I recruitment are similar to a conserved motif in the HIV-1 protein Gag, which has been shown to interact with Tsg101 (Garrus et al., 2001). Therefore, Gag may be mimicking Vps27/Hrs, and, thereby, recruits the ESCRT machinery to an inappropriate membrane to execute viral assembly/budding (presumably bypassing a PI(3)P requirement). Indeed, in a companion paper, Pornillos et al. (2003) demonstrate that fusion of Hrs sequences to a mutant form of Gag lacking its PTAP motif restores viral particle budding. Importantly, these studies indicate that Hrs can effectively recruit the MVB machinery necessary for viral budding. Under these conditions, it will be of interest to examine whether the ESCRT machinery becomes limiting for normal cellular processes, such as receptor down-regulation or antigen presentation, which would provide potential protection of HIV from the immune system. Additional experiments will be necessary to address whether Hrs itself is involved in viral budding. In this regard, future studies may uncover additional proteins that mimic the function of Vps27/Hrs to redirect the ESCRT machinery to other cellular processes.

Materials and methods

Plasmid construction and yeast strains

VPS27 sequences were amplified from genomic DNA and cloned into pRS415 (Sikorski and Hieter, 1989) to create pEE27-2. pRS415*vps27*Δ524-622 and pRS415*vps27*Δ581-622 were constructed by PCR amplification of the relevant regions of the *VPS27* ORF and subcloning into pRS415. The *P581G, T582S* mutations were introduced into *VPS27* by Quikchange™ mutagenesis (Stratagene). The Vps27^{UIM} mutant-encoding plasmid has been described previously (Shih et al., 2002). pEE27-3 carrying a *VPS27-HA* fusion has been described previously (Shih et al., 2002). pDK*vps23-9*, the Vps23^{M85T}-encoding allele, was described previously (Katzmann et al., 2001), as was pEE23-10 that carries wild-type *VPS23* (Babst et al., 2000). pRS416-protein A has been described previously (Katzmann et al., 2001). The plasmid pDKA27 (protein A–Vps27) was created by PCR amplification of *VPS27* coding sequences and cloning into pRS416-protein A. The protein A–Vps27^{UIM} and protein A–Vps27^{VHS} plasmids were made by PCR amplification of either the *vps27^{UIM}* allele or the *vps27^{VHS}* allele and cloning into pDKA27. pMB123 (pRS414, Vps23-prot A) has been described previously (Babst et al., 2000). The plasmid pRS426GFP-Vps27 was constructed by PCR amplification of *VPS27* coding sequences and cloned into pGOGFP426 (Odorizzi et al., 1998). The GFP-Vps27$^{\Delta FYVE}$ encoding plasmid (pMB164) was created by PCR amplification of the relevant domains encoding *VPS27* and sequential cloning into pGOGFP426. The DsRed-FYVE^{EEA1} fusion (pRS425MET3-DsRed-FYVE) was created by PCR amplification of EEA1 FYVE domain–encoding sequences (Burd and Emr, 1998) and cloning into pRS425MET3-DsRed that expresses DsRed.T1 (Bevis and Glick, 2002) under control of the MET3 promoter. pGO45 (GFP-CPS) has been described previously (Odorizzi et al., 1998).

The following yeast strains were used: SEY6210 (*MAT leu2-3,112 ura3-52 his3-200 trp1-901 lys2-801 suc2-9* [Wurmser and Emr, 1998]); TVY614 (6210; *pep4*::*LEU2 prb1*::*hisG prc1*::*HIS3* [Wurmser and Emr, 1998]); EEY6-2 (6210; *vps23*::*HIS3* [Babst et al., 2000]); DKY61 (TVY614; *vps23*::*HIS3* [Katzmann et al., 2001]); MBY21 (6210; *vps27*::*HIS3* [Shih et al., 2002]); MBY52 (TVY614; *vps27*::*HIS3* [this paper]); PHY102 (6210; *vps34*::*TRP1* [Wurmser and Emr, 1998]); DKY54 (6210; *VPS23-GFP*::*HIS3* [Katzmann et al., 2001]); DKY55 (6210; *VPS23-GFP*::*HIS3 vps4*::*TRP1* [Katzmann et al., 2001]); DKY82 (6210; *VPS23-GFP*::*HIS3 vps34*::*TRP1* [this paper]); DKY78 (6210; *VPS23-GFP*::*HIS3 vps27*::*HIS3* [this paper]); DKY79 (6210; *VPS23-GFP*::*HIS3 vps4*::*TRP1 vps27*::*HIS3* [this paper]; SEY18A9 (6210; *vps27Δ pep4*::*LEU2* [Emr lab collection]).

Microscopy

Living cells expressing Vps23-GFP, GFP-Vps27, and/or DsRed-FYVE were harvested at an OD_{600} of 0.4–0.6 and resuspended in PBS for visualization. Visualization of cells was performed on a fluorescence microscope (Axiovert S1002TV; Carl Zeiss MicroImaging, Inc.) equipped with FITC and rhodamine filters, captured with a digital camera (Photometrix), and deconvolved using Delta Vision software (Applied Precision Inc.). Results presented were based on observations of >100 cells.

In vivo biochemical analyses

For the copurification of Ub-pCPS or ESCRT-I (Vps23 and Vps28) with protein A–Vps27, 100 OD_{600} equivalents of cells were harvested, washed in water plus 5 mM *N*-ethylmaleimide (NEM), spheroplasted, and lysed in Hepes lysis buffer (20 mM Hepes-KOH, pH 6.8, 50 mM potassium acetate, 2 mM EDTA plus protease inhibitors: 5 μg/ml antipain, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 10 μg/ml 2-macroglobulin, and 0.1 mM AEBSF) plus 10 mM NEM. Membranes were isolated at 13,000 *g* and resolubilized in lysis buffer plus 0.5% lauryldimethylamineoxide (LDAO; Calbiochem-Novabiochem) by incubation on ice for 10 min. Samples were cleared by centrifugation at 13,000 *g* and supernates were processed to purify protein A as described previously (Katzmann et al., 2001). Material was then visualized by Western blotting with either anti-CPS (Odorizzi et al., 1998), anti-Vps23 (Babst et al., 2000), or anti-Vps28 (Katzmann et al., 2001) antibodies.

In brief, for the copurification of Vps27-HA with Vps23–protein A, 100 OD_{600} equivalents of cells were harvested, washed in water plus 5 mM NEM, spheroplasted, and lysed in Hepes lysis buffer plus 10 mM NEM. Pelletable membrane and soluble cytosolic fractions were generated by centrifugation at 13,000 *g* and equilibrated in lysis buffer plus 0.5% LDAO by incubation on ice for 10 min. Samples were cleared by centrifugation at 13,000 *g* and supernates were processed to purify protein A as described previously (Katzmann et al., 2001). Material was then visualized by Western blotting with anti-HA antibodies.

In vitro binding studies

GST or Ub-GST was purified, glutathione was removed by a desalting column, and the equivalent amounts of the resulting purified proteins were covalently linked to CnBr-activated Sepharose, all according to the manufacturer's instructions (Amersham Biosciences). Cellular extracts were prepared essentially as described previously (Katzmann et al., 2001). In brief, 100 OD_{600} units of cells were glass bead lysed in Hepes lysis buffer plus 5 mM NEM plus 0.5% Triton X-100. Lysates were cleared at 20, 000 × *g* and passed over immobilized GST or Ub-GST. Bound material was washed three times with Hepes lysis buffer plus 0.5% Triton X-100, once with Hepes lysis buffer plus 0.05% Triton X-100, and once with Hepes lysis buffer alone. Elution was performed with 0.5 M, pH 3.4, acetate buffer, eluted material was TCA precipitated, acetone washed, and resolubilized in sample buffer. Approximately 20 OD_{600} units of bound material and 1.0 OD_{600} units of load were subjected to SDS-PAGE and Western blotting with anti-Vps23 (Babst et al., 2000) or anti-HA antisera.

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