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Ubiquitin-Dependent Sorting into the Multivesicular Body Pathway Requires the Function of a Conserved Endosomal Protein Sorting Complex, ESCRT-I

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Summary

The multivesicular body (MVB) pathway is responsible for both the biosynthetic delivery of lysosomal hydrolases and the downregulation of numerous activated cell surface receptors which are degraded in the lysosome. We demonstrate that ubiquitination serves as a signal for sorting into the MVB pathway. In addition, we characterize a 350 kDa complex, ESCRT-I (composed of Vps23, Vps28, and Vps37), that recognizes ubiquitinated MVB cargo and whose function is required for sorting into MVB vesicles. This recognition event depends on a conserved UBC-like domain in Vps23. We propose that ESCRT-I represents a conserved component of the endosomal sorting machinery that functions in both yeast and mammalian cells to couple ubiquitin modification to protein sorting and receptor downregulation in the MVB pathway.

Introduction

The endosomal system coordinates protein transport from both the biosynthetic and endocytic pathways (Gruenberg and Maxfield, 1995). Cell surface receptors that have been internalized and delivered to the endosomal system can either be recycled to the plasma membrane or degraded via delivery to the lumen of the lysosome/vacuole. A critical step in receptor downregulation occurs in late endosomes when the limiting membrane invaginates and buds into the lumen of the organelle to form a multivesicular body (MVB) (Felder et al., 1990; Gruenberg and Maxfield, 1995). During this process, a subset of the membrane proteins within the limiting membrane of the endosome is sorted into these invaginating vesicles. Subsequent fusion of the mature MVB with the lysosome/vacuole results in the delivery of the internal vesicles, along with their associated cargoes, to the lumen of the lysosome/vacuole where they can be degraded by a host of hydrolytic enzymes (Futter et al., 1996). Proteins that remain in the limiting membrane of the MVB are delivered to the limiting membrane of the lysosome/vacuole. This process, referred to as the MVB sorting pathway, thereby sorts proteins destined for the lumen of the lysosome/vacuole away from proteins destined for the limiting membrane of this organelle.

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Perhaps the best example of endosomal sorting in the MVB pathway is the downregulation of activated epidermal growth factor receptor (EGFR) (Sorkin, 1998). Upon binding agonist, activated EGFRs are rapidly internalized and a significant portion of these internalized receptors are sorted into the MVB pathway and degraded following delivery to the lumen of the lysosome (Felder et al., 1990). Entry of EGFR into the MVB pathway is a saturable process and requires the activity of the EGFR kinase, sorting signals in the EGFR tail, and the ubiquitin ligase c-Cbl (Felder et al., 1990; French et al., 1994; Kornilova et al., 1996; Levkowitz et al., 1998, 1999; Yokouchi et al., 1999). c-Cbl is involved in the ubiquitination and subsequent downregulation of activated EGFR. Oncogenic forms of Cbl that are defective for ligase activity result in the recycling of activated EGFR back to the plasma membrane. This continued recycling and prolonged signaling in cells expressing oncogenic forms of Cbl is thought to contribute to tumorigenesis (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). MVB sorting and the subsequent lysosomal degradation of signaling cell surface receptors is therefore a critical mechanism for regulating the potency of agonist-induced signaling.

The MVB pathway is essential for the delivery of biosynthetic and endocytic membrane proteins to the lumen of the yeast vacuole (Odorizzi et al., 1998). The G protein-coupled pheromone receptor Ste2 is downregulated by delivery to the lumen of the vacuole via this pathway (Odorizzi et al., 1998). The vacuolar hydrolase carboxypeptidase S (CPS) is a biosynthetic cargo that requires a functional MVB sorting pathway for delivery to the lumen of the vacuole (Odorizzi et al., 1998). Precursor CPS (pCPS) is synthesized as a type-II transmembrane protein which transits the secretory pathway to the endosomal system, where it is selectively sorted into the invaginating vesicles of the MVB. Delivery to the lumen of the vacuole results in hydrolytic clipping of pCPS to its mature, soluble form (mCPS) (Spormann et al., 1992). Other biosynthetic cargoes, like the hydrolase dipeptidylaminopeptidase B (DPAP B), remain in the limiting membrane of the MVB and after fusion with the vacuole are delivered to the limiting membrane of the vacuole (Roberts et al., 1989).

Little is known about the molecular details of MVB sorting; however, a role for specific lipids like 3′-phosphoinositides and lysobisphosphatidic acid has been suggested (Fernandez-Borja et al., 1999; Kobayashi et al., 1999; Odorizzi et al., 1998). In addition, we have recently demonstrated that the products of a subset of the vacuolar protein sorting (*VPS*) genes, the class E *VPS* genes, are required for protein sorting in the MVB pathway (Odorizzi et al., 1998). Presently, there are 15 known members of the class E *VPS* family, in which mutations confer defects in MVB sorting and exaggerated endosomal compartments referred to as "class E compartments" (Raymond et al., 1992). The class E compartment has been shown to accumulate both biosynthetic and endocytic cargoes (Babst et al., 1997; Odorizzi et al., 1998; Piper et al., 1995). Importantly, yeast

class E proteins have orthologs in other organisms including humans, indicating the molecular mechanism for MVB sorting is highly conserved. For example, the class E Vps protein Vps23 has a mammalian homolog, TSG101 (tumor susceptibility gene 101). Like Vps23, TSG101 is found in a 350 kDa complex (Babst et al., 2000). Consistent with the phenotype of yeast cells deleted for *VPS23*, mammalian cells defective for TSG101 function are impaired both for the delivery of the lysosomal hydrolase cathepsin D and for the downregulation of activated EGFR (Babst et al., 2000). It therefore appears that these proteins function at similar steps in the MVB pathway, but the precise biochemical mechanism of action has remained unclear.

In the present work, we show that ubiquitination of endosomal cargo serves as a signal for sorting into the vesicles that invaginate into the MVB. Furthermore, we present data which support a model in which the Vps23-containing protein complex, ESCRT-I, recognizes ubiquitinated cargo. Taken together, these data suggest that a subset of the Vps class E proteins initiate ubiquitin-dependent MVB sorting by selectively binding ubiquitinated cargo and directing sorting of these cargoes into MVB vesicles.

Results

ESCRT-I Is a 350 kDa Protein Complex Composed of the Vps23, Vps28, and Vps37 Proteins

VPS23 is one of the class E *VPS* genes and is allelic to *STP22*, which was identified by a genetic screen for mutants defective in turnover of misfolded cell surface receptors (Li et al., 1999). We have previously demonstrated that Vps23 is a subunit of a 350 kDa complex that also contains the class E Vps28 protein and an unknown protein or proteins (Babst et al., 2000). In order to identify additional components of this complex, it was purified using a Vps23-protein A fusion protein that fully complemented the protein sorting and morphology defects seen in *vps23*Δ (Babst et al., 2000). Figure 1A depicts a silver-stained gel of the proteins associated with protein A alone or Vps23-protein A fusion using IgG-sepharose affinity chromatography. Western blot analysis demonstrated that two of the bands present in the Vps23-protein A lane corresponded to the Vps23-protein A fusion itself and Vps28 (data not shown). In addition to Vps23 and Vps28, a 25 kDa protein also was detected in the Vps23-protein A lane and not in the control lane. Matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS) was performed on this 25 kDa band. It was identified as the yeast *SRN2* gene product, a small coiled-coil containing protein. *SRN2* was identified as a suppressor of an *rna1-1* allele (Hong et al., 1998) that appears to encode an unstable form of the Rna1 protein (Traglia et al., 1989). The defect in stability of the *rna1-1* gene product may be suppressed by impairing endosomal function. Deletion of *SRN2* yielded a class E phenotype, and complementation analysis revealed that *SRN2* was allelic to the only remaining unidentified class E *VPS* gene from the original VPS screens, *VPS37* (data not shown). Deletion of *VPS37*/*SRN2* not only resulted in a class E Vps phenotype, but also had a dramatic effect on the gel filtration elution profile of both Vps23 and Vps28, dropping the peak size from approximately 350 kDa in a wild-type cell to approximately 250 kDa in a *vps37*Δ (Figure 1B). Two hundred and fifty kilodaltons also corresponds to the size of the complex produced when the only form of Vps23 in the cell has its coiled-coil domain deleted (data not shown), suggesting that Vps37 and Vps23 interact via their coiled-coil domains. A variety of experimental approaches further suggest that the 350 kDa complex contains multiple copies of both Vps28 and Vps37 (data not shown) and a single copy of Vps23 (Babst et al., 2000). Taken together, these data suggest that Vps23, Vps28, and Vps37/Srn2 are the only components of the 350 kDa complex that exist in stoichiometric quantities (see Figure 1A). We refer to this complex as ESCRT-I (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport) because of its role in sorting in the endosomal system.

To analyze the localization of ESCRT-I, a *VPS23-GFP* fusion was constructed that fully complemented the protein sorting and morphology defects in a *vps23*Δ mutant. This fusion was integrated into the chromosome, replacing the endogenous *VPS23* gene, in either wild-type or class E mutant backgrounds. In wild-type cells, the Vps23-GFP fusion localizes to numerous small punctate structures, and also produces a significant cytosolic signal (Figure 2A). In addition, *VPS23-GFP* was integrated into a *vps4*Δ strain. Vps4 is a AAA-ATPase that has been demonstrated to regulate the membrane association of class E Vps proteins, its activity being required for their removal (Babst et al., 1998). In a *vps4*Δ strain, it can be seen that the soluble pool of Vps23-GFP is substantially diminished, consistent with the idea that the ATPase activity of Vps4 is required for the removal of ESCRT-I from endosomal membranes. To verify that the punctate structures represent endosomes, colocalization was performed with the previously described endosomal protein Snf7/Vps32 (Babst et al., 1998). *vps4*Δ cells expressing the integrated *VPS23-GFP* fusion were fixed and subjected to indirect immunofluorescence with anti-Snf7 antibody. It can be seen that Snf7 and Vps23-GFP colocalize, with a portion of Vps23-GFP localizing to the class E compartment and a portion localizing to additional punctate structures probably corresponding to additional endosomes (Figure 2B). As we are unable to detect monomeric Vps23 in cell extracts (Figure 1B), it appears that Vps23 exists exclusively as a component of ESCRT-I and therefore is a suitable indicator for the localization of the complex. From these studies, we conclude that ESCRT-I associates with endosomal membranes and that inactivation of Vps4 stabilizes ESCRT-I association with endosomes. This places the putative site of action of ESCRT-I at the endosome, where MVB formation is occurring.

Analysis of the primary sequence of ESCRT-I components failed to identify any motifs that offered obvious insight into the function of Vps28 and Vps37. However, the presence of a UBC-like (ubiquitin conjugating-like) domain in Vps23 (Babst et al., 2000) indicated a potential role for the protein in ubiquitin modification or recognition. This domain of Vps23 has homology to E2 UBCs, but lacks a critical active site cysteine present in bona fide UBCs. Without this cysteine, Vps23 cannot function as a ubiquitin-conjugating enzyme. In an initial attempt to understand the function of this domain, we randomly mutagenized the UBC-like domain of Vps23 using error-prone PCR mutagenesis and screened for loss-of-func-

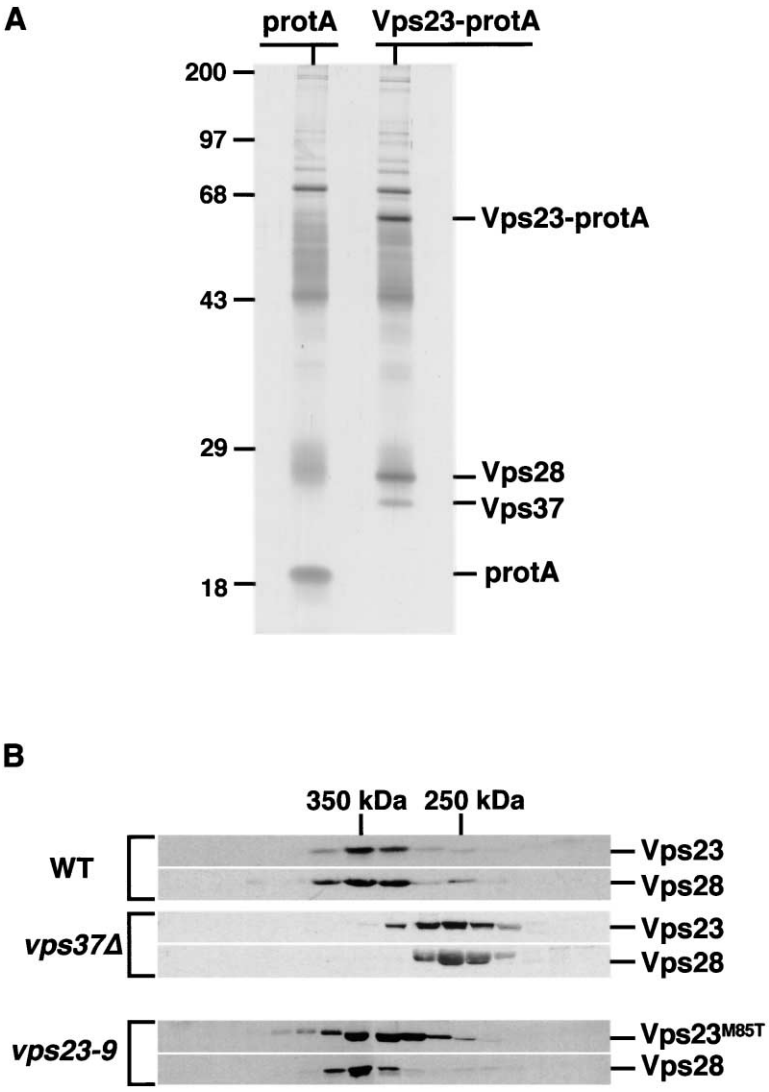

Figure 1. ESCRT-I Is a 350 kDa Complex Composed of Vps23, Vps28, and Vps37

(A) Vps23-protein A (pMB123) was transformed into EEY6 (*vps23*Δ) or pRS416-prot A was transformed into SEY6210 (wild-type), lysates were generated, cleared at 100,000 × g, and protein A or Vps23-protein A was isolated with IgG-Sepharose. Bound material was eluted at low pH and visualized by SDS-PAGE and silver staining.

(B) Gel filtration analysis of cell extracts from SEY6210 (WT), DKY48 (*vps37*/*srn2*Δ), and Vps23^{M85T} (EEY6 with pDK*vps23-9*). Fractions were analyzed with antibodies specific for Vps23 or Vps28.

tion mutations. This screen identified a point mutation within the UBC-like domain of Vps23 (*vps23-9*) that changed the methionine at position 85 to threonine (Vps23^{M85T}). Vps23^{M85T} was found to be functionally inactive, yet the protein was neither destabilized nor defective for assembly into the 350 kDa ESCRT-I complex (Figure 1B).

We reasoned that while the UBC-like domain in Vps23 is presumably not functioning as a UBC (given the lack of the appropriate active site cysteine), it may be capable of interacting with ubiquitin. Given the importance of ubiquitin-dependent downregulation of cell surface receptors and the inability of *vps23*Δ mutants to efficiently sort biosynthetic and endocytic cargo into the MVB pathway, an attractive model is that Vps23 initiates sorting into the MVB pathway by selectively binding to ubiquitinated cargo. A prediction of this model is that biosynthetic MVB cargo would also receive a ubiquitin modification.

Precursor CPS Is Ubiquitinated

We have previously characterized the vacuolar hydrolase CPS as a biosynthetic cargo of the MVB pathway (Odorizzi et al., 1998). Precursor CPS (pCPS) is synthesized as a type-II integral membrane protein. In an endosomal compartment, pCPS is sorted away from other proteins, such as DPAP B, destined for the limiting membrane of the vacuole by inclusion into the internal vesicles of MVBs (Odorizzi et al., 1998). Fusion of MVBs with the vacuole results in the delivery of these internalized vesicles and pCPS (as well as additional cargo proteins) to the lumen of the vacuole. Within the lumen of the vacuole, pCPS is cleaved from its transmembrane anchor by resident hydrolases to yield a mature soluble form (mCPS) (Spormann et al., 1992). To identify candidate *cis*-acting signals involved in initiating entry of pCPS into the MVB pathway, we examined the primary sequence of the cytosolic tail of pCPS. Given the importance of ubiquitination for the MVB-dependent downregulation of a number of cell-surface proteins, the existence of two lysines at positions 8 and 12 of the amino-terminal tail of pCPS represented candidate acceptor sites for covalent ubiquitin modification. To test this hypothesis, we needed to design experiments that allowed the detection of intact pCPS because proteolytic processing of pCPS, as is the case upon delivery to the vacuole,

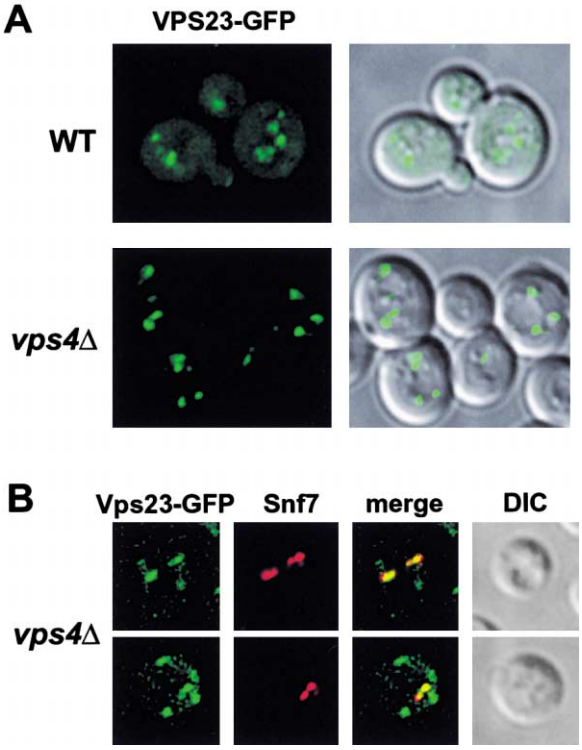

Figure 2. ESCRT-I Localizes to Endosomes

(A) Localization of Vps23-GFP in either DKY54 (wild-type) or DKY55 (*vps4*Δ) living cells by fluorescence and DIC microscopy.

(B) Colocalization of Vps23-GFP and Snf7 in fixed *vps4*Δ cells (DKY55) by fluorescence and DIC microscopy.

removes its transmembrane and small cytosolic tail, the portion of pCPS that would receive the ubiquitin moiety. For this reason, strains that included the triple vacuolar protease mutations, *pep4*Δ *prb1*Δ *prc1*Δ, were used whenever pCPS would be exposed to vacuolar proteases to prevent the removal of the transmembrane and cytoplasmic tail domains of pCPS.

The ubiquitination status of pCPS was addressed by denaturing immunoprecipitations using antibody specific for CPS followed by Western blotting with anti-ubiquitin antibodies. In wild-type cells, a low level of ubiquitinated pCPS (Ub-pCPS) was detected (Figure 3A). Mutations in the *DOA4* gene, which encodes a deubiquitinating enzyme in yeast, result in the accumulation of high molecular weight ubiquitinated substrates within the cell (Swaminathan et al., 1999). We reasoned that if Doa4 was acting to remove ubiquitin from cargoes destined for entry into the MVB pathway, as has been proposed (Amerik et al., 2000), then loss of Doa4 should result in an accumulation of Ub-pCPS. Indeed, performing the anti-CPS immunoprecipitation followed by anti-ubiquitin Western blotting from a *doa4*Δ strain revealed a dramatic increase in the level of Ub-pCPS (Figure 3A). Together, these data demonstrate that pCPS is ubiquitinated and inactivation of the Doa4 deubiquitinase stabilizes this modification. Ubiquitin is a 76 amino acid protein with a molecular weight of approximately 8.5 kDa. Therefore, the apparent size shift of approximately 10 kDa suggests that pCPS receives a single ubiquitin. The presence of a doublet of both pCPS and Ub-pCPS bands is the result of differentially glycosylated forms of CPS. pCPS is modified with one or two N-linked core oligosaccharides in the lumen of the endoplasmic reticulum during its biogenesis (Spormann et al., 1992).

Biosynthetic Ubiquitination of CPS

To address the subcellular compartment in which pCPS was receiving the ubiquitin modification, we utilized mutants that are defective in the biosynthetic transport of pCPS at distinct stages between the endoplasmic reticulum and the vacuole (refer to model in Figure 3B). In either a *gga1*Δ *gga2*Δ mutant or the temperature sensitive for function *sec7-1* mutant at restrictive temperature, both of which block transport out of the Golgi (Costaguta et al., 2001; Franzusoff and Schekman, 1989; Puertollano et al., 2001), we were not able to detect Ub-pCPS (Figure 3A). Consistent with these results, we used pulse-chase analysis and found that Ub-pCPS could be detected after a short pulse-labeling at permissive temperature, but not at the restrictive temperature in *sec7-1* cells. Ub-pCPS was not detected in *gga1*Δ *gga2*Δ at low or high temperature (data not shown). This suggests that pCPS is being ubiquitinated subsequent to exiting the Golgi, or that Golgi function is intimately linked to ubiquitination of pCPS.

We next analyzed mutants that are defective for fusion of transport intermediates with either the endosome or vacuole. Loss of the endosomal t-SNARE Pep12 blocks the delivery of Golgi-derived transport intermediates to late endosomes (Becherer et al., 1996; Robinson et al., 1988). The *pep12*Δ mutant was found to stabilize Ub-pCPS, and this accumulation was not exaggerated in a *pep12*Δ *doa4*Δ double mutant (Figure 3A). Loss of the vacuolar t-SNARE Vam3 has been shown to result in the accumulation of MVBs that are unable to fuse with the vacuole (Darsow et al., 1997). Loss of Vam3 function blocked the accumulation of Ub-pCPS; however, when combined with the *doa4*Δ mutation (*vam3*Δ *doa4*Δ), Ub-pCPS was stabilized (Figure 3A). From this, we conclude that pCPS is ubiquitinated in a *vam3*Δ strain, but the ubiquitin is removed by Doa4 during sorting of pCPS into the MVBs that accumulate in a *vam3*Δ mutant. Loss of both Doa4 and Vam3 function results in sorting of Ub-pCPS into MVBs that cannot fuse with the vacuole. Taken together, these results indicate that pCPS receives a ubiquitin modification subsequent to exiting the Golgi, but prior to delivery to the Pep12-containing endosomes. Furthermore, because Ub-pCPS is stabilized in the *pep12*Δ mutant even in the presence of wild-type Doa4, it suggests that Doa4 is localized to the Pep12-containing endosome or a later endosomal compartment.

As class E Vps proteins are required for proper function of the MVB pathway, loss of class E Vps function may also stabilize Ub-pCPS. In Figure 3A, we show that deletion of two separate class E genes, *vps4*Δ and *vps23*Δ, results in dramatic stabilization of Ub-pCPS. These results clearly indicate that ubiquitin can still be efficiently ligated onto pCPS in the class E mutants, but removal of ubiquitin by Doa4 is severely delayed or inhibited. Furthermore, even though Vps23 contains a

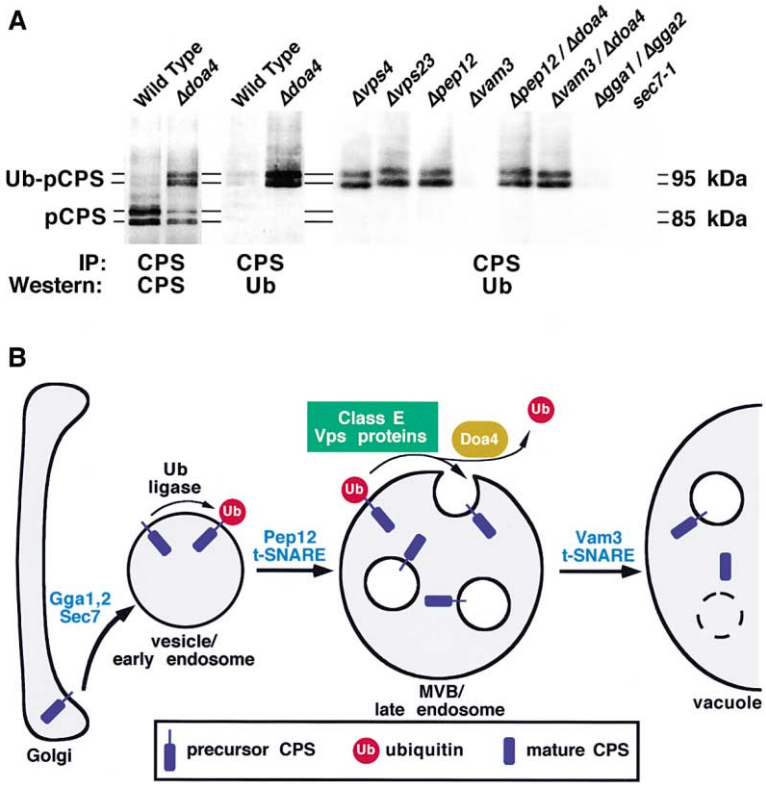

Figure 3. Ubiquitination of pCPS Occurs Prior to a Pep12 Endosome, and Deubiquitination Occurs Prior to Entry into the Vesicles that Invaginate into the MVB

(A) CPS was immunoprecipitated from a variety of strains, followed by Western blotting with either anti-CPS or anti-ubiquitin antibodies. Strains are as follows: Wild-Type (TVY614), *doa4*Δ (DKY51), *vps4*Δ (MBY52), *vps23*Δ (DKY61), *pep12*Δ (CBY31), *vam3*Δ (TDY2), *pep12*Δ/*doa4*Δ (DKY52), *vam3*Δ/*doa4*Δ (DKY53), *gga1*Δ/*gga2*Δ (YCS150), and *sec7-1* (RSY299).

(B) Schematic representation of biogenesis of CPS. Precursor CPS (pCPS) transits the Golgi and receives a ubiquitin modification prior to the Pep12 compartment. In this compartment, the class E Vps machinery initiates protein sorting and formation of MVB vesicles. Prior to entry into the MVB vesicles, Doa4 acts to remove the ubiquitin moiety from pCPS, thereby recycling ubiquitin back into the cytoplasm. Fusion of the MVB with the vacuole is a Vam3-dependent step and results in the delivery of pCPS to the lumen of the vacuole where it is matured via the action of vacuolar hydrolases.

UBC-like domain, this protein is not required for the addition of ubiquitin to pCPS (see *vps23*Δ lane, Figure 3A).

Ubiquitin Modification as a Sorting Signal in the MVB Pathway

The utility of a GFP-CPS fusion for monitoring protein sorting in the MVB pathway has been previously demonstrated (Odorizzi et al., 1998). We used this fusion protein to determine the role of ubiquitination in the trafficking of CPS. Site-directed mutagenesis was utilized to change the two putative ubiquitin-acceptor lysines at amino acid positions 8 and 12 in the cytosolic tail of pCPS to arginines, and the ubiquitination status of both wild-type and mutant forms of the GFP-CPS fusion protein were analyzed. As can be seen in Figure 4A, precursor forms of both the wild-type fusion and the GFP-CPS^{K12R} mutant are substrates for ubiquitin modification. In contrast, both GFP-CPS^{K8R} and GFP-CPS^{K8,12R} are not modified with ubiquitin. In the case of the GFP-CPS fusions that receive ubiquitin modification (WT and K12R), the mobility of the upper doublet of bands suggests that the upper band has been modified with a second ubiquitin moiety, as the molecular weight shift is approximately 10 kDa. This is presumably via a linkage to the first ubiquitin, as it only appears when the lysine at amino acid position 8 is present. It is also worth noting that even in the case of GFP-CPS^{K8R} and GFP-CPS^{K8,12R} mutants, where ubiquitinated forms of these proteins are not detected, it is still possible to detect endogenous Ub-pCPS (Figure 4A). These data demonstrate that K8 in pCPS serves as an acceptor for ubiquitin modification.

The consequence of a block in ubiquitination of the GFP-CPS fusions was examined via fluorescence microscopy. As expected, wild-type GFP-CPS is delivered to the lumen of the vacuole via the MVB pathway, resulting in a GFP fluorescence pattern that is localized to the lumen of the vacuole (Figure 4B). In striking contrast, both GFP-CPS^{K8R} and GFP-CPS^{K8,12R} are mislocalized to the limiting membrane of the vacuole. This was not the case for GFP-CPS^{K12R}, which was localized to the lumen of the vacuole in a manner indistinguishable from the wild-type GFP-CPS fusion protein. Therefore, the data indicate that ubiquitination of pCPS is required for proper sorting of the GFP-CPS fusion into the internal vesicles of the MVB.

To test whether the sequence in the cytoplasmic tail of pCPS that is modified with ubiquitin is not only necessary but also sufficient for sorting in the MVB pathway, we fused the coding sequence for amino acids 5–11 from pCPS (PVEKAPR) to DPAP B, a vacuolar protein that normally traffics to the vacuole limiting membrane. This fusion places the pCPS$_{5\text{-}11}$ sequence in the cytosolic portion of DPAP B between GFP and the amino terminus of DPAP B (GFP- CPS$_{5\text{-}11}$-DPAP B). In Figure 5A, it can be seen that while the wild-type GFP-DPAP B traffics to the limiting membrane of the vacuole, GFP-CPS$_{5\text{-}11}$-DPAP B is delivered to the lumen of the vacuole in wild-type cells. Furthermore, when this fusion protein is expressed in the class E *vps4*Δ mutant, it is now missorted to the limiting membrane of the vacuole, as well as the class E compartment. Thus, as is the case for CPS (Odorizzi et al., 1998), the GFP-CPS$_{5\text{-}11}$-DPAP B fusion traffics to the lumen via the MVB pathway. To test whether the CPS$_{5\text{-}11}$ peptide was conferring the ability to receive ubiquitin onto DPAP B, both GFP-DPAP B and

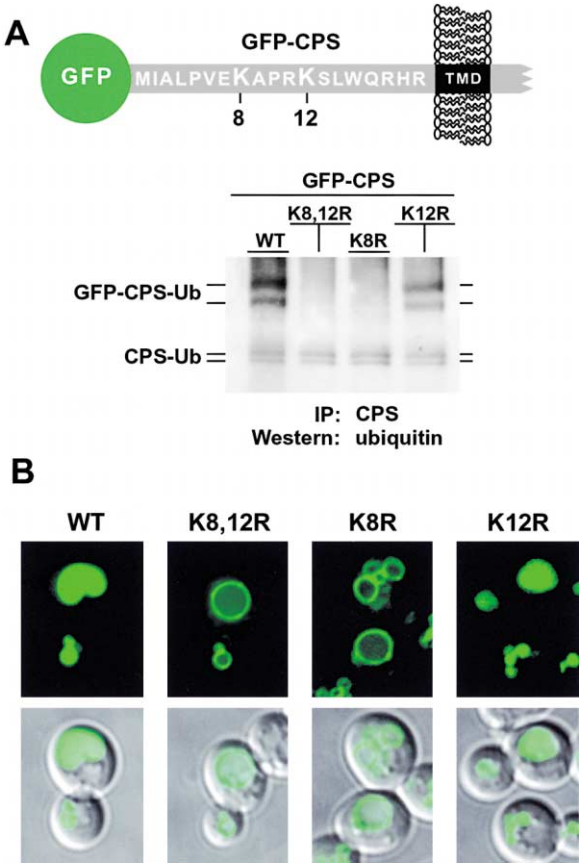

Figure 4. Ubiquitination of pCPS Correlates with Entry into the MVB Pathway

(A) Schematic representation of the cytosolic and transmembrane domains of GFP-CPS and ubiquitination of lysine mutant forms of GFP-CPS. GFP-CPS and K to R mutants were transformed into DKY51, and CPS was immunoprecipitated with anti-CPS antisera and subjected to anti-ubiquitin Western blotting.

(B) Fluorescence and DIC microscopy of yeast cells expressing various forms of GFP-CPS in wild-type cells.

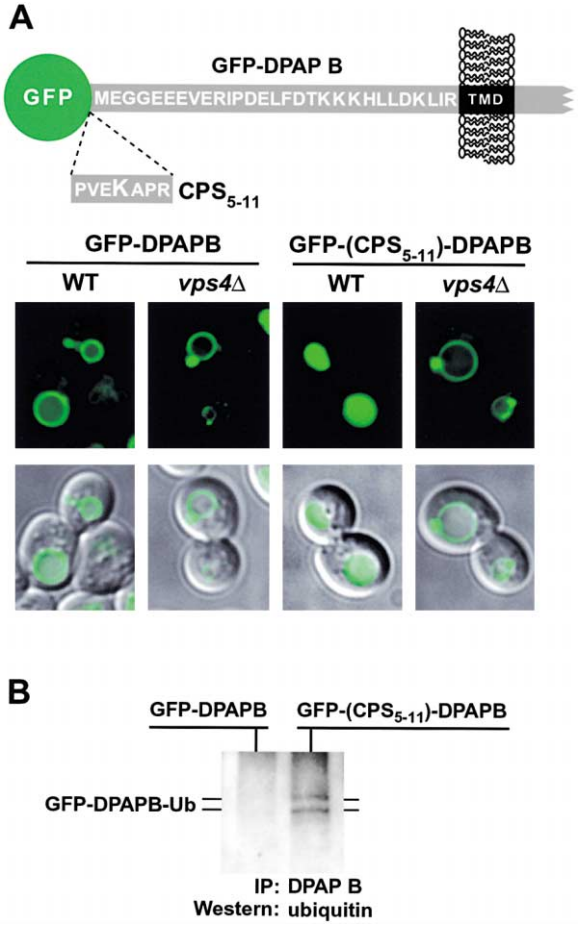

Figure 5. Ubiquitination Is Sufficient for Entry into the MVB Pathway

(A) Schematic representation of the cytosolic and transmembrane domains of GFP-DPAP B (pGO89), as well as the chimera containing the peptide with amino acid residues 5–11 from CPS (GFP-CPS_{5-11}-DPAP B) (pMB225). Fluorescence and DIC microscopy images of yeast cells expressing either pGO89 or pMB225 chimeras in either wild-type (SEY6210) or *vps4Δ* (MBY3) cells. The sequence "PVEKAPR" (CPS_{5-11}) was inserted between GFP and DPAP B, as indicated by the dashed lines.

(B) pGO89 and pMB225 were transformed into DKY51, and DPAP B was immunoprecipitated with anti-DPAP B antisera and subjected to anti-ubiquitin Western blotting.

GFP-CPS_{5-11}-DPAP B were immunoprecipitated using anti-DPAP B antisera and subjected to Western blotting with anti-ubiquitin antibody (Figure 5B). As was the case with GFP-CPS, a doublet is seen for GFP-CPS_{5-11}-DPAP. Once again, the ubiquitination status of the GFP-DPAP B fusion proteins correlates with their sorting phenotype. GFP-DPAP B is not modified with ubiquitin and sorts to the vacuole limiting membrane, while GFP-CPS_{5-11}-DPAP B is ubiquitinated and sorts to the vacuole lumen. We conclude that ubiquitination can serve as a sorting signal for entry into the MVB pathway.

ESCRT-I Interacts with Ubiquitin

It is apparent that proper sorting through the MVB pathway requires both ubiquitination of cargo and the function of class E Vps proteins. Furthermore, because Vps23 contains a UBC-like motif and a mutation in this domain renders the protein functionally inactive, we decided to test whether Vps23 is capable of binding to ubiquitin. We performed protein-protein interaction studies utilizing either GST alone or ubiquitin fused to GST. In this fusion protein, ubiquitin was fused to the amino terminus of GST (a gift from Stefan Jentsch; Koegl et al., 1999). Either GST alone or ubiquitin-GST was purified and then immobilized on Cyanogen Bromide-activated Sepharose beads and incubated with detergent-solubilized extracts prepared from cells producing either wild-type Vps23 or $Vps23^{M85T}$. The beads were washed and bound material was eluted with a low pH wash and subjected to Western blotting using anti-Vps23 or anti-Vps28 antibodies. Figure 6A shows that when extracts from wild-type cells are incubated with ubiquitin-GST beads, it is possible to detect binding of both Vps23 and Vps28. In contrast, no binding of Vps23 or Vps28 is detected when GST alone is used. These data indicate that the Vps23-containing ESCRT-I complex is capable of binding to ubiquitin. Perhaps more compelling is the observation that no binding of either

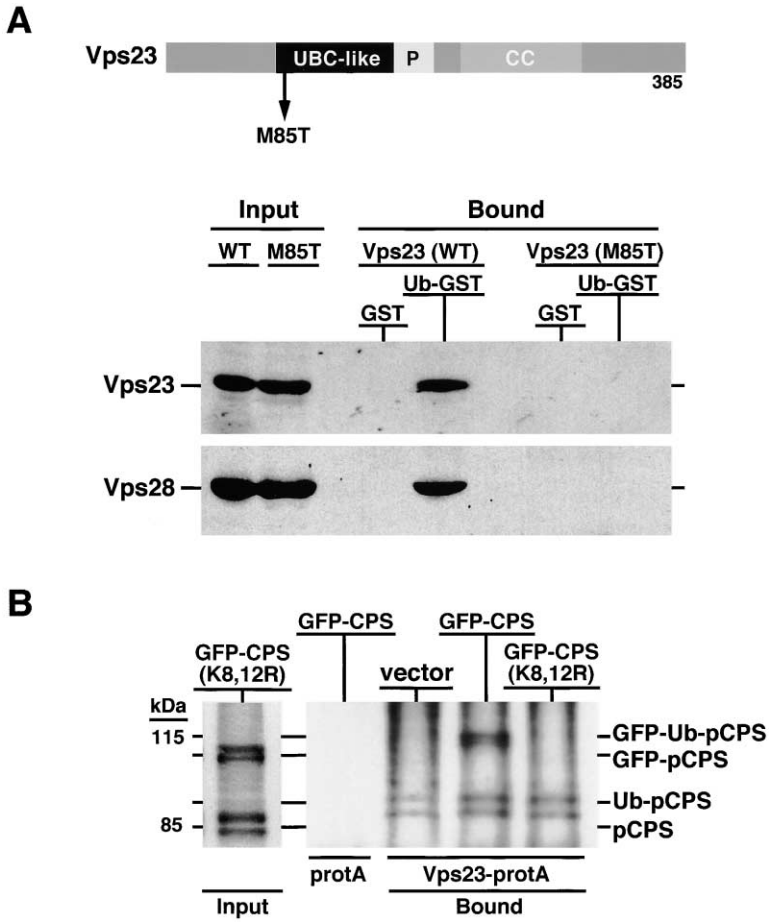

Figure 6. Vps23 Binds Ubiquitinated MVB Cargo

(A) Schematic representation of Vps23 as well as protein-protein interaction data between Vps23 and ubiquitin. GST or ubiquitin-GST was purified and immobilized prior to incubation with extracts from either wild-type cells or cells expressing Vps23^{M85T}. Bound proteins were visualized by Western blotting with either anti-Vps23 or anti-Vps28 antisera. P = proline rich, CC = coiled coil.

(B) Interaction between Vps23 and Ub-pCPS. MBY50 was transformed with plasmids expressing either pRS416-prot A or pMB123. In addition, these strains were transformed with plasmids encoding either GFP-CPS (pGO45) or GFP-CPS^{K8,12R} (pMB208). Membranes were isolated, detergent solubilized, and prot A or Vps23-prot A was isolated using IgG-sepharose. Bound and Input fractions were subjected to SDS-PAGE and analyzed with anti-CPS antibody by Western blot (for simplicity, only the input for pMB208-containing cells are shown, illustrating the molecular weight of ubiquitinated species).

Vps23 or Vps28 is detected when extracts from cells producing Vps23^{M85T} are incubated with ubiquitin-GST. While the simplest interpretation would be that this domain is interacting directly with ubiquitin, the approach used (the source of ESCRT-I in the binding experiments is yeast lysate) does not allow us to make the conclusion that the interaction is direct. However, these data clearly indicate that the UBC-like domain of Vps23 is required for the interaction of ESCRT-I components with ubiquitin, whether via an intermediate protein or a direct interaction.

ESCRT-I Interacts with Ubiquitinated-CPS

To facilitate the detection of an interaction between Vps23 and ubiquitinated cargo, we utilized a class E mutant strain where both ubiquitinated cargo and Vps23 accumulate in an endosomal compartment. The class E *snf7*Δ strain was transformed with either protein A or Vps23-protein A and either GFP-CPS or GFP-CPS^{K8,12R}. Membranes were isolated from these various transformants, solubilized with detergent, and protein A or Vps23-protein A was isolated from the solubilized membranes using IgG-sepharose. Bound material was then subjected to Western blotting with anti-CPS antibody. Figure 6B shows that the wild-type form of GFP-CPS, and not the GFP-CPS^{K8,12R} form, copurifies with Vps23-protein A. Furthermore, it can be seen that endogenous pCPS binds to Vps23-protein A, but not to protein A alone. Vps23 can therefore interact with forms of pCPS that are ubiquitinated, but not to GFP-CPS that is not modified with ubiquitin. The endogenous pCPS and GFP-CPS that are bound to Vps23-protein A migrate at molecular weights of the ubiquitinated forms of these proteins (compare pCPS and GFP-CPS from input with bound in Figure 6B). We also examined the bound fractions from these experiments for GFP-DPAP B, and found that it is not present (data not shown). It has been demonstrated that numerous class E Vps proteins localize to the class E compartment. To rule out the possibility that Vps23 is interacting with pCPS via a large class E Vps protein aggregate, we examined the bound fractions for the presence of two additional class E Vps proteins, Vps24 and Vps22, and were unable to detect their presence (data not shown). Taken together with the in vitro binding studies, these data indicate that Vps23/ESCRT-I binds Ub-pCPS in vivo.

Discussion

MVBs are formed when the limiting membrane of the endosome invaginates and buds into its lumen. Proteins that have been sorted into these vesicles are delivered to the lumen of the lysosome/vacuole upon the fusion of the MVB with this organelle where resident hydrolases (including lipases and proteases) act on both the vesicles and the proteins contained therein. Proteins that

have been retained in the limiting membrane of the MVB are either recycled (e.g., nonubiquitinated cell surface receptors) or delivered to the limiting membrane of the lysosome/vacuole (e.g., DPAP B) upon fusion of the MVB with the vacuole/lysosome. The MVB sorting pathway is therefore critical not only for the function of the lysosome/vacuole (delivery of hydrolases), but also the degradation/downregulation of endocytic cargoes and the recycling of cargoes back to the Golgi and plasma membranes.

In this study, we demonstrate that ubiquitination serves as a sorting signal for entry of cargo into the MVB pathway. Furthermore, we provide evidence that Vps23, a component of the ESCRT-I complex, binds ubiquitin in vitro and is capable of interacting with ubiquitinated cargo in vivo. We therefore conclude that ESCRT-I is acting in the recognition of ubiquitinated cargoes at the endosome and initiating transport of these cargoes into the vesicles that invaginate into late endosomes to form an MVB. Other class E Vps proteins appear to act downstream of this event to regulate the formation of these vesicles (our unpublished data). Our data indicate that cargoes destined for delivery into MVB vesicles are actively sorted away from cargoes that remain in the limiting membrane of the MVB. Mutations in the signal for ubiquitination and MVB sorting result in retention of proteins like pCPS^{K8R} in the limiting membrane of the MVB and vacuole.

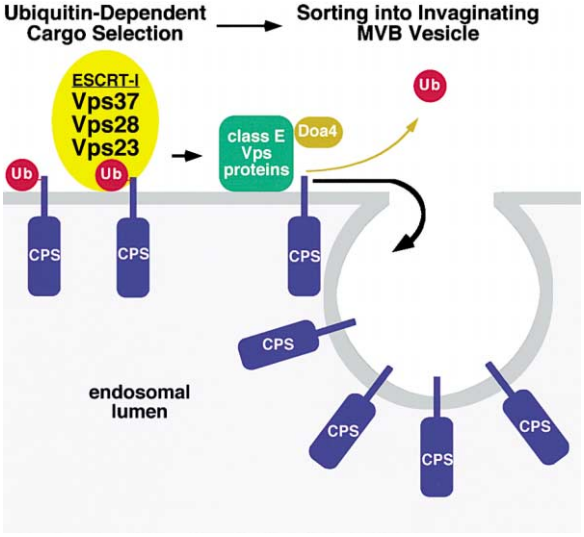

Figure 7. Model for Ubiquitin-Dependent Sorting into the MVB Vesicles

Ubiquitinated cargo is recognized by ESCRT-I, which initiates cargo entry into MVB vesicles. The action of a number of additional class E Vps proteins is required for not only the function of this pathway, but also for recruiting the deubiquitinating enzyme Doa4 to remove ubiquitin from cargo prior to its entry into invaginating vesicles. The concerted action of these proteins results in the sorting of cargo into the MVB pathway.

Ubiquitination of CPS

The finding that pCPS is transiently ubiquitinated has allowed us to gain significant insight into the role of ubiquitin in the delivery of cargoes into the lumen of the vacuole. Ubiquitin has been shown to be involved in a variety of cellular functions including proteasomal degradation, endocytosis, and the regulation of transcription factors (for recent review see Weissman, 2001). Polyubiquitination serves as a signal for degradation via the proteasome for a large number of proteins involved in a variety of cellular functions (Chau et al., 1989; recently reviewed in Weissman, 2001). In addition, reports have suggested that the trafficking fate of two amino acid permeases, Tat2 and Gap1, is regulated via polyubiquitination (Beck et al., 1999; Helliwell et al., 2001); polyubiquitination results in their delivery to the vacuole for turnover under certain nutrient conditions when their transport to the cell surface is not required. Ubiquitin's role in protein trafficking has also been well established as a signal for endocytosis and downregulation of cell surface proteins (reviewed in Hicke, 2001). These studies have indicated that the addition of a single ubiquitin moiety, or in some cases two ubiquitins linked via lysine 63 of ubiquitin itself, is sufficient to induce the internalization of cell surface proteins which are subsequently degraded in the vacuole/lysosome. We now demonstrate that in addition to a role in internalization, monoubiquitination also serves as a signal for entry into the MVB pathway. This provides a potentially satisfying explanation for the mechanism for degradation of ubiquitinated cell surface proteins. Consistent with this idea, Kölling and colleagues recently concluded that limiting levels of free ubiquitin impairs the delivery of the plasma membrane localized ABC-transporter Ste6 to the lumen of the vacuole (Losko et al., 2001).

Based on our analysis of ubiquitination of pCPS in various transport mutants, it appears that ubiquitin is added in a post-Golgi but pre-Pep12 t-SNARE-containing late endosomal compartment. This may represent a transport vesicle or, more likely, an early endosomal compartment. In addition, sorting into MVB vesicles occurs either within the Pep12-containing late endosome or a post-Pep12 compartment, but clearly prior to the Vam3 t-SNARE-containing vacuole compartment. The finding that class E Vps mutants not only block MVB formation, but also stabilize Ub-pCPS indicates that class E Vps proteins are not required for ubiquitination of pCPS, but rather play a role in deubiquitination of pCPS. Taken together with the finding that endosomal association of the Doa4 deubiquitinating enzyme appears to be at least in part regulated via class E Vps proteins (Amerik et al., 2000), these proteins may be acting in concert to couple sorting of ubiquitinated cargo into invaginating vesicles with removal and recycling of free ubiquitin back into the cytoplasm.

Ubiquitination and MVB Sorting

We found that ubiquitin serves as a sorting signal for entry into the MVB pathway. Furthermore, DPAP B, a protein that normally is neither ubiquitinated nor sorted into MVB vesicles, can be directed into these vesicles by addition of the ubiquitination signal from the cytosolic tail of CPS. There is, however, clearly some level of specificity in the ubiquitination of proteins destined for entry into the MVB pathway. The primary sequence "PVEKAPR" enables an as-yet-unidentified ubiquitin ligase to mark a protein (e.g., pCPS or GFP-CPS$_{5\text{-}11}$-DPAP B) for entry into the MVB sorting pathway. DPAP B itself

contains four lysines in its cytosolic tail, none of which receive ubiquitin (Figure 5B). Presumably the specificity is bestowed via specific recognition of the pCPS tail by the ligase machinery. Identification of this ligase will aid in understanding this specificity, as well as the precise subcellular compartment in which pCPS is ubiquitinated.

Role for ESCRT-I in MVB Sorting

Protein-protein interaction studies revealed that the ESCRT-I complex can bind ubiquitin. Intriguingly, Vps23 contains a UBC-like domain and a point mutation in this domain blocked ESCRT-I binding to ubiquitin. This demonstrates that the UBC-like domain of Vps23 is required for this interaction. While it is possible that ESCRT-I interacts with another protein via its UBC-like domain and this protein is responsible for binding ubiquitinated cargo, the simplest interpretation is that ESCRT-I directly interacts with ubiquitin. Attempts to demonstrate a direct interaction between bacterially expressed Vps23 and ubiquitin have failed, but this is not surprising given that Vps23 is part of a 350 kDa complex that includes other Vps proteins, Vps28 and Vps37. Stable interaction of Vps23 with ubiquitin may require assembly of the complete ESCRT-I complex.

Native immunoprecipitations revealed an interaction between Ub-pCPS and ESCRT-I. This interaction between Ub-pCPS and ESCRT-I is consistent with ESCRT-I functioning as a sorting receptor that actively sorts Ub-pCPS into MVB vesicles. While other formal possibilities exist, our studies have not identified any class E Vps proteins (MVB machinery) as being ubiquitinated and thereby serving as targets for ESCRT-I interaction. Nor do we see any defect or change in the ubiquitination pattern of pCPS in a mutant (*vps23*Δ) lacking ESCRT-I function (Figure 3A), consistent with ESCRT-I not serving as a part of the ubiquitin ligase machinery, but rather functioning downstream of ubiquitination.

In summary, we propose the following model (Figure 7). Ub-pCPS, as well as other cargoes (e.g., cell surface receptors, like ubiquitinated Ste2 in yeast), are recognized at the endosome by ESCRT-I. This recognition initiates a cascade of events that ultimately results in the sorting of these cargoes by other downstream class E Vps components into the invaginating MVB vesicles. The end result of this concerted action of the class E Vps proteins is the active separation of cargoes destined to stay in the limiting membrane of the MVB from those destined for delivery into MVB vesicles. Once ESCRT-I has served its specificity function, cargo becomes accessible to Doa4 prior to entry into the invaginating vesicles. Doa4, recruited to the endosome by class E Vps proteins (Amerik et al., 2000), acts upon the ubiquitinated cargo to remove the ubiquitin moiety, thereby allowing it to recycle back into the cytoplasm prior to final entry of pCPS into MVB vesicles.

The role for ubiquitin in sorting cargo at the MVB in yeast parallels a role for ubiquitin in the downregulation of ligand-activated cell surface receptors in mammalian cells. A role for ubiquitination in downregulation of a number of cell surface proteins by delivery to the lysosome/vacuole has been demonstrated (recently reviewed in Hicke, 2001). Our finding that ubiquitination of endosomal cargo serves as a sorting signal for inclusion into MVB vesicles offers an explanation for the mechanism of downregulation. In mammalian cells, downregulation of activated EGFRs is dependent upon the action of the c-Cbl ubiquitin ligase, which tags the signaling receptor for downregulation, rather than recycling (Felder et al., 1990; French et al., 1994; Kornilova et al., 1996; Levkowitz et al., 1998, 1999; Yokouchi et al., 1999). Failure to ubiquitinate and downregulate activated receptors, as in the case of cells expressing dominant-negative oncogenic forms of Cbl, results in receptor recycling, prolonged signaling, and tumorigenesis (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). This is entirely consistent with our observation that failure to ubiquitinate what would normally be an MVB cargo results in its missorting. We also have recently demonstrated that cell lines defective for TSG101, the mammalian homolog of Vps23, display a number of endosome-associated transport defects which include both defects in maturation of the lysosomal hydrolase Cathepsin D and the recycling, rather than degradation, of activated EGFRs (Babst et al., 2000). Again, if the machinery responsible for recognizing ubiquitinated cargo and initiating its entry into the MVB vesicles is defective, recycling of activated receptors would be expected. Lastly, the mammalian form of the AAA-ATPase Vps4, SKD1, has been demonstrated to regulate the endosomal membrane association of both TSG101 and mammalian Vps28, suggesting that the MVB machinery is highly conserved (Bishop and Woodman, 2001). Further characterization of ESCRT-I in both yeast and mammalian cells should provide important insights into the molecular details by which this complex recognizes cargoes destined for the MVB vesicles and escorts them to other class E Vps machineries that ultimately appear to form the invaginating MVB vesicles and sort ubiquitinated cargoes into these vesicles.

Experimental Procedures

Plasmid Construction and Yeast Strains

pGO45 (GFP-CPS) and pGO89 (GFP-DPAP B) have been described (Odorizzi et al., 1998). The plasmids pMB208 (GFP-CPS^{K8,12R}), pMB214 (GFP-CPS^{K8R}), and pMB215 (GFP-CPS^{K12R}) were constructed by PCR-based mutagenesis of pGO45 (Odorizzi et al., 1998). BglII/HindIII cut pGOGFP426 (Cowles et al., 1997b) was ligated with a double-stranded oligonucleotide encoding the amino acid sequence PVEKAPR and the HindIII fragment of pGO89 (Odorizzi et al., 1998), resulting in pMB225 (GFP-CPS$_{5\text{-}11}$-DPAP B). pRS416-protein A has been described elsewhere (Sato et al., 2000), as has pMB123 (pRS414, Vps23-prot A) (Babst et al., 2000). Integrated *VPS23-GFP* was made by the method of Longtine et al. (Longtine et al., 1998). pDK*vps23-9*, the Vps23^{M85T}-encoding allele, was made by random PCR mutagenesis of pEE23-10 (Babst et al., 2000) and DNA sequencing to identify the mutation.

The following yeast strains were used: SEY6210 (*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9* [Robinson et al., 1988]); YCS150 (SEY6210; *gga1*Δ::*TRP1 gga2*Δ::*KanMx* [Costaguta et al., 2001]); TVY614 (6210; *pep4*Δ::*LEU2 prb1*Δ::*hisG prc1*Δ::*HIS3* [Wurmser and Emr, 1998]); EEY6-2 (6210; *vps23*Δ*1*::*HIS3* [Babst et al., 2000]); DKY48 (SEY6210; *srn2*Δ::*HIS3* [this study]); DKY51 (TVY614; *doa4*Δ::*HIS3* [this study]); DKY61 (TVY614; *vps23*Δ::*HIS3* [this study]); TDY2 (SEY6210; *vam3*Δ::*LEU2* [Darsow et al., 1997]); CBY31 (SEY6210; *pep12*Δ::*HIS3* [Burd et al., 1997]); DKY52 (SEY6210; *pep12*Δ::*HIS3 doa4*Δ::*HIS3* [this study]); DKY53 (SEY6210; *vam3*Δ::*LEU2 doa4*::*HIS3* [this study]); MBY52 (TVY614; *vps4*Δ::*TRP1* [this study]); MBY50 (TVY614; *snf7*Δ::*HIS3* [this study]);

DKY54 (6210; *VPS23-GFP*::*HIS3* [this study]); DKY55 (6210; *VPS23-GFP*::*HIS3 vps4*Δ::*TRP1* [this study]); RSY299 (MATα *sec7-1 ade2-1 his3-11,15 leu2-3,112 trp1-*Δ*1 ura3-1* [Franzusoff and Schekman, 1989]).

Microscopy

Fixation and antibody decoration for fluorescence microscopy were performed as described (Babst et al., 1998). Living cells expressing Vps23-GFP were harvested at an OD_{600} of 0.5–0.6 and resuspended in PBS for visualization. Visualization of living or fixed cells was performed on a Zeiss Axiovert S1002TV fluorescence microscope equipped with FITC and rhodamine filters, captured with a Photometrix digital camera, and deconvolved using Delta Vision software (Applied Precision Inc., Issaquah, WA).

Biochemical Analyses

For purification of protein A or Vps23-protein A, 100 OD_{600} units of cells were spheroplasted and lysed as described (Darsow et al., 2001) and purified as described in Sato et al. (2000). Approximately 20 OD_{600} equivalents of eluted material were subjected to SDS-PAGE and visualized by silver staining. For identification of Vps37 by MALDI-mass spectroscopy, 4000 OD_{600} units of *vps23*Δ cells expressing Vps23-protein A were spheroplasted, lysed, and cleared at 100,000 × g as above. Cleared material was loaded onto a DEAE-Sepharose (Amersham Pharmacia) column and bound material was washed with 20 mM Tris (pH 7) plus NaCl in a step-wise fashion until the salt concentration was 150 mM. Bound material was eluted at 170 mM NaCl and eluate was concentrated with a 30 kDa cutoff concentrator (Amicon, Inc., Beverly, MA). Vps23-protein A was then purified and processed as above, the gel was coomassie stained, and the 25 kDa band was cut out and analyzed by MALDI-MS (Scripps Center for Mass Spectrometry, Scripps Research Institute, La Jolla, CA). For the copurification of Ub-pCPS with Vps23-protein A, 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 α_2-macroglobulin, and 0.1 mM AEBSF) plus 10 mM NEM. Membranes were isolated at 13,000 × g and these were resolubilized in lysis buffer plus 1.0% Laurydimethylamineoxide (Calbiochem-Novabiochem Co., San Diego, CA) by incubation on ice for 10 min. Samples were cleared at 13,000 × g and supernates were processed as above. Gel filtration was performed as previously described (Darsow et al., 2001).

Immunoprecipitations

Denaturing immunoprecipitations were carried out essentially as described (Darsow et al., 2001), with the addition of 5 mM NEM to lysis (6 M urea, 1% SDS, 50 mM Tris, pH 7.5, 1 mM EDTA) and antibody incubation buffers (50 mM Tris, pH 7.5, 150 mM sodium chloride, 0.5% Tween-20, and 0.1 mM EDTA). For DPAP B immunoprecipitations, material was treated with Endoglycosidase H as previously described (Cowles et al., 1997a) prior to SDS-PAGE. Anti-DPAP B antisera was a generous gift of Tom Stevens. Material was then visualized by Western blotting with either anti-CPS (Cowles et al., 1997a), anti-GFP (a gift of Charles Zuker), or anti-ubiquitin antibody (Zymed Laboratories Inc., San Francisco, CA). For anti-CPS Western blotting, approximately 0.5 OD_{600} equivalents were subjected to SDS-PAGE, for ubiquitin Western blotting approximately 2 OD_{600} were loaded.

In Vitro Binding Studies

Glutathione S-transferase (GST) or ubiquitin-GST were purified, glutathione was removed by a PD-10 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 Pharmacia). Cellular extracts were prepared essentially as described in Darsow et al. (2001) with the following modifications. 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 36, 000 × g and passed over immobilized GST or ubiquitin-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 or 0.5 OD_{600} units of load were subjected to SDS-PAGE and Western blotting with anti-Vps23 or anti-Vps28 antisera (Babst et al., 2000).

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