CHARACTERIZATION OF CLASS D VPS PROTEINS

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DEDICATION

I would like to thank first my committee, whose patience is immeasurable, and my advisor, Bruce Horazdovsky, whose faith in me persisted even as my own wavered. The same can be said of my mother, who gave up her own scientific career to provide for my brother and me. Her vitality continues to amaze me, and I feel fortunate to call her my friend. Both Malcolm and I will marry this year, and I look forward to the future as we bring up our families together. A degree is, at best, the fifth most important thing to happen to me in Dallas. I have found life-long friends in Tamara, Dara, Lori, Chad, Steve, and Lysandra. Being here has also given me the opportunity to truly grow close to my father and to form a lasting relationship with my younger brothers, Jonathan and Lawrence. Finally, Dallas, of all places, is where I have found my fiancée, Julie, who has given me direction and purpose, and with whom I can't wait to start the rest of my life.

CHARACTERIZATION OF CLASS D VPS PROTEINS

by

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DISSERTATION

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The vacuole of the yeast Saccharomyces cerevisiae is functionally similar to the mammalian lysosome. The components of the VPS (vacuolar protein sorting) system are responsible for proper delivery of vacuolar biosythetic enzymes. Efforts to dissect the genetics of this system have revealed several classes of mutants, each defective in one transport step in the VPS pathway. The Class D VPS proteins are thought to control anterograde traffic between the late Golgi and late endosome. Although most of these proteins have homologues of known function in other systems, two exceptions are the Vps3p and Vps8p proteins. Analysis of Vps3p reveals that it is associated with a high-density structure, possibly a coated vesicle or a large protein complex. The Vps8p protein contains a C-terminal H2 RING finger motif, a domain often associated with E3 ubiquitin ligase activity. *In vitro* analysis reveals that a Vps8p fragment containing this domain has this activity. Deletion of the RING finger reveals that the endocytic marker Ste3p accumulates in an abnormally large late-endosome-derived structure, but that sorting of the soluble vacuolar cargo CPY is relatively unaffected. These results suggest a division of function within the Vps8p molecule.

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LIST OF ABBREVIATIONS

AP	adaptor protein
EGFR	epidermal growth factor receptor
MVB	multi-vesicular body
pCPS	precursor carboxypeptidase S
pCPY	precursor carboxypeptidase Y
SM	Sec1p/Munc18
SNARE	soluble N-ethylmaleimide attachment protein recptor
Ub	ubiquitin
UIM	ubiquitin interaction motif
VPS	vacuolar protein sorting

CHAPTER ONE

Introduction

Membrane traffic in yeast

Saccharomyces cerevisiae continues to serve as an invaluable model for the study of membrane traffic. Work in yeast, far more so than any other organism, has revealed not only the general principles that govern the majority of membrane trafficking events, but also specific examples of how these principles are applied to the regulation of cell physiology.

The three major arms of membrane traffic in yeast are illustrated in Figure 1. The secretory pathway (Figure 1A) is essential for yeast growth. Membrane that allows for the expansion of the daughter bud, proteins required for biogenesis of the cell wall, and molecules necessary for the acquisition of extracellular nutrients are delivered to the cell surface in 100 nm secretory vesicles (Finger and Novick 1998). Secretory proteins are synthesized on the surface of the ER, and cotranslationally inserted into the lumenal space or into the membrane. From there they are transported to the cis-Golgi, and, either by vesicular transport or through maturation of the Golgi cisternae, move to the late Golgi. Here they are sorted into secretory vesicles, which are delivered to and fuse with

the plasma membrane through the action of the exocyst complex and associated proteins.

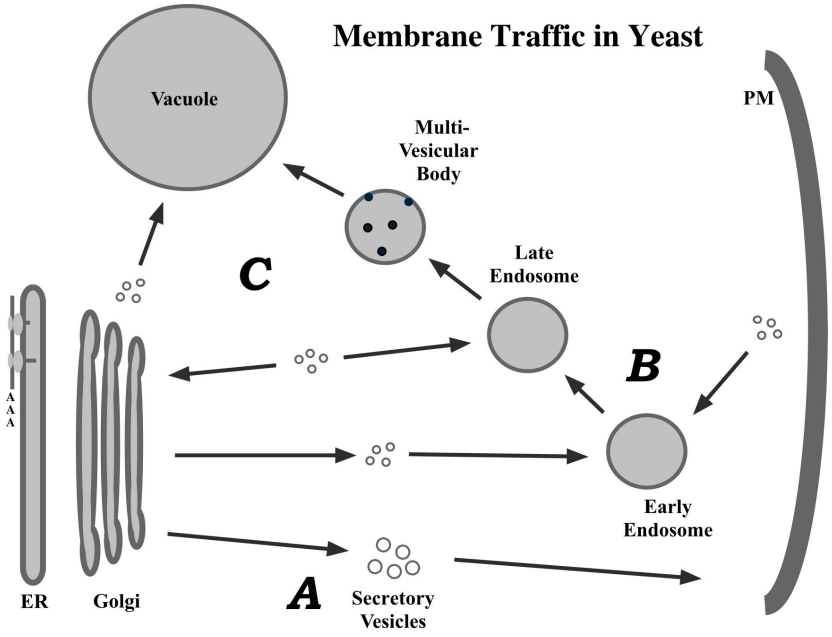

Figure 1 **Membrane Traffic in Yeast.** Although there is extensive overlap, there are three main systems of membrane traffic in yeast. The secretory system (A) is responsible for the delivery of proteins to the extracellular space. The endocytic system (B) controls internalization and delivery to the vacuole of membrane-bound and extracellular fluid phase material. The vacuolar biosynthetic pathway (C) controls delivery of nascent vacuolar proteins. It overlaps with the early stages of the secretory pathway through the late Golgi, and the late stages of the endocytic pathway from the late endosome to the vacuole.

Fluid-phase endocytosis (Figure 1B) is also an essential process (Wendland et al. 1998; Shaw et al. 2001). Nutrient transporters, as wells as receptors for mating factors and other molecules are sorted into small (50 nm) endocytic vesicles and internalized. In addition to transport vesicles, the endocytic system includes specialized organelles known as endosomes. Although there is some debate surrounding the number and nature of endosomal subtypes, most models propose the existence of at least two persistent organelles (Pelham 2002). In yeast, a post Golgi or early endosome receives traffic from

the cell surface and from the TGN (*t*rans-*G*olgi *n*etwork). Like its mammalian counterpart, this is presumed to be a recycling compartment from which internalized material can be quickly returned to the cell surface. Mammalian cells also have a "slow" recycling compartment that typically resides near the nucleus. It is not clear whether yeast have such an organelle, or whether the TGN serves in this capacity. The late or prevacuolar endosome receives traffic from the early endosome and from the TGN, and is the source of retrograde traffic back into the Golgi. The membrane of this organelle invaginates, forming the internal vesicles of the MVB (*m*ulti-*v*esicular *b*ody). This is a short-lived structure that quickly matures into or fuses with the yeast equivalent of the mammalian lysosome, the vacuole.

The VPS System

The vacuole is a large, acidified organelle that, while not essential for growth, is important for a wide variety of cellular processes including osmoregulation, pH and ion homeostasis, degradation of endocytic and autophagic material, and storage of metabolites. The vacuolar biosynthetic pathway (Figure 1C) is responsible for delivery of most of the newly-made proteins required for vacuolar function (Horazdovsky et al. 1995). It intersects with the proximal portion of the secretory pathway up to the point of the late Golgi. From there, vacuolar proteins are separated from secretory

traffic, packaged into transport vesicles, and delivered to the vacuole through the action of the VPS (*v*acuolar *p*rotein *s*orting) system. The VPS system itself controls several arms of membrane traffic between the Golgi and vacuole. Clathrin-coated transport vesicles containing proteins destined for the lumen of the vacuole are first delivered to the late endosome, merging with endocytic traffic from the plasma membrane. Another branch of the VPS system retrieves Golgi material from the late endosome. Finally, some of the proteins bound for the limiting membrane of the vacuole are delivered in transport vesicles by a route that bypasses the late endosome.

In addition to a signal sequence that allows for contranslational translocation into the lumen of the ER, soluble vacuolar proteins contain sequences that specify delivery to the late endosome and vacuole. It is the characterization of these sequences that eventually allowed for the genetic dissection of the VPS system. Johnson, et al. (Johnson et al. 1987) discovered that the N-terminal 50 amino acids of the soluble vacuolar protease CPY (*c*arboxy*p*epetidase *Y*) could confer vacuolar localization to any protein to which it was fused. Lack of this sequence caused accumulation of CPY in the late Golgi, non-specific incorporation into secretory vesicles, and secretion into the extracellular space. Bankaitis, et al (1986) took advantage of this phenomenon to devise a screen to isolate mutants that are defective in sorting of proteins to the vacuole. They fused the N-terminus of CPY to invertase, a normally secreted enzyme that converts sucrose to fructose and glucose. By expressing this construct in a mutant lacking the wild-type version of invertase, a strain was created that could only grow on sucrose if the CPY-invertase fusion was for some reason secreted. Since it contains the CPY pro-sequence,

in an otherwise wild-type background the fusion protein is delivered to the vacuole and degraded. However, mutation of the genes that control the VPS system cause CPY-invertase to accumulate in the Golgi and to be secreted, allowing these mutants to grow on sucrose as a sole carbon source.

In this way, thousands of mutants in genes controlling VPS function were isolated, which fell into over forty complementation groups. Other screens and selections identified mutants that were deficient for vacuolar proteases or for vacuolar inheritance. In all, at least 54 genes have been implicated in sorting of proteins to the vacuole, not including

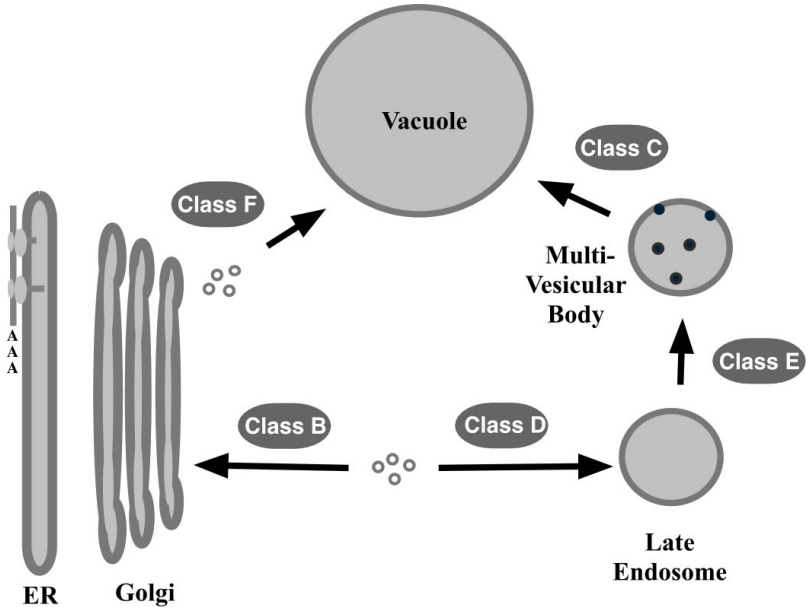

Figure 2 **The VPS Classes.** Characterization of the vacuolar and endosomal morphology of the *vps* mutants has led to their classification into six groups. The Class D and B mutants affect anterograde and retrograde transport, respectively, between the Golgi and late endosome. The Class E mutants affect MVB formation, the Class C mutants affect fusion of transport intermediates at the vacuole. Class A mutants have normal vacuolar morphology.

genes that also play roles in essential functions such as ER to Golgi transport. In an effort to handle such genetic complexity, the VPS mutants were classified based on their vacuolar morphology as observed by electron microscopy. As the mutants have become better characterized, it has become apparent that mutants of the same class usually affect the same part of the VPS pathway (Figure 2). These pathways and the affected genes and proteins are now often referred to by the class designation of the mutant.

The VPS classes and CPY sorting

The membrane morphologies of the VPS mutants fall into six classes, designated A through F. The class A mutants have no visibly altered vacuolar or endosomal morphology. Although the functions of many of the class A genes remain unclear, it is suspected that the lack of structural defects in class A mutants is due to the fact that each affected gene controls delivery of only one or several cargo molecules, and that their mutation does not cause general problems of membrane flow.

For example, mutants in the Class A gene *VPS10* are defective for incorporation of precursor CPY into transport vesicles at the Golgi, but do not affect vacuolar morphology. The formation of these vesicles is thought to require clathrin coat formation (Brodsky et al. 2001; Deloche et al. 2001). The clathrin heavy and light chains form a multimeric lattice that coats regions of membrane during vesicle formation. Association

with the various AP (*a*dapto*r p*rotein) complexes controls clathrin coat assembly in different regions of the cell. The adaptor complexes, in turn, coordinate vesicle formation though direct or indirect binding of cargo proteins, phospholipid binding and modifying enzymes, and signaling molecules. Formation of CPY-containing transport vesicles is thought to require the non-traditional adaptor proteins Gga1p and Gga2p (Mullins and Bonifacino 2001). These are redundant for function, such that mutation of both genes is required to observe a CPY sorting defect. The GGA proteins contain an N-terminal VHS (*V*ps27p, *H*RS, *S*TAM) domain that likely interacts with the cytoplasmic tail of Vps10p, while other domains interact with the clathrin lattice. This system is recapitulated in mammals. Glycans on lysosomal enzymes are modified with mannose 6-phosphate (M6P), which is recognized in the late Golgi by the M6P receptor (Doray et al. 2002). The cytoplasmic tail of this receptor interacts with mammalian GGA proteins, driving their incorporation into AP-1 and clathrin-coated transport vesicles (Doray et al. 2002).

The Class B genes are thought to be responsible for retrograde transport between the late endosome and the Golgi (Horazdovsky et al. 1997). Mutants in these genes mislocalize Vps10p to the vacuole and fail to retain Golgi resident proteins. Two Class B proteins, Vps5p and Vps17p, are similar to the mammalian SNX (*s*orting *nex*in) proteins. The SNX family controls endosomal trafficking of a number of cell surface receptors, including the EGF (*e*pidermal *g*rowth *f*actor) receptor (Kurten et al. 1996). In yeast, Vps5p and Vps17p heterodimerize and associate with late endosomal membranes. There

they anchor a complex containing Vps35p, which is known to interact with the cytoplasmic tails of retrograde traffic cargo, probably including Vps10p (Nothwehr 2000). How this complex mediates retrograde traffic is unclear. It is associated with membranes of high density, possibly transport vesicles, and has been suggested to form a coat complex (Seaman et al. 1997).

The class E mutants have normal vacuolar morphology and an enlarged, acidified late endosome-derived structure known as the class E compartment, but only moderately affect CPY sorting. Recent work has revealed that the class E genes are responsible for the formation of internal vesicles from late endosomal membranes, giving rise to MVBs (Katzmann et al. 2001; Babst et al. 2002; Babst et al. 2002). Incorporation of ubiquitinated membrane proteins into these vesicles (discussed below) results in their delivery to the vacuolar lumen, and disruption of class E function causes such cargo to accumulate on the limiting vacuolar membrane and in the Class E compartment.

The Class F mutants affect the subunits of the AP-3 adaptor complex (Odorizzi et al. 1998). These participate in the formation and delivery of Golgi-derived vesicles that fuse directly with the vacuole, bypassing the late-endosomal intermediate. These vesicles are thought to contain cargo destined for the limiting membrane of the vacuole, such as ALP (*al*kaline *p*hosphatase). The fusion of such vesicles, MVBs, and all transport intermediates at the vacuole is controlled by the Class C VPS proteins (discussed further

below). Because of this, Class C mutants display the most severe and pleiotropic phenotypes, including only vestigial vacuoles or no vacuoles at all.

The Class D VPS proteins: Rabs and SNAREs in membrane traffic

The class D mutants display enlarged vacuoles and severe missorting of CPY and other soluble proteases. They affect the genes that are thought to control anterograde vesicular traffic between the late Golgi and the late endosome (Horazdovsky et al. 1995). Identification of these genes has revealed that many of them share homology with other proteins known to be involved in membrane transport, in particular those responsible for

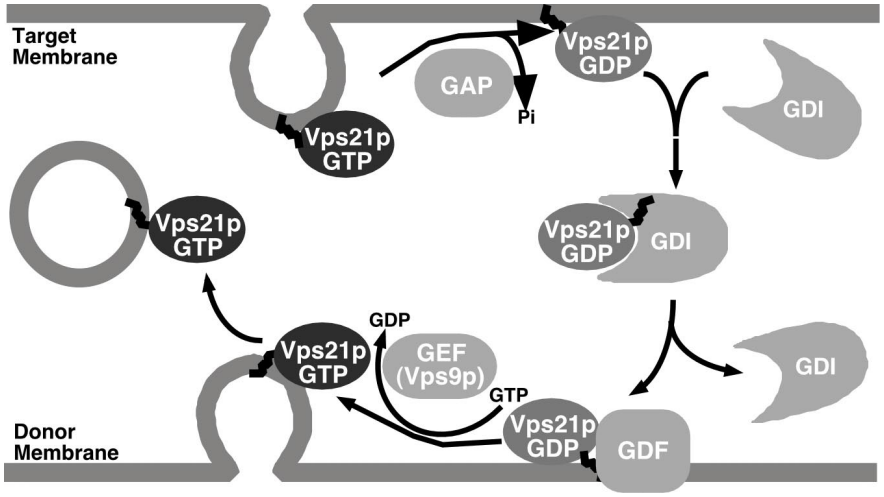

Figure 3 **The Rab Cycle in Membrane Traffic.** After a fusion event, Rab proteins such as Vps21p are extracted from target membanes in their GDP-bound state. They are then transported back to the donor membane where they are incoporated into a new vesicle. Exchange factors such as Vps9p replace the GDP nucleotide with a new molecule of GTP, preparing the Rab for another round of membrane fusion.

vesicular docking and fusion events. Among these is the Rab GTPase, Vps21p. The GTP hydrolysis cycle of Rab proteins coordinates vesicular docking and fusion reactions [Figure 3, (Pfeffer 2001)]. Rab proteins, anchored to membranes by lipid modification of C-terminal CAAX motifs, promote fusion of vesicles or other donor membranes only while in their GTP-bound, active state. After fusion, the GTP nucleotide is hydrolyzed to GDP. Since Rabs typically have very low intrinsic GTP hydrolysis rates, this reaction may be catalyzed by a GAP (*G*TPase *a*ctivating *p*rotein). The resulting GDP-bound form of the Rab is extracted from the target membrane by the soluble GDI (*G*DP *d*issociation *i*nhibitor) protein, which recycles it to a new donor membrane. After re-insertion, the Rab is incorporated into a new transport intermediate, such as a vesicle or a tubule. At some point during transit of this compartment to the target membrane, the GDP nucleotide is replaced by a molecule of GTP through the action of a Rab GEF (*g*uanine nucleotide *e*xchange *f*actor). The resulting GTP-bound, active Rab is thought to be required for the formation of SNARE and effector protein complexes, which mediate fusion.

The participation of Rabs in several transport steps has been studied carefully, particularly in yeast, but the precise molecular events that cause Rab activation and subsequently membrane fusion remain unclear. Each transport step appears to have a multimeric complex required for tethering incoming vesicles to their target membranes (Whyte and Munro 2002). Several of these contain nucleotide exchange factors that activate Rab proteins, while others appear to act downstream of Rab activation. Of the

latter group, the yeast exocyst is required for fusion of secretory vesicles at the plasma membrane mediated by the Rab Sec4p. It contains subunits that interact with sites of vesicle fusion as well as with activated Sec4p on secretory vesicles. The components of the exocyst share distant sequence similarity with the yeast COG (*c*onserved *o*ligomeric *G*olgi) and GARP (*G*olgi-*a*ssociated *r*etrograde *p*rotein) complexes. Although its function is controversial, the COG complex is thought to tether COPI-coated vesicles at the early Golgi (Suvorova et al. 2002). The GARP complex associates with activated vesicular Ypt6p Rab as a tether in endosome to late Golgi retrograde traffic (Siniossoglou and Pelham 2001).

Several tethering complexes act upstream of Rab activation. *In vivo*, the TRAPP-I complex is associated with early Golgi membranes. It is thought to be required for tethering because, *in vitro*, it can bind to ER-derived transport vesicles and catalyze nucleotide exchange on the Ypt1p Rab, activating it for fusion (Wang et al. 2000). The TRAPP-II complex, which contains many of the same components, is thought to perform a similar function for late Golgi-derived vesicles (Sacher et al. 2001).

The Class C VPS complex contains a core of Pep3p and Pep5p that associates with several other factors required for docking and fusion at the vacuole. On the target vacuolar membrane, the Class C complex binds Vps39p, the exchange factor for the Ypt7p Rab (Wurmser et al. 2000). Similar to TRAPP, it is thought to activate Rab proteins on the donor membrane while acting as a tether. Analysis of homotypic

vacuolar fusion *in vitro* suggests that the Class C complex is also required downstream of Ypt7p activation (Seals et al. 2000). This function is likely mediated by its association with Vam3p, a member of the syntaxin family of SNARE (*SNA*P *re*ceptor) proteins. Similarly, the COG and GARP complexes are thought to provide links between activated Rabs and SNARE proteins.

SNAREs facilitate membrane fusion by providing the motive force required to overcome the energetic barrier associated with bringing two lipid bilayers in close contact (Weber et al. 1998). This is accomplished through the formation of highly stable complexes between SNARE proteins anchored on opposite membranes. After fusion, SNARE complexes are resolved through the ATP-dependent action of NSF (*N*-ethylmaleimide-*s*ensitive *f*actor) and α-SNAP (*s*oluble *N*SF *a*ttachment *p*rotein). These are general factors that act on any of the numerous SNARE complexes formed throughout the cell. Structural analysis of SNARE complexes reveals that each contains a four helix bundle, with three helices contributed by SNAREs on the target membrane (t-SNAREs), and one by a SNARE on a vesicle or donor membrane (v-SNARE). SNAREs typically inhabit characteristic organelles, and it has been suggested that recognition of particular SNAREs from opposite membranes provides specificity to membrane fusion events. Countering this idea, however, are reports that SNAREs are somewhat promiscuous in complex formation, and that they can be functionally interchangeable (Gotte and Gallwitz 1997; Bhattacharya et al. 2002). Although it is clear that a particular SNARE protein can be involved in several different fusion events, it is probable that each event requires

formation of a distinct SNARE complex, adding to the specificity achieved by tethering complexes.

One of the Class D VPS proteins is a member of the syntaxin family of SNARE proteins: the late endosomal and vacuolar resident t-SNARE Pep12p. It participates in membrane fusion of Golgi-derived transport vesicles with the late endosome through the formation of a SNARE complex including Vti1p, Ykt6p and either Vam7p or Tlg1p (Dilcher et al. 2001). Formation of this complex is thought to be regulated by the Class D Rab protein, Vps21p. Along with Ypt52p and Ypt53p, Vps21p is one of the three yeast orthologues of the mammalian Rab5 protein, which has been implicated in a variety of membrane trafficking events in the endosomal system (Woodman 2000).

Although no tethering complex per se has been identified in Golgi to endosome traffic, Tall et al. (Tall et al. 1999) demonstrated that activated Vps21p can bind directly to the

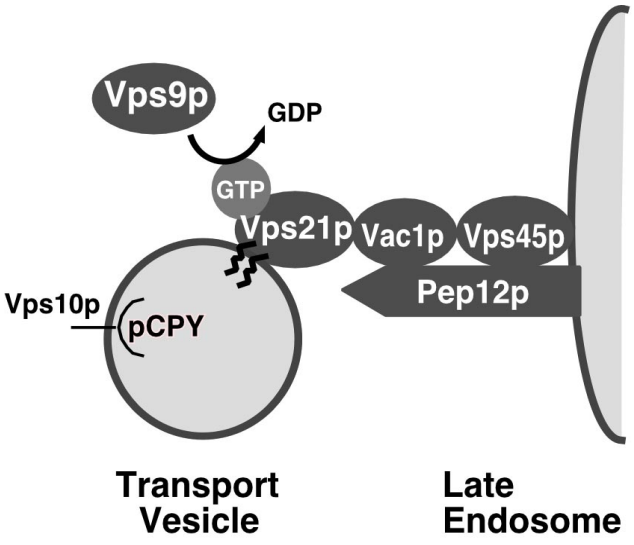

Figure 4 **The Class D Rab Complex**. Vps9p stimulates guanine nucleotide exchange on vesicle-associated Vps21p. Active Vps21p is then competent for interaction with Vac1p, which, in turn, interacts with the SM protein, Vps45p. The formation of this complex is thought to stimulate formation of SNARE complexes involving Pep12p, possibly through direct interaction.

endosomal protein Vac1p, possibly coordinating the formation of a tethering complex (Figure 4). Vac1p contains two FYVE finger domains that are thought to drive its association with PI 3P-rich endosomal membranes. There it binds Vps45p, the Class D representative of the SM (*S*ec1p/*m*unc18) protein family. There are only four such proteins in yeast, but one family member is required for all membrane fusion events (Jahn 2000). Sec1p participates in fusion at the plasma membrane, Sly1p resides in the ER and Golgi, and Vps33p interacts with the Class C complex at the vacuole. These proteins associate with syntaxin-related SNAREs, and are thought to regulate SNARE complex formation. It has been proposed that SM/syntaxin interactions serve to inhibit formation of inappropriate and untimely SNARE complexes, and that this inhibition is released when the appropriate signal is received from an activated Rab.

Mounting evidence suggests, however, that SM proteins do not inhibit SNARE complex formation, and that they play a positive, rather than inhibitory, role in membrane fusion. Despite the fact that they are both required for transport between the Golgi and late endosome, whether Vps45p binds directly to Pep12p is a point of controversy (Dulubova et al. 2002). So, if stimulation of SNARE complex formation involving Pep12p is a consequence of Vps21p activation, other factors than just Vac1p and Vps45p may be involved.

Acting upstream of Vps21p, another Class D protein, Vps9p, serves as its nucleotide exchange factor (Hama et al. 1999). As of yet, Vps9p has not been associated with a

protein network analogous to other large complexes with Rab exchange activity, such as the Class C complex and TRAPP. Analysis of the minimal sequences in Vps9p required for exchange activity revealed a conserved Rab5 GEF domain found in numerous proteins from yeast to humans. These include the Rin proteins, which are thought to stimulate endocytosis of activated tyrosine receptor kinases (Tall et al. 2001), and the alsin protein, which is mutated in a form of familial ALS (Hadano et al. 2001; Yang et al. 2001).

Given their involvement in biosynthetic traffic into the late endosome, it is not surprising that Vps21p and Pep12p have been implicated in endocytic traffic to this compartment. Mutants in both genes demonstrate kinetic delays in vacuolar delivery of the endocytic marker, Ste3p (Gerrard et al. 2000a; Gerrard et al. 2000b). A null mutant of the Class D gene *VPS8* displays a similar delay in Ste3p processing (Luo and Chang 2000), though defective vacuolar function in this mutant calls this result into question. Other studies of *vps8* mutants have revealed a number of phenotypes common to all of the Class D *vps* mutants (Chen and Stevens 1996; Horazdovsky et al. 1996). These include enlarged vacuoles and defects in sorting of cargo that pass through the late endosome, such as pCPY. The requirement for *VPS8* in CPY sorting can be bypassed by overexpression of Vps21p. Analysis of Vps8p reveals that it is a 134 kD protein with a particularly short half life (< 30 minutes), and that associates with a high speed membrane pellet. In keeping with the genetic interaction with *VPS21*, Vps8p dissociates from this membrane

fraction and is soluble in a *Δvps21* mutant. Importantly, these genetic interactions were not observed with *VPS45* or *PEP12*.

Phosphoinositides and membrane traffic

Many membrane trafficking events and even organelle identity depend on recognition of the various phosphorylated forms of phosphatidylinositol (PI) (De Camilli et al. 1996). The majority of Golgi phosphoinositides are modified at the 4 position on the inositol ring (PI 4P), while the plasma membrane contains mostly PI 4,5$P_{2,}$ and the endosomal system is marked by PI 3P and PI 3,5P_2. Proteins involved in membrane traffic contain domains that specifically recognize the various forms of these lipids. For instance, many proteins implicated in endocytosis at the plasma membrane contain ENTH (*e*psin *N*-*t*erminal *h*omology) domains, which bind PI 4,5P_2 (De Camilli et al. 2002). These proteins often contain other domains that link them to the endocytic machinery, including clathrin, the clathrin adaptor proteins, and the cytoplasmic tails of membrane proteins that are targeted for endocytosis.

In yeast and animals, PI 3P is found mostly in the endosomal system and the vacuole, and mutation of Vps34p, the only PI 3-kinase in yeast, generates a severe Class D VPS phenotype. Accordingly, several protein families in yeast and humans involved in endosomal and vacuolar trafficking contain domains that bind PI 3P. One such domain is the PX domain, which is found in the mammalian SNX proteins and in the yeast proteins

Vps5p and Vps17p (Sato et al. 2001). As discussed above, these proteins are implicated in endosomal sorting of cell surface receptors and in retrograde traffic between the endosome and Golgi.

Another PI 3P-binding motif is the FYVE (*F*AB, *Y*OTB, *V*ac1p, *E*EA1) RING finger domain, which is found in several Rab effector proteins. In mammals, the FYVE protein EEA1 (*e*arly *e*ndosomal *a*ntigen) protein is recruited to early endosomes by PI 3P and Rab5, where it is essential for fusion of endosomal membranes *in vitro* (Christofordis et al. 1999). Rabenosyn-5 contains two FYVE domains, targeting it to Rab 5 and Rab 4-containing endosomal membranes, and possibly serves as a link between the complexes coordinated by these GTPases (de Renzis et al. 2002). The closest yeast relative of Rabenosyn-5 is the Class D VPS protein, Vac1p. As mentioned above, Vac1p is a member of the Class D Rab complex that contains Vps21p and Vps45p.

Ubiquitin and membrane traffic

Ubiquitin is a 76 amino acid protein found in all eukaryotic organisms. It is extremely well conserved; there are only three amino acid substitutions between the yeast and human proteins. As its name and its conserved sequence would suggest, ubiquitin is involved in numerous processes throughout the cell. Rather than acting independently or as part of a protein complex, it is used as a posttranslational modifying group that alters

the stability or function of target proteins (Hershko and Ciechanover 1998). The C-terminal glycine of ubiquitin is linked through an isopeptide bond to the ε-amino groups of substrate lysine residues. After the initial modification, polyubiquitin chains can be formed by modification of lysines in ubiquitin itself. Chain formation usually involves lysine 48 of ubiquitin, although conjugates involving other lysines have been observed. Proteins marked with polyubiquitin are recognized by the cap complex of the 26 S proteasome and quickly degraded. This mechanism is used to dispose of misfolded proteins in the ER, and to control the stability of numerous structural and signaling molecules.

All ubiquitination events rely on the sequential action of the enzymes of the ubiquitin cascade. The E1 "activating" enzyme recognizes and is covalently modified with a molecule of ubiquitin in an ATP-dependent reaction. There is only one E1-encoding gene in yeast, indicating that the activating enzyme is a very general factor. After activation, ubiquitin is transferred to an E2 "conjugating" enzyme. These enzymes have somewhat more specificity, as there are 13 family members in yeast encoded by the *UBC* genes. Finally, E3 ubiquitin "ligases" coordinate with E2 enzymes to catalyze the final transfer of ubiquitin to target substrates.

Modification with ubiquitin is typically associated with degradation by the proteasome. In fact, the proteasomal cap complex only recognizes polyubiquitin chains of at least four units in length (Thrower et al. 2000). It has recently become clear that

monoubiquitination can act as a signal in much the same way as other post-translational modifications, such as phosphorylation. Just as SH2 and PTB domains recognize phosphorylated proteins, motifs specialized for interaction with monoubiquitinated proteins are also being uncovered (Hicke 2001). As the number of monoubiquitinated targets and proteins that recognize them grows, it is becoming apparent that they participate in a complex signaling network that controls many aspects cellular physiology. With respect to membrane traffic, these molecules can be separated into two general categories: cargo molecules and proteins that participate in the trafficking machinery.

Monoubiquitination is a common way of controlling the fate of transmembrane cargo proteins. In much the same way that polyubiquitination of soluble proteins leads to degradation by the proteasome, monoubiquitination of membrane proteins diverts proteins into trafficking pathways that lead to lysosome/vacuole, which in most cases leads to their degradation (Hicke 2001). However, this pathway is also used to direct vacuolar biosynthetic proteins, such as precursor CPS (*c*arboxy*p*eptidase *S*), to their proper destination. Although membrane cargo can be ubiquitinated at numerous sites in the cell, it is a particularly common mechanism for regulating the abundance of receptors on the cell surface, both positively and negatively. Recognition of ubiquitin modifications is required at multiple steps in this process.

Ubiquitination of activated receptors can induce a negative feedback loop that leads to receptor degradation. One of the most well characterized monoubiquitination events involves the mammalian EGF receptor tyrosine kinase. Upon activation by ligand, autophosphorylation of EGFR initiates a signaling cascade that promotes cell proliferation. The phosphorylated receptor is bound and targeted for monoubiquitination by the c-Cbl E3 ligase. This directs internalization and diversion of ubiquitinated receptors to late endosomes, while unmodified receptors are recycled to the cell surface (Levkowitz et al. 1998). At the late endosome, ubiquitinated receptors are recognized by the human homologue of Vps23p, the TSG101 (*t*umor *s*usceptibility *g*ene 101) protein, which is thought to direct their incorporation into the internal vesicles of MVBs, and thereby promote degradation in the lumen of the lysosome (Babst et al. 2000; Bishop et al. 2002). Disruption of this process can cause a failure to downregulate EGFR signaling and can contribute to carcinogenesis. The viral oncogene v-Cbl, which cannot ubiquitinate EGFR because it lacks the E3 ligase domain, promotes recycling of activated receptors, prolonging their half-life and overstimulating proliferation signals (Levkowitz et al. 1999). Knockdown of TSG101 expression also causes increased recycling of EGFR, and retention of active, ubiquitinated receptors on endosomal membranes (Bishop et al. 2002).

In yeast, a variety of cell surface receptors and transporters are subject to regulation by monoubiquitination (Hicke 2001). These include the mating factor receptors, Ste2p and Ste3p (discussed in Chapter 4), the uracil permease Fur4p, the tryptophan permease

Tat2p, the galactose permease Gal2p, the **a**-factor export protein Ste6p, and the ABC transporter Pdr5p . The abundance of all of these proteins on the plasma membrane is subject to regulation by monoubiquitin in a way that is appropriate for each metabolic system. A particularly interesting example is the regulation of the general amino acid permease, Gap1p (Soetens et al. 2001). When grown on a poor nitrogen source, such as proline or urea, Gap1p is expressed abundantly on the cell surface. Upon transfer of cells to a preferred nitrogen source, such as glutamate or NH_4^+, Gap1p is quickly ubiquitinated, which results in its internalization and degradation in the vacuole. As cells continue to grow in rich nitrogen, newly synthesized Gap1p is polyubiquitinated in the late Golgi, causing it to be delivered directly to the vacuole, completely bypassing the plasma membrane. Polyubiquitination requires Bul1p and Bul2p; in the absence of these proteins, Gap1p is delivered to the cell surface even in nitrogen-rich growth conditions (Helliwell et al. 2001). Bul1p and Bul2p are thought to act by altering the activity of the Rsp5p E3 ubiquitin ligase towards Gap1p. Rsp5p and its mammalian homologue Nedd4 are broad specificity E3 ligases responsible for the monoubiquitination, and thereby trafficking, of many of the substrates discussed, including the EGFR. There are also indications that it affects endocytosis through ubiquitination of cytoskeletal elements (Kaminska et al. 2002).

In yeast, it has been demonstrated that monoubiquitin acts as a general endocytosis signal, containing all the information necessary to induce internalization of plasma membrane proteins (Shih et al. 2000). The regions surrounding phenylalanine 4 and

isoleucine 44 are thought to comprise the surfaces that transmit this signal (Sloper-Mould et al. 2001), and the molecular mechanisms that control ubiquitin-mediated trafficking are beginning to be elucidated. Monoubiquitinated proteins are recognized by specialized domains (Buchberger 2002), including the UIM (*u*biquitin *i*nteracting *m*otif), the UBA (*ub*iquitin *a*ssociating domain), the CUE domain (identified based on homology to the Cue1p protein), and the UBC-like domain (identified based on homology to E2 ubiquitin conjugating enzymes). All of these are found in numerous proteins involved in membrane traffic. An intriguing feature of many of the proteins containing these domains is that, in many cases, they not only bind monoubiquitin, but are themselves monoubiquitinated.

This is true of several of the proteins involved in clathrin-mediated endocytosis. At the plasma membrane, the AP-2 clathrin adaptor complex is required for budding of many types of endocytic vesicles. Among the proteins that interact with this complex are the Eps15 and Eps15R (*E*GF receptor *p*athway *s*ubstrate) proteins (Confalonieri and Di Fiore 2002), which have themselves been implicated in endocytosis. Each contains a UIM that binds ubiquitin and that targets the protein for monoubiquitination in response to treatment with EGF (Polo et al. 2002). The epsin proteins bind Eps15 and Eps15R (through ubiquitin-independent mechanisms), and contain clathrin-binding motifs as well as similarly EGF-responsive UIMs (Polo et al. 2002). How ubiquitin binding and ubiquitination of these molecules contribute to endocytosis is unclear. It has been proposed that UIMs interact with ubiquitinated receptors, linking them to AP-2

complexes and to clathrin (Riezman 2002). Indeed, *in vitro* studies suggest that isoleucine 44 of ubiquitin, a critical residue for endocytosis, is required for its interaction with UIM domains (Beal et al. 1998). However, a direct association between any UIM protein and an activated receptor, or any ubiquitinated protein, has yet to be uncovered. This fact, coupled with the strong correlation between UIMs and monoubiquitination, has led some to speculate that some UIMs may not, in fact, be responsible for recognition of other ubiquitinated proteins at all (Buchberger 2002). Rather, they propose that they mark proteins containing them for ubiquitination, and that intramolecular interactions between the ubiquitin moiety and the UIM domain prevent formation of a polyubiquitin chain, as has been described for the HRS (*h*epatocyte growth factor *r*egulated-tyrosine kinase *s*ubstrate) protein (Shekhtman and Cowburn 2002). Such a mechanism could also regulate the availability of UIMs and ubiquitin modifications for intermolecular interactions with other proteins.

Epsins and other proteins involved in endocytosis contain ENTH domains, which localize them to the PI 4,5P_2-rich plasma membrane. The UIM protein HRS and its yeast homologue Vps27p, however, contain FYVE zinc finger domains that target them to the endosomal system. These proteins are components of the Class E VPS machinery responsible for incorporation of ubiquitinated membrane cargo into MVBs. In yeast, this process begins when ubiquitinated proteins are recognized by the ESCRT-I (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport) complex, consisting of Vps37p, Vps28p, and Vps23p (Katzmann et al. 2001). Vps23p contains a large region of homology to the E2

ubiquitin conjugating enzymes known as the UBC-like domain, which is required for a direct binding of Vps23p to monoubiquitinated membrane cargo. Cargo-associated ESCRT-I is then assumed to activate the ESCRT-II and ESCRT-III complexes (Babst et al. 2002; Babst et al. 2002). These, along with a host of other Class E VPS proteins, cause invagination of endosomal membranes and formation of internal vesicles by unknown mechanisms. Ubiquitin is removed from cargo molecules during this process by the ESCRT-III-associated ubiquitin protease, Doa4p. The role of the UIM-containing Vps27p/HRS protein in MVB formation remains unclear. Although it is certainly required for this process, Vps27p has not been found to be physically associated with any of the Class E proteins. Vps27p/HRS also contains a VHS domain, the cargo association domain of the GGA proteins required for vesicle formation at the late Golgi.

Budding of retroviral particles from infected cells is a topologically similar process to MVB formation, and there is evidence to suggest that retroviruses co-opt the Class E machinery for their own purposes (Garrus et al. 2001). Viral particle formation can be disrupted by depletion of ubiquitin or mutation of the Nedd4 E3 ligase. The retroviral Gag proteins that control budding are thought to interact with Nedd4, and formation of HIV-1 particles requires Gag recruitment of the Vps23p orthologue TSG101 (VerPlank et al. 2001).

CHAPTER TWO

Localization of the Class D VPS protein, Vps3p

Introduction

Vps3p is the only Class D protein for which no enzymatic or functional role has been ascertained. It is predicted to be a soluble protein, with no obvious structural features, nor any regions of readily detectable homology to any other proteins. Only one study has been directed specifically toward understanding the function of Vps3p. Raymond et al. (1990) determined that electrophoretic mobility of Vps3p suggests that it is a low-abundance protein with a molecular weight of approximately 140 kD, quite different from the predicted mass of 117 kD. This is presumably due to glycosylation at several predicted sites. They also analyzed a *vps3* deletion mutant and a temperature sensitive point mutant, and found that they had many of the phenotypes now commonly associated with the Class D *vps* mutants. These include defects in vacuolar morphology, acidification and inheritance, along with a defect in CPY maturation. Unpublished results by this same group also indicate that Vps3p is associated with a pelletable cellular constituent.

Results

Vps3 is associated with a high-speed pellet through a saturable anchor

Fractionation of cell extracts by differential centrifugation can provide insight into the intracellular localization of a protein of interest. In a protocol commonly used by yeast cell biologists, mechanically lysed cells are centrifuged gently (500 xg) to remove unbroken cells, generating a supernatant of starting material (SM). The SM is centrifuged at 13,000 xg to generate the medium-speed pellet (P13) and supernatant (S13) fractions. The P13 contains larger membranous structures, including the plasma membrane, the vacuole, and the endoplasmic reticulum. The S13 is then centrifuged at 100,000 xg to generate the high-speed pellet (P100) and supernatant (S100) fractions. The P100 contains smaller membranous structures, such as the Golgi, endosomes, and vesicles, and very large protein complexes such as the cytoskeleton. The S100 or cytosol is the soluble fraction of the cytoplasm.

These fractionation patterns have been determined by following the distribution of marker proteins that are known to be associated with various organelles. So, for instance, the alkaline phosphatase protein (ALP) is known to reside in the vacuolar membrane, and can detected exclusively in the P13 (Horazdovsky et al. 1997). Likewise, the CPY sorting receptor, Vps10p, is thought to reside mainly in the late Golgi, and fractionates to the P100 (Horazdovsky et al. 1997). The analysis of many other marker proteins using

this protocol has allowed for the designations described above, making differential centrifugation a useful tool for determining subcellular localization.

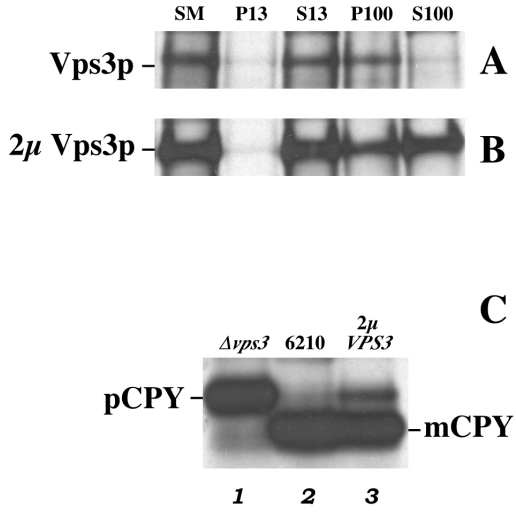

Figure 1 **Vps3p is associated with a saturable component of the P100**. Vps3p immunoprecipitated from fractionated extracts of SEY6210 (A) and SEY6210 containing pRS424-*VPS3* (B). C. CPY immunoprecipitaed from JHRY20-2C (lane 1), SEY6210 (lane 2) and SEY6210 with pRS424-*VPS3* (lane 3) and analyzed by autoradiography of a 10% gel.

In order to determine the subcellular localization of Vps3p, wild-type cells were metabolically labeled with ^{35}S methionine and cysteine and subjected to the fractionation protocol. Vps3p was immunoprecipitated from each fraction and detected by gel electrophoresis and autoradiography. Vps3p fractionates to the high-speed P100 (Figure 1A), indicating that it is associated with a small membrane or a large protein complex. Fractionation of cells overexpressing Vps3p reveals that a great majority of the excess protein is in the S100 fraction (Figure 1B). This suggests that there exists some anchor that is required to maintain the association of Vps3p with the P100. Overexpression of Vps3p overwhelms the capacity of this anchor, and the excess protein remains soluble. This anchor could be another membrane-associated protein, or Vps3p could be directly associated with the membrane through a saturable lipid binding site. It is interesting to

note that overexpression of Vps3 causes a slight defect in maturation of CPY (Figure 1C). It is possible that this represents the dominant-negative effect of solublized Vps3p, or of over-occupancy of its membrane-anchored binding partners.

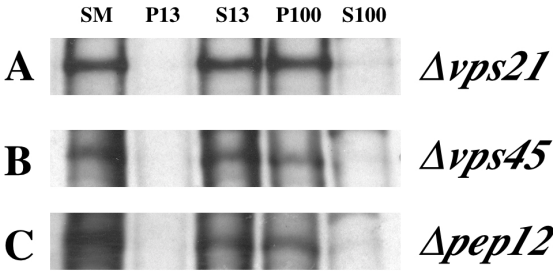

Figure 2 **Association of Vps3p with the P100 is stable in Class D *vps* mutants**. Vps3p immunoprecipitated from ^{35}S-labeled, fractionated extracts of CBY10 (A), CCY120 (B), and CBY31 (C), and visualized by autoradiography

Any of the several other Class D VPS proteins that associate with the P100 would be likely candidates for a protein that anchors Vps3p, including Vps21p, Vps45p, and Pep12p. All of these proteins are associated at least in part with the late endosome, the proposed site of action for the Class D proteins. If one of these was bound to Vps3p, it might be expected that in their absence, Vps3p would not associate with the P100. To test this, Vps3p was immunoprecipitated from fractionated extracts of *Δvps21* (Figure 2A), *Δvps45* (Figure 2B), and *Δpep12* (Figure 2C) mutants. The fractionation pattern of Vps3p is unchanged in each case, suggesting that none of these proteins serve as the saturable anchor that mediates Vps3p association with the P100.

Vps3p is associated with a dense component of the P100

Another strategy used to determine subcellular localization is density gradient analysis. In this technique, a suspension of membranes is layered onto a bed of sucrose (or some other gradient-forming solution) and centrifuged to equilibrium for (18 or more hours at > 150,000 xg). All of the particles in the suspension equilibrate to the section of the gradient that is equivalent to their intrinsic density. This density is a function of the protein to lipid ratio of a given membrane structure. So, protein-rich structures such as coated transport vesicles and Golgi membranes will fractionate to the more dense regions of the gradient, while relatively protein-poor membranes such as endosomes and vacuoles will fractionate to less dense regions.

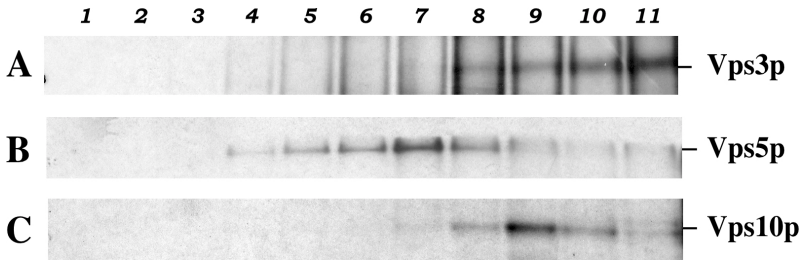

Figure 3 **Vps3p associates with a dense component of the P100**. P100 isolated from 50 OD of ^{35}S-labelled SEY6210 was equilabrated on a gradient of 9-37% Accudenz. The gradient was fractionated and Vps3p (A), Vps5p (B), and Vps10p (C) were immunoprecipitated from each fraction and visulized by autoradiography.

To determine the density of the Vps3p-associated structure, the P100 was collected from 50 OD of ^{35}S-labelled cells, resuspended, and subject to density gradient analysis. Immunoprecipitation of Vps3p from each fraction reveals that it segregates to the bottom-most, densest region of the gradient (Figure 3A). High-density structures that fractionate to the P100 include coated vesicles and Golgi membranes.

To assess the distribution of these structures in this particular gradient, Vps10p, a late Golgi-associated membrane protein (Cooper and Stevens 1996), and Vps5p, which is thought to be associated with transport vesicles (Horazdovsky et al. 1997), were immunoprecipitated from the same gradient fractions. Vps10p equilibrates to the middle of the gradient, and overlaps only slightly with Vps3p (Figure 3B). Although Vps5p is associated with structures more dense than the Golgi and overlaps with Vps3p distribution somewhat, Vps5p peaks in fraction 8 (Figure 3C), while Vps3p peaks at the bottom of the gradient, suggesting that they are associated with different structures. This fractionation pattern might be expected to result from pelleting of aggregated membranes at the bottom of the gradient tube. The peaks of Vps5p and Vps10p higher in the gradient, however, suggest that this is not occuring. Although they were not analyzed on this particular gradient, late endosomal markers such as Pep12p are always associated with relatively low density membranes (Horazdovsky et al. 1997). Fractionation of Vps3p with high density structures indicates that it is not associated with late endosomes.

Extraction of Vps3p from the P100 with detergent and salt

Testing the effects of various agents on the association of Vps3p with the P100 provides another way of understanding the nature of the anchor. Treatment of extracts with high concentrations of salt disrupts interactions that rely on electrostatic contacts. This technique strips membranes of nearly all peripherally associated proteins, leaving only integral membrane and lipid-anchored proteins associated with the pelletable fractions. Treatment with the non-ionic detergent Triton X-100 solublizes most membranes. Sterol- and sphingolipid-rich regions such as caveolae and lipid rafts are resistant to this treatment, however, and resistance to solublization in Triton X-100 is often used as a way of demonstrating that a protein is associated with such membranes (Maxfield 2002).

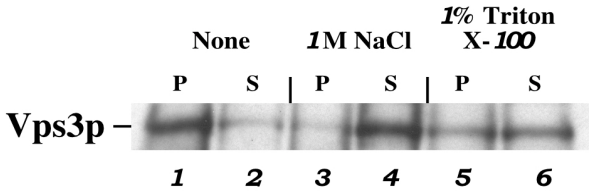

Figure 4 **Extraction of Vps3p with salt and detergent**. ^{35}S-labeled Extracts of SEY6210 were lysed in standard lysis buffer (lanes 1 and 2), or lysis buffer containing 1 M NaCl (lanes 3 and 4) or 1% Triton X-100 (lanes 5 and 6). Lysates were fractionated as described, and the P100 (P) and S100 (S) fractions were analyzed for Vps3p content by immunoprecipitation and autoradiography.

In an effort to gauge the effects of these treatments on the association of Vps3p with the P100, labeled spheroplasts were lysed in buffer containing either 1M NaCl or 1% Triton X-100 and fractionated as above. Vps3p is pelleted almost entirely in the P100 in untreated

extracts (Figure 4, lanes 1 and 2), while it is almost completely solublized in extracts treated with 1M NaCl (lanes 3 and 4).

This is consistent with a peripheral membrane association not mediated by a lipid anchor. Vps3p is only partially solublized by treatment with Triton X-100, however, which can be explained in one of several interesting ways. Either it is associated with a detergent insoluble membrane, or, alternatively, it is bound to a large proteinaceous component of the P100 such as the cytoskeleton.

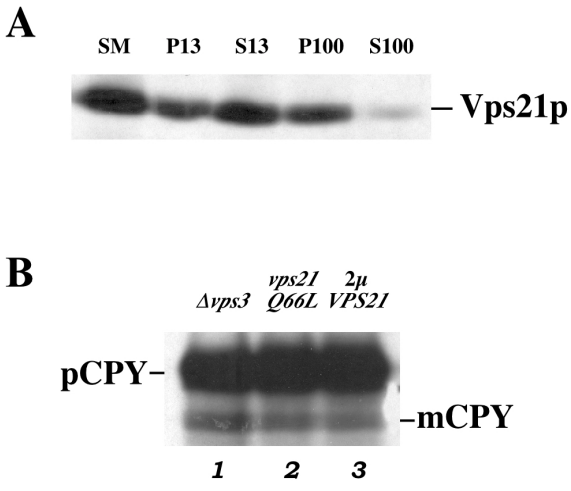

Figure 5 **Testing for genetic interactions between *VPS3* and *VPS21***. A. ^{35}S-labeled JHRY20-2C was fractionated and analyzed for Vps21p by immunoprecipitation and autoradiography. B. CPY was immunoprecipitated from ^{35}S-labeled extracts of JHRY20-2C (lane 1), JHRY20-2C with pRS416-*vps21Q66L* (lane 2), and JHRY20-2C with pRS426-*VPS21* (lane 3) and visualized by autoradiography.

Testing for Genetic Interactions between *VPS3* and *VPS21*

As discussed above, Rab proteins coordinate the fusion of large protein complexes that stimulate docking and fusion of transport vesicles. The Class D *vps* phenotype of the *Δvps3* mutant suggests that Vps3p may be involved in the docking and fusion events regulated by Vps21p. For this reason, we might expect to observe genetic interactions between the *VPS21* and *VPS3*

genes. A genetic interaction between *VPS21* and another Class D gene of unknown function, *VPS8*, suggests that Vps8p acts upstream of Vps21p in the delivery of Golgi-derived transport vesicles to the late endosome (see Chapter 4). Overexpression or activation of Vps21p can bypass the requirement for Vps8p in CPY sorting (Chapter 4, Figure 5). We tested for a similar interaction with *VPS3* by overexpressing Vps21p (Figure 5A, lane 2) or expressing at wild-type levels an activated allele of *VPS21*, *vps21Q66L* (lane 3) in a *Δvps3* mutant, and assaying these strains for CPY maturation. The CPY sorting defect of a *Δvps3* mutant (lane 1) is not corrected in either case.

As described in the Introduction, the membrane association of Vps21p changes as it progresses through vesicular transport cycle. Since mutation of *VPS3* is thought to affect the Class D machinery, it might be expected to change the distribution of Vps21p. Fractionation of *Δvps3* mutant reveals that Vps21p is distributed normally in this strain (Figure 5B). Several Class D genes demonstrate genetic interactions with *VPS21* that confirm their close functional relationship (Tall et al. 1999). While mutants in *VPS3* and *VPS21* share similar Class D *vps* phenotypes, Vps3p and Vps21p do not have a functional association that can be revealed by the experiments described in Figure 5.

Discussion

The partial solubility of Vps3p in Triton X-100 suggests that it may be associated with a non-membranous element of the P100. The most prominent proteinaceous component of this fraction is the actin cytoskeleton. The idea that Vps3p is associated with the cytoskeleton is not unfounded. Vps3p, like all of the Class D VPS proteins, is thought to be involved in vesicular transport between the Golgi and the late endosome, and translocation of vesicles and organelles in a variety of cellular settings involves movement along actin filaments (Stamnes 2002).

While studying the *Δvps3* mutant, Raymond et al. were among the first to recognize the vacuolar inheritance defect that is now known to be shared by all Class D mutants. Daughter buds in yeast inherit vacuoles and other organelles through a partitioning process. Tubulation and vesiculation of the parental vacuoles generates extensions and smaller vacuoles that are moved into the growing bud along actin cables (Catlett and Weisman 2000). The class V myosin Myo2p is the motor protein that drives this process. Why the Class D mutants are defective for vacuolar segregation is unclear. It is interesting, however, that the Class B and the Class C mutants, all of which have vacuolar protein targeting defects as severe or worse than the Class D mutants, do not have vacuolar inheritance defects. The inheritance defect in the Class D mutants, then, is not simply a secondary effect of dysfunctional protein targeting to the vacuole (as is, for instance, the vacuolar acidification defect). It has been suggested that late endosomes

play a critical role in formation of the "segregation structures" required for vacuolar inheritance, and that disruption of traffic into them in Class D mutants generates a secondary inheritance defect. Alternatively, it is possible that the Class D proteins are directly involved in vacuolar movement during cell division as well as vesicular movement between the Golgi and endosome.

The vacuolar morphology of the Class D mutants might also indicate a link with the cytoskeleton. Wild-type vacuoles are morphologically complex and dynamic organelles, with multiple lobes and tubular extensions. It is likely that these properties are maintained and driven through interaction with the actin cytoskeleton. Interestingly, it has been noted that the simple, rounded vacuolar morphology observed in Class D mutants might be the expected result if vacuoles were freed of their cytoskeletal contacts, or if movement along actin structures was perturbed (Raymond et al. 1990).

Such models linking Class D proteins with membrane movement and vacuolar structure suggest that one or several of them should be directly or indirectly linked to the actin cytoskeleton. The most likely contact point would be the molecular motor that drives vacuolar movement, the Myo2p protein. Indeed, Myo2p and other Class V myosins have been demonstrated to interact with Rabs and Rab complexes to drive organelle transport (Itoh et al. 2002). Mutational analysis of *MYO2* reveals that it encodes a multi-function protein. Point mutations in four closely spaced amino acids in its C-terminal cargo-binding domain specifically disrupt vacuolar inheritance, leaving its other, essential

functions intact (Catlett et al. 2000). If these amino acids represent the point of contact between the cytoskeleton and the Class D proteins, it might also be expected that such mutations would also affect sorting of proteins into the late endosome. No such effects have yet been reported, however.

If the Class D proteins do contact the cytoskeleton, what is the evidence that Vps3p provides this connection? Vps3p is associated with a very dense fraction of the P100 (Figure 3), and is only partially solublized in Triton X-100 (Figure 4), suggesting that it may be bound to a proteinaceous structure, potentially the cytoskeleton. Vps3p remains associated with this structure in the absence of Vps21p, Vps45p, and Pep12p. Vps21p is attached to Golgi-derived transport vesicles and to late endosomes by a geranyl-geranyl modification, while Pep12p is attached to the late endosome by virtue of its hydrophobic tail. These two proteins are the membrane anchors for the Class D proteins, so their disruption would be expected to solublize other components. Indeed, deletion of *VPS21* has been reported to disrupt the association of Vps8p with the P100 (Horazdovsky et al. 1996). That the pattern of Vps3p fractionation is unaffected in the absence of Vps21p or Pep12p suggests that neither mediates the association of Vps3p with the P100. The process of elimination presents the actin cytoskeleton as a likely candidate for the localization of the Vps3p protein.

How might this be verified experimentally? The Myo2p protein seems a likely place to look for connections between Class D proteins and the cytoskeleton. As suggested

above, *myo2* mutants that specifically affect vacuole inheritance might also affect Golgi to endosome sorting. In this model, Vps3p would be expected to physically associate with Myo2p, and with other components of the Class D machinery. As has been demonstrated in other systems, actived Rabs are often associated with cytoskeletal elements. Therefore, as a link to the cytoskeleton, Vps3p might be expected to associate with the active form of Vps21p. Figure 5A demonstrates that the requirement for Vps3p in CPY sorting cannot be bypassed by overexpression of Vps21p. This is consistent with Vps3p acting downstream of Vps21p activation. Curiously, Vps21p fractionation is not altered in a *Δvps3* mutant (Figure 5B), suggesting that Vps21p is present at normal levels in all phases of its transport cycle. Disruption of cytoskeletal contacts would be expected to trap Vps21p on Golgi-derived vesicles incapable of transport to the late endosome, resulting in a redistribution of P13 material to the P100.

Although there are clearly no closely related proteins in yeast or other organisms, comparison of Vps3p with protein sequence databases reveals that it has limited homology to the yeast protein Uso1p. Uso1p is required for tethering of COPI-coated vesicles to Golgi membranes, and consists of a globular head group and a very long coiled-coil domain. Homodimers of Uso1p are thought to form long rods of over a hundred nanometers in length (Yamakawa et al. 1996) that interact at either end with vesicle and Golgi anchors (Sonnichsen et al. 1998). It has been proposed that Golgi-bound dimers of Uso1p "search" the surrounding space for incoming vesicles and tether any that they encounter. The mammalian golgins and the endosomal EEA1 protein have

similar coiled-coil structures, and perhaps serve analogous functions. Although it is not predicted to form coiled-coils, regions of Vps3p share weak similarity to the coiled-coil domains of Uso1p. If other parts of the protein were found to associate with vesicle and endosomal anchors, one might predict that Vps3p acts as a vesicle tether.

Materials and Methods

Strains and Reagents

The strains and reagents used were identical to those listed Chapter 3, Materials and Methods, with the exception of the JHRY20-2C *vps3Δ1* strain (*MATα, his3-Δ200, leu2-3, leu2-112, ura3-52 vps3Δ1::LEU2*) (Raymond et al. 1990), CBY10 (SEY6210 *vps21Δ1::HIS3*) (Horazdovsky et al. 1994), CBY31 (SEY6210 *pep12Δ1::HIS3*) (Burd et al. 1997), and CCY120 (SEY6210 *vps45Δ2::HIS3*) (Cowles et al. 1994). PRS424-VPS3 was obtained from G.Tall.

Pulse-Chase Metabolic Labeling of Spheroplasts with ^{35}S

Logarithmically growing cells (OD_{600} = 0.5 – 1.0) were harvested by centrifugation, and resuspended at 10 OD/mL in 100 mM Tris pH 9.4, 10 mM DTT, and incubated at room temperature for 10 minutes. Cells were pelleted and resuspended at 10 OD/mL in spheroplasting buffer (YNB (*y*east *n*itrogen *b*ase), 2% glucose, complete amino acids, 1

M sorbitol, 20 mM Tris pH 7.5). 1 µL/OD of freshly prepared zymolyase solution (10 mg/mL zymolyase) was added to the cells, and the tubes were incubated at 30°C for 10 minutes with shaking. The cells were pelleted and resuspended at 10 OD/mL labeling medium (YNB, 2% glucose, complete amino acids, 1 M sorbitol) and again incubated at 30°C for 10 minutes with shaking. 3 µL EasyTag Express ^{35}S Protein Labeling Mix was added per OD of spheroplasts, and incubation was continued for 15 minutes. 40 µL of chase solution (0.2% yeast extract, 5 mM methionine, 1 mM cysteine) was added per mL of cell suspension, and the incubation was continued for an additional 30 minutes before placing on ice.

Subcellular Fractionation

20 OD of pulse-chase labeled spheroplasts were pelleted, resuspended in 1 mL ice cold lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, protease inhibitors), and lysed with 8 strokes in a cold 2 mL dounce homogenizer. Unbroken cells were removed by centrifugation for 5 minutes at 500 xg, and 250 µL of the supernatant was collected as "Starting Material". The remaining supernatant was centrifuged for 10 minutes at 13,000 xg, and 250 µL of this supernatant was collected as "S13". The pellet was resuspended in 750 µL lysis buffer, and 250 µl of this was collected as "P13". 250 µL of the remaining supernatant was centrifuged at 100,000 xg for 1 hr, and the supernatant was collected as "S100", while the pellet was collected as the "P100". 1 mL of cold 10% trichloroacetic acid (TCA) was added to each fraction, and they were vortexed and put on ice for 30 minutes. The precipitates were then pelleted by centrifugation at 13,000 xg for

5 minutes. The supernatant was removed and the pellets were sonicated briefly in two washes of cold acetone. All traces of acetone were removed by aspiration and vacuum drying for 5 minutes. The dry pellet was resuspended in 100 µL SDS cracking buffer (1% SDS, 50 mM Tris pH 7.5) by sonication, and incubated at 65°C for ten minutes. 1 mL of Tween IP buffer (0.5 % Tween 20, 50 mM Tris pH 7.5) was added, and samples were vortexed cleared twice by centrifugation at 13,000 xg for 10 minutes. 10 µL of 100 mg/mL BSA and 5 µL of the appropriate rabbit antiserum was added to each sample, which were then placed on a rocking platform overnight at 4°C. 75 µL of 15% suspension of Protein A-Sepharose beads was added to each sample, and rocking was continued for at least 90 minutes. The beads were washed twice in Tween IP buffer, and once in TBS (50 mM Tris pH 7.5, 150 mM NaCl). After removal of the last wash, the beads were resuspended in 50 µL urea sample buffer (8 M urea, 5% SDS, 10% glycerol, 1% β-mercaptoethanol) and incubated at 65°C for 15 minutes. The beads were pelleted, and 40 µL of the supernatant was analyzed by gel electrophoresis followed by autoradiography. Fractionation of lysates extracted with NaCl and Triton X-100 was performed as above, except the lysis buffer contained either 1 M NaCl or 1% Triton X-100.

Density Gradient Analysis

50 OD of ^{35}S-labelled spheroplasts were prepared as above, and lysed in 5 mL cold HEPES lysis buffer (20 mM HEPES-KOH pH 7.0, 50 mM potassium acetate, 1 mM EDTA, 0.2 M sorbitol), and lysed with 8 strokes in a 7 mL dounce homogenizer. The

lysate was cleared by centrifugation a 13,000 xg for 10 minutes, and a P100 was generated by centrifugation at 100,000 xg for 1 hr. The P100 was resuspended in 1 mL HEPES lysis buffer by passing it through a 30 gauge needle three times, and layered onto a Nycodenz step gradient in a Beckman 14 x 89 mm UltraClear tube consisting of the following steps: 1 mL 37%, 1.5 mL 31%, 1.5 mL 27%, 1.5 mL 23%, 1.5 mL 20%, 1.5 mL 17%, 1 mL 13%, 1 mL 9%. All Nycodenz solutions were prepared in HEPES lysis buffer. The gradient was centrifuged overnight at 170,000 xg in a Beckman SW41 swinging bucket rotor. 11 1 mL fractions were collected by pipetting from the top of the gradient. Each fraction was homogenized by vortexing, and its density determined using a refractometer. 100 µL of TCA was added to each fraction, and they vortexed and held on ice for 30 minutes. The precipitates were collected by centrifugation at 13,000 xg for 10 minutes, and washed once in 10% TCA. Further washing, solublization, and immunoprecipitation of the fractions was performed as described above in “Subcellular Fractionation”.

CHAPTER THREE

The H2 RING Finger of Vps8p has E3 Ubiquitin Ligase Activity that is not Required for Sorting of Ubiquitinated Cargo in the Late Endosome

Introduction

RING fingers and the ubiquitin cascade

In comparison to other Class D VPS proteins, the role of Vps8p in membrane traffic is relatively unclear. Analysis of its domain structure reveals that it contains two copies of the clathrin heavy chain repeat, which is present in seven copies in the arm region of the clathrin heavy chain. The role of this domain in other proteins containing one or two copies is not well understood. A potential enzymatic function is suggested by C-terminal RING (*r*eally *i*nteresting *n*ew *g*ene) finger domain. RING fingers are defined by four pairs of cysteines and histidines that coordinate two molecules of zinc (Figure 1A). Other than the zinc-binding residues, there are virtually no sequence requirements for RING finger domains, and the loops between the first two pairs and last two pairs can vary widely in size. They are different from zinc fingers in that the zinc atoms are coordinated in a "cross-brace" pattern rather than a tandem pattern (Figure 1B). RING fingers with a histidine at only the four position are known as HC RINGs, while those with histidines at both the four and five positions are known as H2 RINGs. In virtually

every case where it has been tested, H2 RING fingers demonstrate E3 ubiquitin ligase activity (Fang et al. 2003).

There are several families of E3 ligases. The HECT (*h*omologous to *E*6-AP *c*arboxy-*t*erminus) domain containing ligases include the very general Rsp5p enzyme, which is responsible for ubiquitination of numerous targets at the plasma membrane and elsewhere. HECT domain E3s form a covalent intermediate with a molecule of ubiquitin that is subsequently transferred to the target substrate. RING finger E3 ligases, however, act in concert with at ubiquitinated E2 conjugator enzyme to catalyze transfer to the

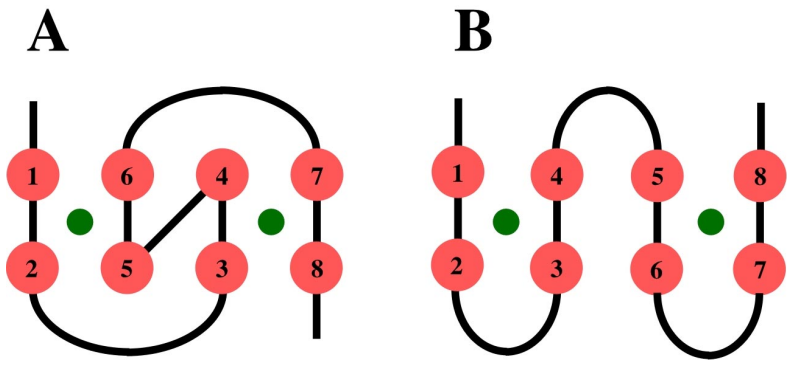

Figure 1 **Zinc-binding motifs.** A. In a RING finger, two molecules of zinc are coordinated by eight cysteine and histidine residues in a cross-brace pattern. B. In zinc fingers, zinc atoms are coordinated in a tandem arrangement.

target substrate. RING E3s, such as c-Cbl and APC (*a*naphase *p*romoting *c*omplex), are often very large or are part of multi-subunit complexes that provide substrate recognition and that regulate E3 ligase activity (VanDemark and Hill 2002).

Monoubiquitination and vacuolar delivery of CPS

One potential target for Vps8p ubiquitin ligase activity is the soluble vacuolar protease CPS. CPS is synthesized as a type II transmembrane precursor protein with a short, N-terminal cytoplasmic sequence that is targeted for ubiquitination on lysine 8. After delivery from the Golgi to the late endosome through the action of the Class D VPS proteins, this modification allows for the recognition of precursor CPS by the Class E VPS machinery, which directs its incorporation into the internal vesicles of MVBs (Katzmann et al. 2001). When these structures fuse with vacuoles, the transmembrane and cytoplasmic segments of precursor CPS are removed by proteolysis, releasing the mature, active protease into the lumen of the vacuole. Mutation of the target lysine or of Class E genes leads to defects in MVB formation, and ubiquitinated precursor CPS (Ub-pCPS) accumulates on the limiting membrane of the vacuole.

In Class E mutants, Ub-pCPS also accumulates in an abnormal perivacuolar structure known as the Class E compartment (Rieder et al. 1996). This mutant organelle is an abnormally enlarged form of the late endosome. It is acidified, enzymatically active, and electron microscopy reveals that it has a multi-lamellar structure. The formation of this compartment in Class E mutants gives rise to a secondary phenotype, a weak CPY sorting defect that results from destabilization of the CPY sorting receptor, Vps10p. As discussed, Vps10p recognizes precursor CPY in the late Golgi, allowing it to be packaged into transport vesicles bound for the late endosome. When these vesicles arrive at their

destination, the receptor releases its cargo and is recycled back to the late Golgi, where it binds a new molecule of precursor CPY. In wild-type conditions, it is known that a single Vps10p molecule can complete this cycle many times, as the half-life of a nascent receptor is at least several hours (Cooper and Stevens 1996). Class E *vps* mutants, however, destabilize Vps10p. This is thought to be the result of exposure of cycling Vps10p to the acidified, enzymatically active Class E compartment. Indeed, overexpression of Vps10p counteracts this effect and can complement the CPY sorting defect of a Class E mutant (Piper et al. 1995).

Results

The H2 RING finger of Vps8p has E3 Ubiquitin Ligase Activity

Since H2 RING finger motifs are strongly correlated with E3 ubiquitin ligase activity, Vps8p was tested for this property. An 85 amino acid C-terminal fragment of Vps8p was purified and assayed for ubiquitin ligase activity *in vitro*. This segment was selected in order to ensure a structurally stable polypeptide, as it contains the RING domain and one predicted α-helix N-terminal to the RING domain. The most straightforward way to observe E3 activity is to reconstitute the entire ubiquitin cascade. A complete ubiquitination reaction contains partially purified E1 activator enzyme, E2 conjugator enzyme, E3 ligase, and ubiquitin along with a substrate and ATP. The very general yeast

E1 enzyme, Uba1p, and E2 enzyme, Ubc4p were used for this assay. Since the *in vivo* target of the putative Vps8p ubiquitin ligase activity is not known, the appearance of high molecular weight, non-specifically polyubiquitinated proteins was used as an indicator of E3 activity.

When reactions lacking E1, E2 or ubiquitin were analyzed by blotting for ubiquitinated proteins, no high molecular weight products were detected (Fig 2A, lanes 1-3). Reactions lacking RING peptide generated small amounts of ubiquitinated proteins (lane 4), consistent with the background transfer activity intrinsic to the E2 protein, while RING

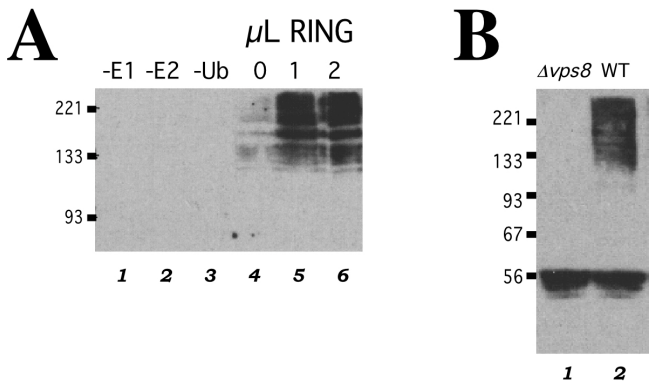

Figure 2 **Vps8p has E3 ubiquitin ligase activity *in vitro*.** A. 10 µL ubiquitination assays were set up as described in Materials and Methods, incubated at room temperature for 60 minutes, and diluted in 40 µL of urea sample buffer. 20 µL of each reaction was then analyzed by electrophoresis and western blotting for ubiquitinated proteins. B. Vps8p was immunoprecipitated from native lysates of PSY83 (lane 1) SEY6210 (lane 2) onto Protein A-Sepharose beads, which were then added to 20 µL ubiquitination reactions lacking RING protein. These reactions were incubated at room temperature with gentle vortexing for one hour and then diluted in 60 µL urea sample buffer. 20 µL of each reaction was then analyzed by electrophoresis and western blotting for ubiquitinated proteins.

protein greatly stimulated this activity (lanes 5-6). This contradicts current models of RING protein function, which postulate that RING domains by themselves do not directly affect E2 ubiquitin transfer activity. Rather, they suggest that RING-containing proteins act as scaffolds that serve to bring E2 enzymes into close contact with substrate proteins.

It was next important to establish the E3 ligase activity of full-length Vps8p, although attempts to purify it from bacteria and insect cells were unsuccessful. Vps8p immunoprecipitated from native extracts was assayed instead. Vps8p immunoprecipitates on Protein A-Sepharose beads were added to *in vitro* ubiquitination reactions in lieu of RING protein, and these reactions were analyzed for ubiquitinated products. Immunoprecipitates from wild type extracts demonstrated E3 activity (Fig 2B, lane 2), while those from extracts of strains deleted for the *VPS8* gene did not (lane 1). That both the C-terminus of Vps8p and immunoprecipitates of full length protein demonstrate E3 ubiquitin ligase activity in the *in vitro* assay suggest that Vps8p has this activity *in vivo*.

The *vps8ΔRING* Mutant Contains an Abnormal Endosomal Compartment

In order to understand the role of the RING motif in VPS function, we recreated the partial deletion of Vps8p described by Horazdovsky et al. (Horazdovsky et al. 1996) and introduced it into the *Δvps8* strain. The *vps8ΔRING* mutation removes the C-terminal 32 amino acids of the protein, including the last five zinc-coordinating residues of the RING motif. The vacuolar and endosomal morphology of these strains was assessed by staining cells with the lipophilic fluorescent dye FM 4-64 (Vida and Emr 1995). This dye is incorporated into the plasma membrane and follows the endocytic pathway to vacuoles, which, in a wild-type strain, appear as one to five bright red rings per cell when viewed by fluorescence microscopy. Abnormalities in vacuolar and endosomal structure caused by disruptions in the endocytic and VPS pathways are readily observed using this technique.

The SEY6210 strain is wild-type for VPS function, and displays the normal vacuolar morphology (Figure 3A). Consistent with previously described results (Horazdovsky et al. 1996), a *Δvps8* mutant contains fewer, enlarged vacuoles (Figure 3B). This mutant also displays a densely-staining, perivacuolar structure. The deletion mutant strain carrying either the wild-type *VPS8* gene (Figure 3C) or the *vps8ΔRING* mutant (Figure 3D) has normal vacuolar morphology and number. The *vps8ΔRING* mutant, however, also contains the abnormal perivacuolar structure observed in the *Δvps8* mutant.

Given the involvement of Vps8p and other Class D proteins in membrane traffic through the late endosome (Gerrard et al. 2000; Gerrard et al. 2000), it was reasoned that the abnormal perivacuolar structure observed in the *Δvps8* and *vps8ΔRING* mutants may be derived from late endosomal membranes. In order to test this possibility, we stained with FM 4-64 strains carrying a CFP (*c*yan-*f*luorescent *p*rotein) -tagged version of the late endosomal resident t-SNARE, Pep12p. The most dense regions of FM 4-64 perivacuolar staining correspond to areas of Pep12p-CFP fluorescence (Figure 4A) in the *vps8ΔRING* mutants (Figure 4B). This suggests that the abnormal structure is late endosome-derived, and that these mutants have an endosomal trafficking defect.

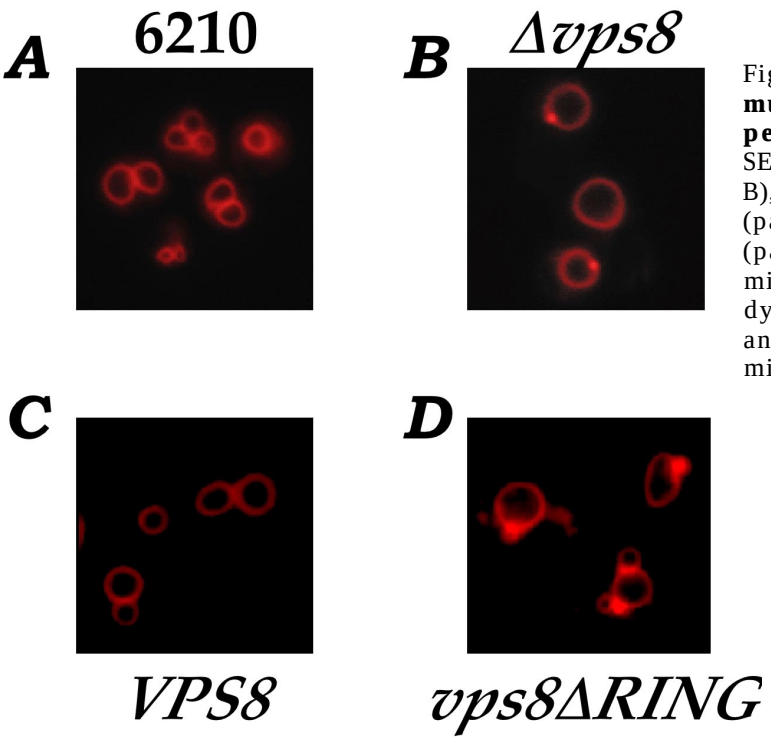

Figure 3 **The *vps8ΔRING* mutant contains an abnormal perivacuolar compartment.** SEY6210 (panel A), PSY83 (panel B), and PSY83 carrying pPS71 (panel C) or pRS414*vps8ΔRING* (panel D), were stained for 15 minutes with FM 4-64, chased in dye-free medium for 60 minutes and visualized by fluorescence microscopy.

Ubiquitination and vacuolar targeting of CPS are unaffected in the *vps8ΔRING* mutant

The abnormal endosomal structure (Figures 2 and 3) and relatively weak CPY sorting defect (Horazdovsky et al. 1996) seen in the *vps8ΔRING* mutant are reminiscent of similar defects in the Class E *vps* mutants. As mentioned above, the Class E proteins control MVB formation by recognizing ubiquitinated membrane proteins and directing their incorporation into internal vesicles. Disruption of Class E function causes the formation of the Class E compartment and a mild CPY sorting defect. It was reasoned that the phenotypic similarities observed in the Class E and *vps8ΔRING* mutants occur

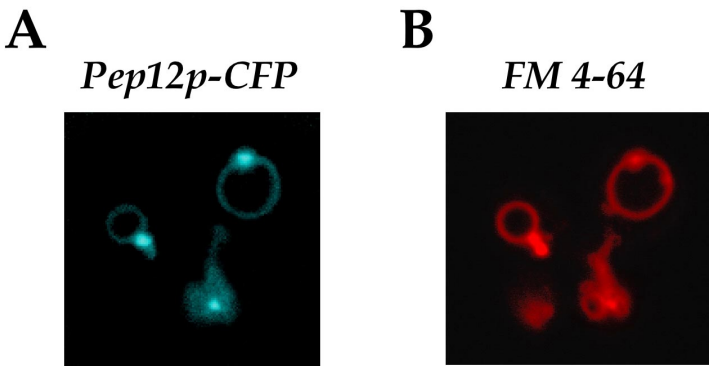

Figure 4 **The abnormal compartment in *vps8ΔRING* is derived from the late endosome.** PSY83 carrying pRS414*vps8ΔRING* and pRS416*PEP12-CFP*, was stained for 15 minutes with FM 4-64, chased in dye-free medium for 60 minutes, and visualized for CFP (panel A) and FM 4-64 (panel B) fluorescence.

because mutation of the RING domain reveals a Class E *vps* phenotype that has previously gone unnoticed in a *vps8* deletion mutant, which causes a more severe Class D phenotype. Class D mutants affect a wide variety of cargo that pass through late

endosome, while the Class E mutants are thought only to affect ubiquitinated membrane cargo such as CPS.

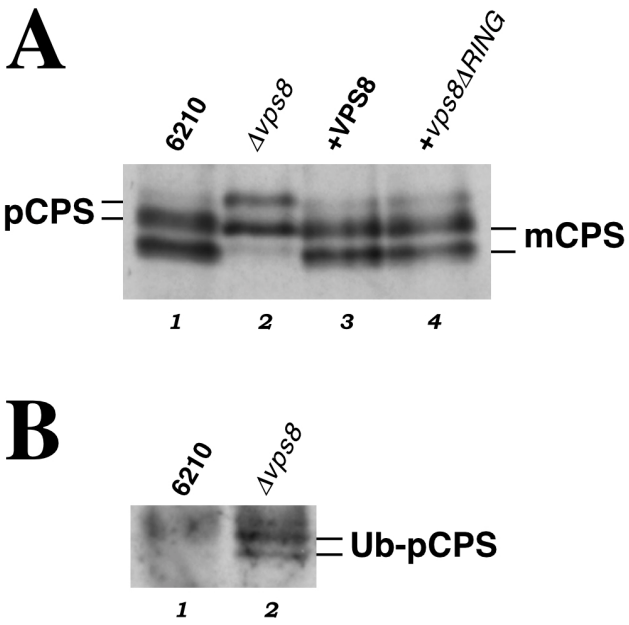

Figure 5 **CPS is processed normally in *vps8* mutants**. A. CPS immunoprecipitated from ^{35}S-labelled SEY6210 (lane 1), PSY83 (lane 2), and PSY83 carrying pPS81 (lane 3) or pRS414*vps8ΔRING* (lane 4), and analyzed by autoradiography of a 7.5% gel. B. Steady-state CPS immunoprecipitated from SEY6210 (lane 1) and PSY83 (lane 2), and western blotted for ubiquitinated proteins.

Such a phenotype in the *vps8ΔRING* mutant could result from a defect in ubiquitination of membrane cargo proteins such as CPS, or of some component of the Class E VPS machinery itself. In either case, the *vps8ΔRING* mutant would be expected to have a defect in CPS sorting to the vacuole. In order to test this possibility, CPS was immunoprecipitated from ^{35}S-labelled cells. In a wild-type strain, CPS migrates as a doublet because it has two differentially glycosylated forms (Fig 5A, lane 1). In *Δvps8*, as in any Class D mutant, CPS is not delivered to the lumen of the vacuole, the short, N-terminal transmembrane and cytoplasmic segments are not cleaved, and the protein accumulates in its higher molecular weight precursor forms (lane 2). This defect can be fully complemented by addition of either the wild type *VPS8* gene (lane 3) or the

vps8ΔRING mutant (lane 4). This suggests that the E3 ligase activity of Vps8p is not required for effective delivery of CPS to the vacuole.

Furthermore, Vps8p is not required for ubiquitination of CPS. Ub-pCPS is virtually undetectable in wild-type cells since it represents only a very small portion of the total pool of CPS protein, and because it has a very short half-life. Ubiquitinated forms can only be visualized in mutant strains that accumulate the precursor form of the molecule, such as Class D and Class E *vps* mutants (Katzmann et al. 2001). Accordingly, western blotting for ubiquitinated proteins in a CPS immunoprecipitate from a wild type extract reveals no Ub-pCPS (Fig 5B, lane 1). Ub-pCPS can be immunoprecipitated from extracts of a *Δvps8* mutant, however (lane 2), suggesting that Vps8p does not play a role in its ubiquitination.

The Abnormal Endosomal Structure Observed in the *vps8ΔRING* Mutant is Distinct from the Class E Compartment.

Destabilization of Vps10p in Class E mutants is thought to result from exposure to the enzymatically active Class E compartment (Rieder et al. 1996). In order to determine whether the abnormal endosomal structure observed in the *vps8ΔRING* mutant shares this property of the Class E compartment, the stability of Vps10p was measured by immunoprecipitation from cells labeled with ^{35}S amino acids in a 45-minute pulse-chase

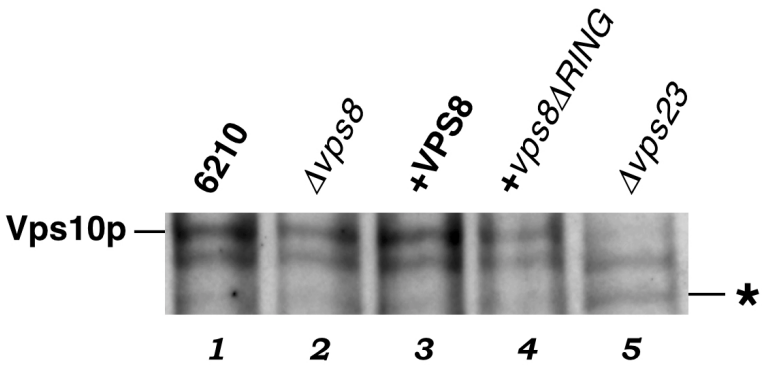

Figure 6 **Vps10p is stable in *vps8* mutants**. Vps10p was immunoprecipitated from ^{35}S-labelled SEY6210 (lane 1), PSY83 (lane 2), and PSY83 carrying pPS81 (lane 3) or pRS414*vps8ΔRING* (lane 4), and SEY6210*Δvps23* (lane 5) and analyzed by autoradiography of a 7.5% gel. The proteolyzed form of Vps10p is marked by an asterisk at the right.

protocol. In a Class E mutant such as *Δvps23* (Figure 6, lane 5), destabilization of Vps10p can be observed as the disappearance of the full-length protein coupled with the appearance of a proteolytic product (indicated by an *). Although they contain an abnormal endosomal structure similar in morphology to the Class E compartment, neither *Δvps8* (lane 2) nor the *vps8ΔRING*(lane 4) mutant destabilized Vps10p relative to a wild-type (lane 1) or complemented strain (lane 3).

Discussion

Figure 1 suggests that Vps8p has E3 ubiquitin ligase activity *in vitro*. It is important to question, however, whether the appearance of a high molecular weight smear of ubiquitinated proteins is actually indicative of E3 ligase activity. In typical *in vitro*

assays, ubiquitination is observed as the appearance of a “ladder” of target proteins with the modified species spaced one ubiquitin unit apart, or 8.5 kD. This ladder is then visualized by blotting for the target protein, eliminating the background of ubiquitinated side products. Although this approach was tried with several substrates, a ladder of ubiquitinated products was never observed (data not shown). What, then, are the ubiquitinated proteins observed in Figure 2? They are the results of non-specific ubiquitination of the proteins involved in the reaction itself, and of bacterial proteins carried over from their rather crude purifications. All of the proteins used in these reactions are 6xHis fusions purified in one step on Ni-Agarose. Such preparations are always highly contaminated, so it is not possible to determine the identity of the ubiquitinated products in Figure 2.

The observation that the RING domain alone is sufficient to catalyze ubiquitination *in vitro* is not without precedent. For example, the RING domain of the c-Cbl protein is sufficient E3 activity for autoubiquitination *in vitro* (Joazeiro et al. 1999). What is the mechanism by which the RING finger confers E3 ligase activity? The recent discovery that U-box proteins exhibit ubiquitin ligase activity suggests that the cross-brace structure is an important feature (Meacham et al. 2001). The U-box is predicted to adopt a conformation similar to the RING finger through formation of intramolecular interactions between conserved residues (Aravind and Koonin 2000). This occurs in the absence of zinc, indicating that, in RING fingers, these atoms most likely do not participate in reaction intermediates. It is generally thought that RING domains function in ubiquitin

transfer by forming complexes with E2 enzymes, bringing them into closer contact with target molecules by virtue of interactions through other parts of the E3 ligase (Zheng et al. 2000). The observation that ubiquitination of non-specific substrates can be stimulated by a short peptide that lacks substrate recognition domains indicates, however, that the Vps8p RING operates by stimulating E2 ubiquitin transfer activity.

Figure 2B demonstrates that Vps8p immunoprecipitates from wild-type extracts contain ubiquitin ligase activity. Analysis of these immunoprecipitates by silver staining (data not shown) reveals that they contain a large number of proteins, raising the possibility that the activity is due to one of these contaminants. That it is not observed in immunoprecipitates from a *Δvsp8* extract suggests that the activity does not associate non-specifically with Protein A-Sepharose beads, nor with antibodies in the Vps8p antiserum. It is possible, however, that the activity is not due to Vps8p itself, but rather some protein that co-immunoprecipitates with Vps8p.

Co-immunoprecipitation with other factors may, in fact, enhance the ubiquitin ligase activity of Vps8p. Several RING-containing proteins only exhibit E3 activity in the context of large regulatory complexes, while others form homo- or heterodimers mediated by interaction between RING domains, which can be required for activity (Fang et al. 2003). In the case of Vps8p, the latter mechanism is a particularly interesting possibility. Identification of protein complexes in yeast indicates that Vps8p associates with two other H2 RING proteins: Pep3p (Vps18p), Pep5p (Vps11p) (Gavin et al. 2002).

E3 ligase activity has not been demonstrated for any of these molecules, and a more complete discussion of the implications of these associations follows in Chapter 4.

When considered in combination, the *in vitro* assays utilizing RING peptide and immunoprecipitated protein suggest that Vps8p has bona fide ubiquitin ligase activity. What is the target of this activity? In many instances, E3 ligases catalyze their own ubiquitination in addition to modification of other proteins. A notable example is the activity of the mammalian Mdm2 protein, which results in proteasomal degradation of the p53 protein, but also of itself (Fang et al. 2000). Certainly, then, Vps8p itself should be considered a prime candidate as a target for its own activity. As discussed above, monoubiquitination has become recognized as a major mode of regulating membrane trafficking events. Given the phenotype of *vps8* mutants, it seems likely that Vps8p will be involved in monoubiquitination of proteins involved in these processes. Although it is not clear whether they participate in endosomal traffic, the yeast epsins, Ent1p and Ent2p, as well as the yeast homologue of Eps15, Ede1p, are potential targets for Vps8p activity. Indeed, given the strong correlation between ubiquitin binding and ubiquitination, any protein containing a UIM or similar domain should be considered a candidate. It is possible, of course, that rather than modifying proteins with monoubiquitin, Vps8p modifies the stability of substrate proteins by polyubiquitination. No proteins involved in yeast membrane traffic are known to be regulated in this manner, however.

The phenotypes initially observed in strains carrying the *vps8ΔRING* mutation suggested that a target for Vps8p activity may be involved in the Class E VPS pathway. A weak CPY sorting defect and the appearance of an abnormal, late endosome-derived compartment in this mutant (Figures 3 and 4) are reminiscent of the Class E *vps* phenotype. Furthermore, the Class E proteins are known to recognize ubiquitinated proteins and direct their incorporation into MVBs, raising the possibility that Vps8p ubiquitinates cargoes that follow this route, such as CPS. CPS sorting and ubiquitination are normal in the *vps8ΔRING* mutant, however (Figure 5).

Two of the proteins involved in the Class E machinery, Vps23p and Vps27p, contain domains known to bind ubiquitin and that are also targeted for ubiquitination. As a component of the ESCRT-1 complex, Vps23p is thought to provide initial recognition of ubiquitinated cargo molecules through its UBC-like domain (Katzmann et al. 2001), while the function of the UIM protein Vps27p is unclear. It was reasoned that defects in ubiquitination of these proteins in the *vps8ΔRING* mutant could be responsible for the formation of the abnormal endosome by the same mechanism that causes the formation of the Class E compartment. Aberrant proteolysis in the Class E compartment observed in *Δvps27* is thought to contribute to destabilization of the CPY sorting receptor, Vps10p (Piper et al. 1995). Consistent with previous results, the stability of Vps10p in *vps8* mutants (Figure 6) suggests that the abnormal endosome seen in these strains is quite distinct from the Class E compartment, however, and that the Class E proteins are not likely targets for Vps8p activity.

What is the cause of this abnormal endosomal structure, then? The Class D mutants are usually associated with defects in transport to the late endosome. Interestingly, overexpression of Vps21p generates a similar, "coalesced" endosome phenotype (Gerrard et al. 2000a). This also occurs as a result of Rab5 overexpression in mammalian cells, and is thought to be the consequence of increased levels of endosome-endosome fusion (Bucci et al. 1992). In this context, it is hard to explain why depletion of Vps8p would cause such a phenomenon, especially given that it is thought to act as a positive factor upstream of Vps21p activation (Horazdovsky et al. 1996). A *vps8* deletion mutant would be expected to decrease levels of homotypic endosome fusion, not increase them.

Several Class D mutants accumulate membranous structures, thought to be Golgi-derived transport vesicles incapable of fusion at the endosome, stranded throughout the cytoplasm (Cowles et al. 1994; Horazdovsky et al. 1994; Becherer et al. 1996). Why, then, would a Class D mutant such as *Δvps8* cause enlargement, rather than diminution, of the late endosome? The Class E mutants are presumed to have enlarged endosomes because defects in the endosomal maturation process prohibits formation of new vacuoles or fusion with existing ones. Perhaps the job of Vps8p, and possibly other Class D proteins as well, is not finished once Golgi-derived transport vesicles have reached their targets. They may also be involved in endosomal maturation or transport out of endosomes. The putative association of Vps8p with the Class C VPS complex suggests that this indeed may be the case. In Chapter 4, an endosome to vacuole transport defect is revealed in the

vps8ΔRING mutant, indicating that, indeed, Vps8p participates at two steps in biosynthetic traffic to the vacuole.

Materials and Methods

Strains and Reagents

Bacterial strains used in this study were DH5a (Invitrogen) and HMS174 DE3 (Novagen). The *Saccharomyces cerevisiae* strains used in this study were SEY6210 (*MATα, trp1, lys2, leu2, his3, ura3, suc2Δ9*) ((Robinson et al. 1988), PSY83 (SEY6210; *vps8Δ1*::HIS3) (Horazdovsky et al. 1996). The bacterial strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µ/ml) and kanamyacin (50 µg/ml) when necessary (Miller 1972). Yeast strains were grown in media containing 2% peptone, 1% yeast extract and 2% glucose (YPD) or synthetic media supplemented with appropriate amino acids as required (Sherman 1979). Thermostable DNA polymerases, restriction endonucleases and DNA modifying enzymes were purchased from Invitrogen (Carlsbad, CA), Roche Molecular Biochemicals (Indianapolis, IN) or New England Biolabs (Beverly, MA). EasyTag Express ^{35}S Protein Labeling Mix was purchased from PerkinElmer Life Sciences (Boston, MA). Protein A Sepharose was purchased from Amersham Biosciences (Piscataway, NJ). Zymolyase 100T was purchased from Seikagaku (Tokyo, Japan). Glass beads (0.5 mm) were purchased from Biospec Products, Inc. (Bartlesville, OK). SuperSignal West Femto Maximum Sensitivity Substrate was

purchased from Pierce Biotechnology Inc. (Rockford, IL). FM4-64 was purchased from Molecular Probes Inc. (Eugene, OR). Pronase was purchased from (Calbiochem-Novabiochem Corp., La Jolla, CA. Potato acid phosphatase was purchased from (Boehringer Mannheim Corp., Indianapolis, IN).

Plasmid Construction

pRS424-*vps8ΔRING* was constructed by removing the NcoI fragment from pPS81 (Horazdovsky et al. 1996), filling in the overhanging ends by treatment with Klenow, and religating the resulting blunted ends. pRS426-*VPS21* and pRS416-*vps21Q66L* were obtained from G. Tall.

Purification of the Vps8p C-terminal RING peptide

A fragment encoding the C-terminal 85 amino acids of Vps8p was amplified by PCR from pPS83 and ligated blunt into SmaI-cut pBlueScriptKS(+). The BamHI/SalI fragment of this clone was ligated into pET28b in frame with the 5' 6x-His tag to generate pET28b-RING. Synthesis of RING protein was induced in 1 L of HMS174 DE3 containing pET28b-RING by addition of isopropyl-b-D-thiogalactoside (IPTG) to 500 μM. Cells were collected after 4 hours of protein production at 37° and protein was purified using 1 mL Ni-Agarose per the recommendations of the manufacturer.

Ubiquitination Assay

Complete reactions contained 1 µL of the yeast E1 activating enzyme Uba1p (a gift of Hongtao Yu), 0.5 µL of the yeast E2 conjugating enzyme Ubc4p (Hongtao Yu), 1 µL of the cytoplasmic tail of Vps10p (50 ng/µL), 1 µL ubiquitin (5 µg/µL), varying amounts of RING peptide, and XB buffer (10 mM HEPES pH 7.7, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM ATP) to 10 µL. Reactions were mixed on ice, placed at room temperature for 1 hr, stopped by addition of 40 µL urea sample buffer, and heated at 65°C for 10 minutes. 20 µL of each reaction was analyzed by gel electrophoresis and western blotting for ubiquitin-containing proteins.

Native Immunoprecipitation/Ubiquitination Assay

15 OD of cells were spheroplasted as described, pelleted, and lysed by resuspension in 3 mL cold Tween IP buffer containing protease inhibitors. The lysates were cleared by centrifugation at 13,000 xg for 10 minutes, and 30 µL of 100 mg/mL BSA and 15 µL of α-Vps8p serum (Horazdovsky et al 1996) was added to the supernatants, which were incubated at 4°C for 90 minutes with rocking. 30 µL of a 15% suspension of Protein A-Sepharose beads was added, and incubation was continued for an additional 90 minutes. The beads were then pelleted at 4°C and washed three times in 1 mL of cold Tween IP buffer, and three times in 1 mL cold XB buffer. The beads were resuspended in 30 µL, and 5 µL was added (in lieu of RING peptide) to each premixed, double-sized ubiquitination reaction (20 µL final volume). The reactions were mixed continuously at low speed on a vortexer at room temperature for 1 hr. The reactions were stopped by

addition of 60 µL urea sample buffer, and heated at 65°C for 10 minutes. 20 µL of each reaction was analyzed by gel electrophoresis and western blotting for ubiquitin-containing proteins.

Immunoprecipitation of CPS and Vps10p

Cells were spheroplasted and labeled as described in Chapter 2, Materials and Methods - Pulse-Chase Metabolic Labeling of Spheroplasts with ^{35}S. Vps10p and CPS was immunoprecipitated from extracts of labeled spheroplasts lysed by precipitation in 10% TCA. Further processing of these samples was done as described for TCA-precipitated subcellular fractions in Chapter 2, Materials and Methods – Subcellular Fractionation.

Immunoprecipitation and Detection of Ub-pCPS

CPS was immunoprecipitated from unlabelled spheroplasts as above, and Ub-CPS was detected as described in Chapter 2, Materials and Methods - Western Blotting.

CHAPTER FOUR

A Ste3p endocytic trafficking defect in the *vps8ΔRING* mutant reveals a division of function within Vps8p

Introduction

The a-factor receptor, Ste3p

Sexual conjugation in yeast is mediated by pheromonal signaling between cells of the two mating types, **a** and α. **a** strains secrete the peptide **a** mating factor and display the α -factor receptor on their cell surface, while α strains synthesize α -factor and the **a**-factor receptor. Activation of either receptor initiates a number of responses, most notably the formation of a mating projection, which arises through polarized growth towards the source of the pheromonal stimulus. These responses require a variety of signaling and transcriptional regulation events. Efforts to identify strains incapable of mating have revealed the *ste* (*ste*rile) mutants (Hartwell 1980), which are defective at multiple points in these pathways. The receptors for α -factor, Ste2p, and for **a**-factor, Ste3p, are the only examples of seven transmembrane domain, G protein-coupled receptors in yeast. Activation of either receptor initiates the same downstream signaling cascade, a process carried out not by the affiliated G_{α} subunit, but rather by the $G_{\beta\gamma}$complex (Whiteway et

al. 1990). Free $G_{\beta\gamma}$ is thought to stimulate recruitment to the membrane of the scaffold protein, Ste5p (Whiteway et al. 1995), and its associated MAP kinase module, the only one of its kind in yeast.

Ste3p can follow two distinct endosomal trafficking pathways, depending on the presence or absence of **a**-factor (Chen and Davis 2000). In the absence of ligand, cell surface receptor is internalized, delivered to the late endosome, and incorporated into the internal vesicles of MVBs before degradation in the lumen of the vacuole. The initial internalization step requires phosphorylation of Ste3p at multiple serines and threonines in a C-terminal PEST-like sequence. In other proteins, PEST sequences regulate degradation by the proteasome by serving as recognition sites that drive phosphorylation-dependent polyubiquitination. Likewise, phosphorylation of the PEST-like sequence by the redundant type I casein kinases Yck1p and Yck2p is required for monoubiquitination of Ste3p by an unknown E3 ubiquitin ligase (Feng and Davis 2000). Fusion of this 36 amino acid segment to the normally stable plasma membrane ATPase, Pma1p, is sufficient to cause its internalization and delivery to the vacuole (Roth et al. 1998). The Ste3p C-terminus contains three lysines that are targeted for ubiquitination, two of which fall within the PEST-like sequence. These are redundant for function, such that only mutation of all three prevents internalization and transport to the vacuole (Roth and Davis 2000). Indeed, it has been shown that the requirements for ubiquitination and for the PEST-like sequence in this process can be eliminated by expressing mutant Ste3p that is fused in frame at its C-terminus with ubiquitin. As discussed earlier, in addition to its

role in endocytosis, ubiquitination of Ste3p is also required for incorporation into the internal vesicles of MVBs.

Activated Ste3p is phosphorylated by an unknown kinase at multiple residues in the C-terminal cytoplasmic domain outside of the PEST-like sequence, which, in contrast to activated Ste2p, requires the activity of most of the downstream signaling network (Feng and Davis 2000). Ste3p is also diverted away from the vacuolar trafficking pattern and into a recycling pathway, thereby sensitizing cells to further stimulation. This recycling pathway is completely independent of ubiquitination of the receptor and of the function of the PEST-like sequence (Chen and Davis 2002).

Results

Endocytic trafficking of Ste3p is disrupted in a *vps8ΔRING* mutant

In the absence of the **a**-factor ligand, Ste3p is synthesized, transported to the cell surface, internalized, and delivered to the vacuole fairly quickly, giving the protein a half-life of only 20 minutes. A common way to assay for endocytic function in yeast is to arrest protein synthesis using cyclohexamide, and to follow the disappearance of the remaining pool of Ste3p as it makes its way through the endocytic system and is degraded in the

vacuole. In wild-type cells, this pool is degraded with a half-time of 20 minutes, while it is stabilized indefinitely in mutants that affect endocytic trafficking or Ste3p ubiquitination.

In order to determine whether Vps8p E3 ligase activity plays a role in the delivery of ubiquitinated cargo from the cell surface to the vacuole, we analyzed Ste3p endocytic trafficking in the *vps8ΔRING* mutant. Growing cultures were treated with cyclohexamide, and samples were withdrawn at the indicated time points and analyzed for Ste3p content. Cells carrying a deletion of *VPS8* have proteolytically defective

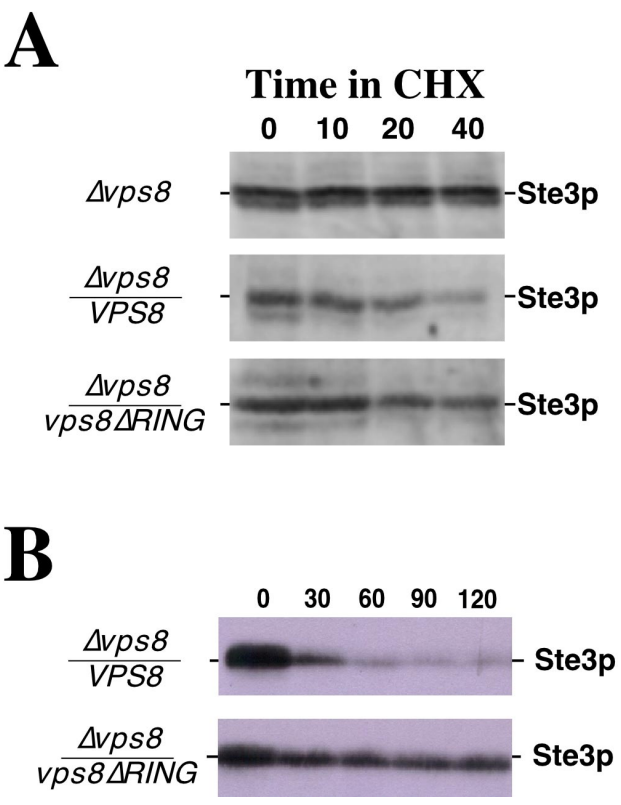

Figure 1 **Ste3p is stabilized following treatment with cyclohexamide in the vps8ΔRING mutant**. A. Logarithmically growing cultures of PSY83 (top row), PSY83 containing pPS81 (middle row) and PSY83 containing pRS414-*vps8ΔRING* (bottom row) were treated with cyclohexamide for the indicated times and analyzed for Ste3p content by western blotting. B. PSY83 containing pPS81 (top row) and PSY83 containing pRS414-*vps8ΔRING* (bottom row) were treated as in A.

vacuoles, so that even if endocytic cargo could be effectively transported there, it would not be degraded in this strain. Accordingly, Ste3p is completely stabilized after treatment with cyclohexamide in *Δvps8* (Figure 1A, top row), while it is degraded with normal kinetics if this strain is complemented with the wild-type gene (middle row). In the strain carrying the *vps8ΔRING* mutant, the majority of Ste3p is stabilized after 40 minutes (bottom row). This result is consistent if the experiment is carried out over a longer time course (Figure 1B). The near wild-type CPY maturation kinetics observed in this mutant indicate that vacuolar proteolytic capacity is normal. This suggests, then, that a defect in the trafficking mechanism, rather than vacuolar proteolytic deficiency, is responsible for stabilization of Ste3p after treatment with cyclohexamide in the *vps8ΔRING* mutant.

Ste3p does not accumulate at the cell surface in the *vps8ΔRING* mutant

As discussed above, modification with ubiquitin is required at two points in the endocytic trafficking of Ste3p. Monoubiquitination events at lysines 424, 432, or 453 are important for the initial internalization step (Roth and Davis 2000), and it is possible that Vps8p E3 ubiquitin ligase is responsible for these modifications. In this case, it would be expected that the *vps8ΔRING* mutant would be defective in this step, and accumulate Ste3p at the cell surface. In order to determine whether the stabilized pool of Ste3p observed in Figure 1 represents its abnormal retention at the plasma membrane, the *vps8ΔRING* mutant was tested using a protease “shaving” assay. In this protocol, cells were

metabolically labeled with ^{35}S amino acids and chased with cold amino acids for 90 minutes, providing enough time for nascent labeled protein to be internalized, but within the observed time course of the Ste3p sorting defect in the *vps8ΔRING* mutant. These cells were then treated with an extracellular protease that degrades any protein remaining on the cell surface. This experiment was carried out in strains defective for vacuolar proteolysis, so that any internalized proteins would be protected from degradation in the vacuole. The Ste3p from these cells was then immunoprecipitated and detected by autoradiography.

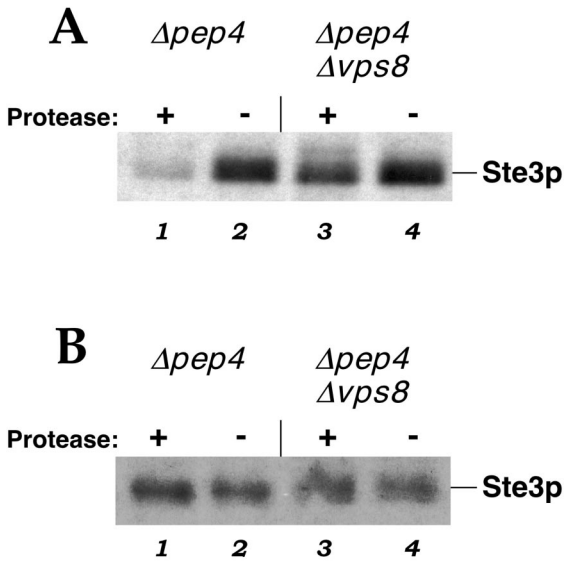

Figure 2 **Ste3p does not accumulate at the plasma membrane in the *Δvps8* mutant.** A. TVY1 (lanes 1 and 2) and TVY1*Δvps8* (lanes 3 and 4) were labeled for 15 minutes with 35S amino acids, fixed in azide and fluoride, treated with extracellular protease, and Ste3p was immunoprecipitated and detected by autoradiography. Loss of material from the sample analyzed in lane 1 resulted in reduced signal. Nonetheless, lane 3 serves as an adequate control for exposure of Ste3p to digestion by treatment for protease. B. As in A, except cells were chased in cold amino acids for 60 minutes prior to fixation.

Figure 2 indicates that Ste3p is protected from degradation by extracellular protease in the *vps8ΔRING* mutant. In Panel A, the cells were treated with protease immediately after labeling, without a chase period, so that some of the labeled, nascent protein is likely to still be on the cell surface. As expected in these cells, a significant fraction is accessible to extracellular protease (Figure 2A, lanes 1 and 3). Although there appears to be a general

loss of Ste3p in the "chased" cells (Fig 2B), whatever protein is left (lanes 2 and 4) is not accessible to extracellular protease (lanes 1 and 3). This holds true for the *vps8ΔRING* mutant strain, suggesting that its endocytic Ste3p trafficking defect represents a block at some point after the initial internalization step.

Ste3p accumulates in the abnormal endosomal structure in the *vps8ΔRING* mutant

A GFP-tagged version of Ste3p was used to ascertain the point at which the *vps8ΔRING* mutant affects endocytic trafficking. To visualize the position relative to the vacuole of Ste3p stabilized by treatment of cells with cyclohexamide, strains carrying the Ste3p-GFP construct were first labeled with a 15 minute pulse of FM 4-64 (discussed in Chapter 1). This was followed with a 90-minute chase period in cyclohexamide, and visualization by fluorescence microscopy.

Because of its compact β-barrel structure, GFP is resistant to proteolysis in the vacuole. It is therefore possible to observe the accumulation of Ste3p-GFP in the vacuolar lumen in wild-type cells (Fig 3, row A), and in the *Δvps8* mutant complemented with the wild-type gene (row C). In both the uncomplemented deletion mutant (row B) and the *vps8ΔRING* mutant (row D), however, Ste3p-GFP accumulates in the abnormal late endosomal structure previously observed in Chapter 3, Figure 3.

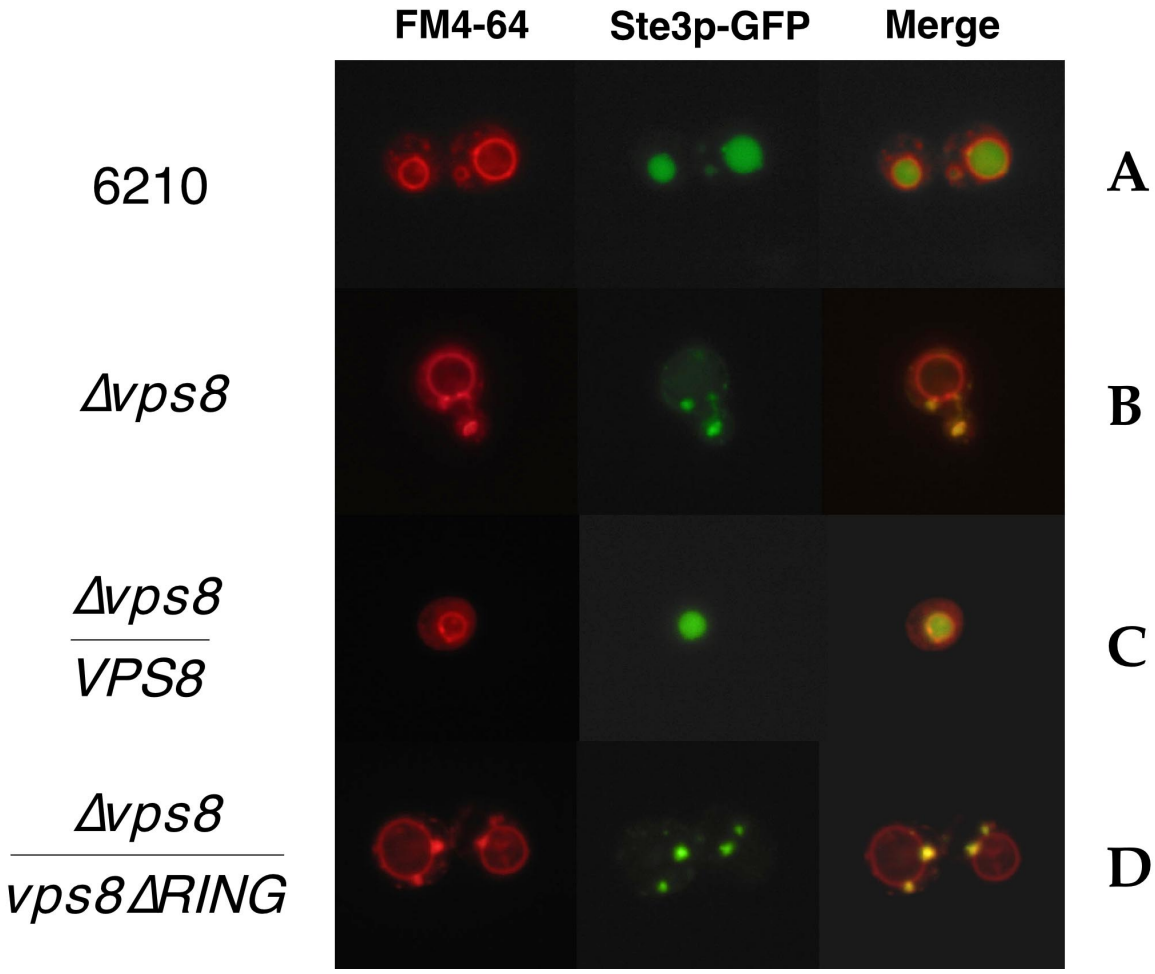

Figure 3 **Ste3p accumulates in the abnormal late endosome of the *vps8ΔRING* mutant**. SEY6210 (row A), PSY83 (row B), and PSY83 carrying pPS81 (row C) or pRS414-*vps8ΔRING* (row D) were treated for 15 minutes with FM 4-64, and chased for 90 minutes in dye-free medium containing cyclohexamide. Cells were then fixed in azide and fluoride and visualized for FM 4-64 (left column) and GFP (middle column) fluorescence.

Ste3p is ubiquitinated in the *Δvps8* mutant

The trafficking phenotype observed in Figure 3 could be explained by a deficiency in ubiquitination of Ste3p caused by the *vps8ΔRING* mutant. It was therefore important to establish whether the Ste3p sorting defect correlated to a change in its ubiquitination status. Detecting the ubiquitinated forms of Ste3p is complicated by several factors. First, these forms of Ste3p represent only a very small fraction of the total pool of Ste3p protein. To compound this problem, they are also multiply phosphorylated, making detection of discrete molecular weight species difficult. To circumvent this issue, extracts were treated with acid phosphate before analysis for Ste3p content. The *Δvps8* mutant contains all three ubiquitinated species (Figure 4, lane 4), indicating that, while the E3 ubiquitin ligase activity of Vps8p is required for proper endocytic trafficking of Ste3p, it is not a direct substrate.

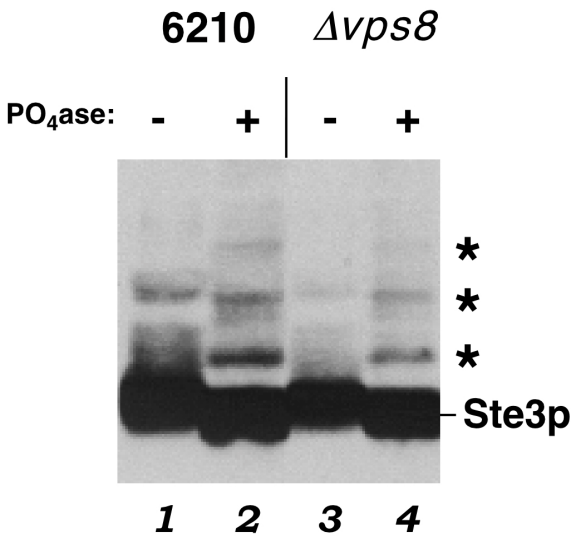

Figure 4 **Ste3p is ubiquitinated in the *Δvps8* mutant**. Extracts of SEY6210 (lanes 1 and 2) and PSY83 (lanes 3 and 4) were treated (lanes 2 and 4) or mock treated (lanes 1 and 3) with potato acid phosphatase and blotted for Ste3p. The three ubiquitinated forms of Ste3p are indicated by asterisks on the right.

The role of Vps21p in Vps8p-mediated CPY and Ste3p sorting

A better understanding of the role of Vps8p in CPY and Ste3p sorting can be gained by a closer inspection of the relationship between Vps8p and the Class D Rab protein, Vps21p. As mentioned above, Horazdovsky et al. (Horazdovsky et al. 1996) reported that deletion of Vps21p disrupted the association of Vps8p with the high-speed membrane pellet in subcellular fractionation experiments. They also noted that overexpression of Vps21p could partially bypass the requirement for Vps8p in CPY sorting. These results suggest a close functional relationship between Vps8p and Vps21p.

This idea is borne out by a result obtained by Pam Marshall. She demonstrated that the requirement for Vps8p in CPY sorting can be bypassed by activation of Vps21p (Figure 5A). In the *Δvps8* mutant, most immunoprecipitated CPY protein is in the precursor form, while some is proteolyzed to an aberrantly processed form (lane 1). This phenotype is approximately 50% corrected by expression of the GTPase defective *vps21Q66L* mutant (lane 2). These data indicate that Vps8p acts upstream of Vps21p activation in the delivery of Golgi-derived transport vesicles to the late endosome, and that this requirement can be obviated by expression of constituitively active Vps21p.

An important question, then, is whether the requirement for the activity of the Vps8p RING domain in Ste3p endocytic trafficking can also be bypassed by activation of Vps21p. To determine this, *vps8ΔRING* mutant strains expressing wild-type and GTPase-defective Vps21p were analyzed for Ste3p sorting using the stabilization assay. In comparison to the wild-type protein, the activated protein does not alleviate the Ste3p trafficking defect associated with the *vps8ΔRING* mutant (Figure 5B). This suggests that Vps8p action in Ste3p trafficking is independent of its activity upstream of Vps21p in CPY sorting.

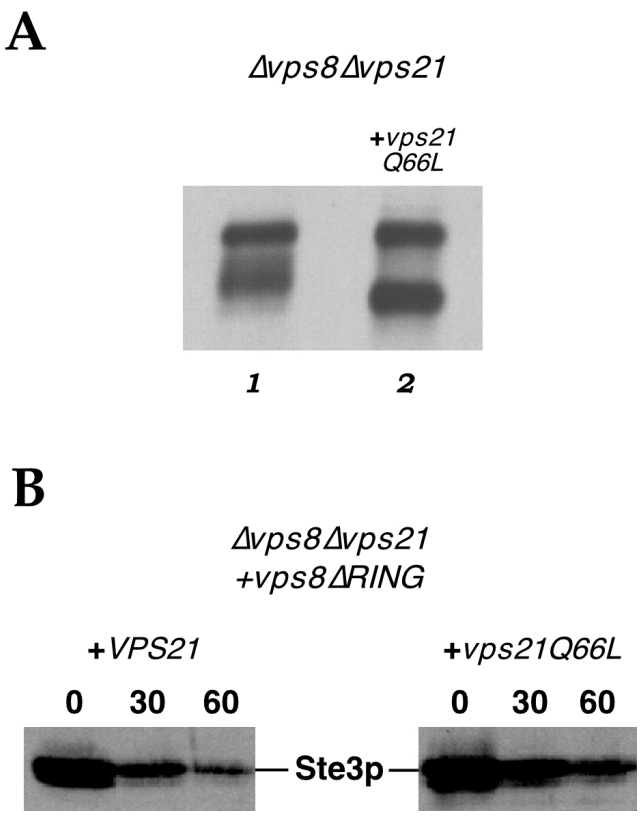

Figure 5 **Activated Vps21p bypasses the requirement for Vps8p in CPY sorting, but not the requirement for the Vps8p RING finger in Ste3p sorting**. A. CPY was immunoprecipitated from ^{35}S labeled whole-cell extracts of SEY6210*Δvps8Δvps21* (lane 1) and SEY6210*Δvps8Δvps21* containing pRS416-*vps21Q66L*(lane 2) and visualized by autoradiography. B. SEY6210*Δvps8Δvps21* containing pRS414-*vps8ΔRING* and either pRS416-VPS21 (left) or pRS416-*vps21Q66L* (right), were treated with cyclohexamide and samples were collected at the indicated times. Ste3p content was analyzed in these samples by extraction and western blotting.

Discussion

Consistent with their role at the late endosome, mutants of the Class D genes *VPS21* and *PEP12* have been reported to affect endocytic traffic (Gerrard et al. 2000a; Gerrard et al. 2000b). Vacuolar processing of Ste3p is delayed at the permissive temperature in ts (*t*emperature *s*ensitive) mutants of *PEP12* and *VPS21*, although less severely than in the *vps8ΔRING* mutant. Additionally, *Δpep12* and *Δvps21* strains accumulate Ste3p in endosomal intermediates, though these structures appear to be distinct from the abnormally enlarged late endosomes observed in mutants of *VPS8* (Chapter 3, Figure 3). There is also previous evidence to suggest that deletion of *VPS8* can also cause an endocytic defect. Using assays similar to those described in the present study, Luo and Chang (Luo and Chang 2000) reported a kinetic delay in delivery of FM 4-64 and Ste3p to the vacuole in a *Δvps8* strain. The observation that Ste3p is stabilized in a deletion mutant, however, is somewhat suspect given that the vacuoles in this strain are severely proteolytically impaired. One interpretation of this result is that the stabilized pool is not the result of an endocytic trafficking defect, but rather a defect in vacuolar proteolysis. The *vps8ΔRING* mutant has functional vacuoles, however, as evidenced by near wild-type levels of CPY maturation. It is likely, then, that the Ste3p stabilization phenotypes seen in both the *Δvps8* and *vps8ΔRING* mutants (Figure 1) are due to membrane trafficking defects. Figure 2 demonstrates that in a *Δvps8* mutant, Ste3p is internalized normally, placing this defect between plasma membrane and the vacuole. Figure 3B

indicates that a *Δvps8* mutant strain accumulates GFP-Ste3p in a late endosomal structure, indicating the Vps8p protein is not required for entry of endocytic material into this compartment. This counters prevailing models, which suggest that the Class D proteins are required for traffic into the late endosome from all sources (Gerrard et al. 2000a). Transit of Ste3p out of the endosome, however, does require Vps8p, and appears to involve the activity of the RING domain, as GFP-Ste3p accumulates in the late endosome in the *vps8ΔRING* mutant (Figure 3D).

Failure to ubiquitinate Ste3p at any of the C-terminal lysyl acceptor sites results in its accumulation at plasma membrane (Roth and Davis 2000). If the *vps8ΔRING* mutant is defective for ubiquitination of Ste3p, a similar phenotype would be expected. That Ste3p is internalized normally in a *Δvps8* mutant (Figure 2) suggests that Vps8p is not required for its ubiquitination. This is verified by the fact that all three forms of Ste3-Ub can be detected in *Δvps8* (Figure 4). One of these sites can accept a di-ubiquitin moiety, however, so it remains a possibility that one of the other acceptor lysines is a target for Vps8p activity, and that a Ste3p ubiquitination defect of the *vps8ΔRING* mutant is masked by ubiquitination at the other two sites.

The differential effects of the *vps8ΔRING* mutant on CPY and Ste3p sorting imply a division of function within the Vps8p molecule. This is confirmed by the nature of the genetic interaction between mutants of *VPS8* and *VPS21* (Horazdovsky et al. 1996). The requirement for Vsp8p in CPY sorting is bypassed in cells expressing the GTPase

defective *vps21Q66L* mutant (Figure 5A). This suggests that Vps8p acts upstream of Vps21p activation in Golgi to endosome traffic. However, the inability of activated Vps21p to bypass the Ste3p sorting phenotype of the *vps8ΔRING* mutant (Figure 5B) indicates that Vps8p is participating in another process that requires the RING domain, but that is independent of activated Vps21p. Such a model is supported by the observation that Vps9p, another factor that acts upstream of Vps21p, exhibits a similar division of function.

PEST-like sequences similar to the one in the Ste3p C-terminus have been identified in at least three other proteins, including the uracil permease, Fur4p, and the **a**-factor export protein, Ste6p (Roth et al. 1998). Since it appears that Vps8p is not responsible, ubiquitination of these proteins, as well as CPS, is most likely carried out by the very general HECT-domain E3 ubiquitin ligase, Rsp5p. Substrates of Rsp5p include Ste2p, the general amino acid permease Gap1p, and, as has been demonstrated recently, the exchange factor for the Vps21p Rab GTPase, Vps9p (Davies et al. 2003). Vps9p is targeted for ubiquitination at its C-terminus, probably within its C-terminal CUE domain. The CUE domain was originally identified as a region of the Cue1p protein that interacts with the ubiquitinated E2 enzyme, Ubc7p, and is thought to act as a general ubiquitin interaction motif (Biederer et al. 1997). The correlation between ubiquitination and ubiquitin binding holds in this case as well. Davies et al. demonstrate that immobilized Vps9p can bind free ubiquitin, and that mutation of conserved methionine 419 in the CUE domain abolishes this activity.

Removal of the CUE domain from Vps9p or mutation of M419 causes a very mild CPY sorting phenotype, appearance of an abnormal endosomal structure, and a Ste3p processing delay. As with the Vps8p protein, these data support a role for Vps9p separate from its function upstream of Vps21p in CPY sorting. The similar phenotypes observed in the *vps8ΔRING* and *vps9ΔCUE* mutants suggest that, in fact, Vps8p and Vps9p may be participating in these alternate processes together. This scenario seems very plausible given that the domains of Vps8p and Vps9p involved have E3 ubiquitin ligase activity and ubiquitin binding activity, respectively. Attempts to demonstrate a physical association between Vps8p and Vps9p have failed, however (data not shown), perhaps because ubiquitination of Vps9p is required for such a complex to occur.

In several cases, H2 RING fingers have been designated as "interaction" domains responsible for formation of complexes not known to be related to ubiquitination events (Bartoli et al. 2001; Elion 2001). Although it may be that in such cases ubiquitination events merely have yet to be uncovered, it is entirely possible that the RING finger of Vps8p is also a simple interaction domain that does not have ubiquitin ligase activity *in vivo*. The non-specific, *in vitro* polyubiquitination activity of the RING domain and immunoprecipitated Vps8p may be artifacts of the H2 RING finger structural motif, and may not be indicative of genuine E3 ligase activity. The answer to this question will only be provided with the discovery of a specific, *in vivo* substrate.

Materials and Methods

Strains and Reagents

All strains and reagents are identical to those described in Chapter 3, with the following exceptions: TVY1 (SEY6210Δ*pep4::LEU*), TVY1Δ*vps8*(TVY1Δ*vps8::HIS*), and SEY6210Δ*vps8*Δ*vps21* (SEY6210Δ*vps8::HIS*, Δ*vps21::NEO*).

Plasmid Construction

pRS424 Ste3p-GFP was a gift of B. Davies. It was constructed by fusing the C-terminus of the GFP open reading frame to the N-terminus of Ste3p, with transcription driven by the *STE3* promoter.

Labeling with FM 4-64

Cells from 1 mL of logarithmically growing cultures (OD_{600} = 0.5 to 1.0) were incubated with 50 µL of 16 µM FM 4-64 (pulsed) in prewarmed YPD for 15 minutes at 30°C. 1.2 mL of prewarmed YPD (chase) was added and incubation was continued for 1 hr. Cells were pelleted at low speed, resuspended in 50 µL YPD and placed on ice. Cells were held on ice until 2-3 µL per slide was used for standard fluorescence microscopy. For FM 4-64 labeling of cells expressing Ste3p-GFP, 5 OD of logarithmically growing cells containing pRS426 STE3-GFP were pulsed in 100 µL, and chased for 90 minutes in 2 mL YPD containing 3.125 µg/mL cyclohexamide. 1 mL of these cells was added to an equal volume of ice cold stop solution (20 mM NaFl, 20 mM NaN_3) and put on ice for 30

minutes. Cells were pelleted and resuspended in 100 µL cold stop solution and held on ice for microscopy.

Protease Shaving Assay

5 OD of logarithmically growing cells were pelleted and spheroplasted as described. Spheroplasts were resuspended in 1 mL YNB/glucose with complete amino acids containing 1M sorbitol and pulse-labeled with 15 µL EasyTag Express ^{35}S Protein Labeling Mix for 15 minutes at 30°C. 45 µL chase solution (0.2% yeast extract, 5 mM methionine, 1 mM cysteine) was added, and 500 µL of the cell suspension was collected and added to an equal volume of ice cold protease buffer (20 mM NaFl, 20 mM NaN_3, 1M sorbitol, 50 mM Tris pH 7.5) and held on ice. 500 µl of the remainder was "chased" for 60 minutes at 30°C, added to an equal volume of cold protease buffer, and held on ice for 30 minutes. Each of the samples was split in half, and the cells were pelleted and resuspended in 100 µL protease buffer, with or without 25 U/mL of Pronase, and incubated at 37°C with shaking for 60 minutes. The cells in each sample were then washed 4 times in 1 mL protease buffer containing protease inhibitors, precipitated in protease buffer containing 10% trichloroacetic acid (TCA), and held on ice for thirty minutes. The cells were pelleted at high speed for 1 minute, and sonicated briefly in two washes of cold acetone. All traces of acetone were removed by aspiration and vacuum drying for 5 minutes. The dry pellet was resuspended in 100 µL SDS cracking buffer (1% SDS, 50 mM Tris pH 7.5) by sonication, and incubated at 65°C for ten minutes. 1 mL of Tween IP buffer (0.5 % Tween 20, 50 mM Tris pH 7.5) was added, and samples were vortexed cleared twice by centrifugation at 13,000 xg for 10 minutes. 3µL of each

lysate was removed for scintillation counting. 10 µL of 100 mg/mL BSA and 1 µL α-Ste3p serum was added to each sample, which were then placed on a rocking platform overnight at 4°C. 75 µL of 15% suspension of Protein A-Sepharose beads was added to each sample, and rocking was continued for at least 90 minutes. The beads were washed twice in Tween IP buffer, and once in TBS (50 mM Tris pH 7.5, 150 mM NaCl). After removal of the washes, the beads were resuspended in 50 µL urea sample buffer (8 M urea, 5% SDS, 10% glycerol, 1% β-mercaptoethanol) and incubated at 37°C for 15 minutes. The beads were pelleted, and 20 µL of the supernatant was analyzed by gel electrophoresis followed by autoradiography.

Ste3p stabilization assay

Logarithmically growing cells were pelleted and resuspended at 2 OD/mL in YNB containing 2% glucose and complete amino acids. 1.25 µL/OD of cyclohexamide (1 mg/mL) was added to each culture, and immediately a 500 µL zero time sample was collected and added to 500 µL stop solution (20 mM NaFl, 20 mM NaN_3) on ice. 500 µL samples were collected and mixed with stop solution at the indicated time points. The cells were pelleted, flash frozen in liquid nitrogen, and thawed in 100 µL urea boiling buffer (8M urea, 5% SDS, 1% β-mercaptoethanol). 100 µL of acid-washed glass beads was added, and the samples were vortexed at high speed for 10 minutes. 100 µL of urea sample buffer was added, and the samples were incubated at 65°C for 10 minutes and cleared by centrifugation at 13,000 xg for 10 minutes. 10 µL was analyzed by gel electrophoresis and western blotting for Ste3p.

Phosphatase Treatment

5 OD of logarithmically growing cells were pelleted, flash frozen in liquid nitrogen, and thawed in 100 µL urea boiling buffer (8M urea, 5% SDS, 1% β-mercaptoethanol). 100 µL of acid-washed glass beads was added, and the samples were lysed by vortexing at high speed for 10 minutes. The lysates were cleared by centrifugation at 13,000 xg for 10 minutes. 2 µL of each lysate was diluted 1 mL of acid phosphatase buffer (20 mM sodium citrate, pH 6.0), and 75 mU of potato acid phosphatase (diluted to 15 mU/µL in acid phosphatase buffer) was added. Reactions were incubated at 30°C for 1 hr, BSA was added to 2 mg/mL, and reactions were precipitated by addition of 100 µL TCA and incubation for 30 minutes on ice. Precipitated proteins were then washed twice by sonication in cold acetone, and vacuum dried for 5 minutes. The dried pellet was resuspended by sonication in 40 µL urea boiling buffer and incubated at 65°C for 10 minutes. 40µL of urea sample buffer was added, and the samples were vortexed and cleared by centrifugation at 13,000 xg for 10 minutes. 40 µL of each sample was analyzed by gel electrophoresis and western blotting for Ste3p.

Western Blotting

Gels were blotted overnight in a mini Trans-Blot apparatus (BioRad) onto nylon-reinforced nitrocellulose according to manufacturer's instructions. Blots were rinsed in Tris-buffered saline (TBS, 50 mM Tris pH 8.0, 150 mM NaCl) and blocked in TBSTM (TBS containing 5% nonfat dry milk, 0.05% Tween 20, 1 mM NaN_3) for a minimum of 1 hr at room temperature or overnight at 4°C with rocking or shaking. The blocking

solution was changed for new TBSTM containing primary antibody for a minimum of 2 hr at room temperature or overnight at 4°C with rocking or shaking. Rabbit α-Ste3p serum and mouse α-ubiquitin antibody (Zymed) were used at a 1:1000 dilution. The blots were then washed 3 times for 10 minutes each in TBST (TBS containing 0.5% Tween 20), and incubated for a minimum of 90 minutes with TBSTM containing a 1:1,500 dilution of goat α-rabbit (for Ste3p detection) or goat α-mouse (for detection of ubiquitinated proteins) IgG conjugated to HRP. The blots were again washed 3 times for 10 minutes each in TBST, and rinsed twice with TBS. The blots were then incubated in a 1:5 dilution of SuperSignal West Femto Maximum Sensitivity Substrate for 3 minutes, blotted dry, placed in a sandwich of clear plastic film, and exposed to X-ray film.

CHAPTER FIVE

Discussion and Future Experiments

Proteomic scale efforts to identify protein complexes in yeast have placed Vps8p not within a complex of Class D VPS proteins, as might be expected, but rather in a complex of Class C VPS proteins (Gavin et al. 2002). As discussed earlier, the Class C proteins are involved in fusion of a variety of transport intermediates at the vacuole. The involvement of ubiquitination in this process is supported by the observation that Pep3p and Pep5p also contain C-terminal H2 RING finger domains, though the roles of these domains in Class C function remain to be determined. The idea that the RING domain of Vps8p plays a role in fusion of certain endosomes with the vacuole fits well with the observation that Ste3p accumulates in enlarged endosomal structures next to the vacuole in the *vps8ΔRING* mutant. Since biosynthetic traffic of CPS and CPY are unaffected however, one must invoke a model whereby two separate populations of late endosomes co-exist: one containing biosynthetic cargo and the other containing endocytic cargo. These compartments would be expected to have many of the same markers, such as Pep12p and Vps21p, but would differ in their requirements for fusion with the vacuole. Plasma membrane-derived endosomes would require a Class C complex containing a functional Vps8p RING finger, while biosynthetic endosomes would not require the

RING finger. Although this model of parallel endosomal pathways goes against current thinking, which would have endocytic and biosynthetic traffic intersecting at a common late endosome, any model has to account for the Ste3p-specific effect of the *vps8ΔRING* mutant.

There is evidence to suggest that the Class C proteins also function in Golgi to endosome traffic. Peterson and Emr (2001) identified a ts mutant of *PEP5* that affects CPY maturation, but not ALP maturation, and that has a Class D vacuolar morphology at the restrictive temperature. They also uncovered genetic interactions between this *pep5* mutant and ts mutants in *VPS21*, *VPS45*, and *VAC1*, and went on to show that Vac1p can physically associate with Pep5p in a pull-down assay. Srivastava et al. (2000) independently demonstrated genetic interactions between *PEP3*, *PEP12* and *VAC1*, and two-hybrid interactions between Vac1p and both Pep3p and Pep5p. As previously discussed, Vac1p associates with a Class D complex containing Vps21p, Pep12p and Vps45p, while Pep3p and Pep5 are part of a similar vacuolar complex including the Rab Ypt7p, the t-SNARE Vam3p, and the SM protein Vps33p (Seals et al. 2000).

Where does Vps8p fit into this scheme? Woolford et al. (1998) identified a mutant of *VPS8* that suppresses the CPY sorting defect associated with a *pep5* mutant, but not the defects in other routes to the vacuole. This result indicates the involvement of a Vps8p-Class C complex in Golgi to endosome sorting (Figure 1). As discussed in the Introduction, the TRAPP complexes link Ypt1p to its exchange factor, and at the vacuole,

the Class C complex ties Ypt7p to Vps39p (Wurmser et al. 2000). By analogy, then, it seems likely that the Class C complex also connects Vps21p to its upstream factors, Vps8p and Vps9p. In this scenario, Vps8p could serve as a bridge between the Class C complex and Vps9p. Thus, a *Δvps8* mutant can be bypassed by overexpressing or activating Vps21p, relieving the requirement for these upstream factors. It is important to note that overexpression of Vps9p does not bypass the requirement for Vps8p in CPY sorting. In the context of this model, this would indicate that association of Vps9p with the Class C complex is required for exchange activity towards Vps21p. Indeed, the *in vitro* exchange activity of Vps9p is not robust (Esters et al. 2001), and it has been suggested that ancillary factors are likely to be required for efficient activity *in vivo*.

Given its involvement in activation of Ypt7p and putative role in CPY sorting, it seems likely the core Class C complex of Pep3p and Pep5p also acts upstream of Vps21p in

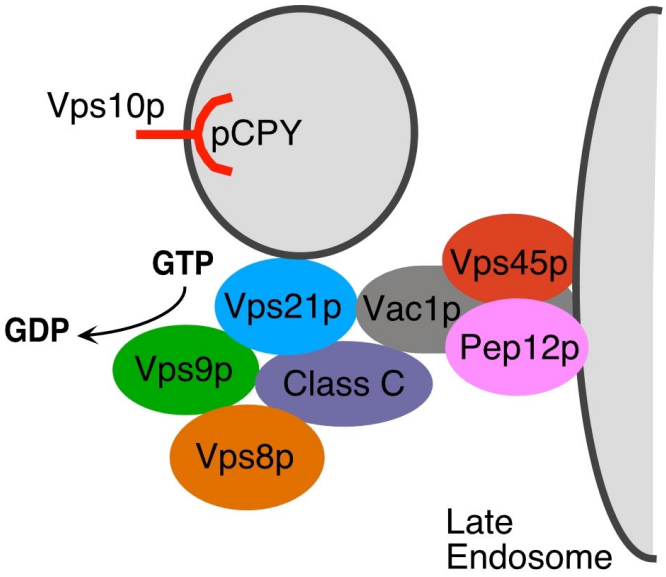

Figure 1 **A Class D tethering complex acts upstream of Vps21p in Golgi to endosome vesicular traffic.** The Class C complex linked to Vps8p and Vps9p would stimulate exchange of GDP for GTP on Vps21p anchored on Golgi-derived transport vesicles. Deletion of either the RING or the CUE domain does not impair this process enough to significantly affect activation of Vps21p, however, allowing association of Vps21p with the late endosomal complex and vesicle fusion to proceed.

association with Vps9p and Vps8p. Whether these participate together in a Rab activation and tethering complex, analogous to the vacuolar Class C complex and TRAPP, remains to be determined. Such a Class D tethering complex would be linked to the endosome through a Pep5/Vac1p association, and to the vesicle through a Vps9p/Vps21p interaction. This would lead to activation of Vps21p, allowing it to associate with Vac1p and Vps45p. This model predicts an association of Vps8p with the Class C complex, and large-scale efforts to identify protein complexes in yeast suggest that this indeed the case. These results should be verified, and it should be determined which elements of the complex contact Vps8p directly, and whether this association is mediated by the RING domain.

It is also expected that Vps9p would associate with the Class D tethering complex, possibly through Vps8p. Attempts to demonstrate a physical association between Vps8p and Vps9p have failed, however (data not shown), perhaps because CUE-directed ubiquitination of Vps9p is required for such a complex to occur. Vps39p binds the Class C complex through Pep5p, so perhaps Vps9p would also be linked to the Class D complex through Pep5p. Experiments to test these possibilities using ubiquitinated and unmodified Vps9p are essential.

What are the consequences of Vps9p monoubiquitination? The CUE domain could participate in an intramolecular interaction with the ubiquitin moiety, similar to HRS. This issue is best addressed through NMR or crystalographic analysis of Ub-Vps9p.

Monoubiquitination could also enhance its relatively weak Rab exchange activity, either by inducing a conformational change, or by allowing it to associate with other factors, such as the Class D complex, that upregulate exchange on Vps21p. Experimental answers to these questions require the isolation of Ub-Vps9p. Although it might be difficult to obtain adequate amounts of this protein from yeast cells, it seems probable that it could be generated in *in vitro* reactions that reconstitute the ubiquitin cascade. Alternatively, an in-frame fusion of monoubiquitin with the C-terminus of Vps9p may have similar properties to native Ub-Vps9p. Analogous constructs of Ste3p mimic the effects of its monoubiquitination *in vivo*.

The evidence connecting Vac1p with Pep5p suggests that Vac1p may function by a model proposed by De Renzis et al. (de Renzis et al. 2002) for a human protein with very

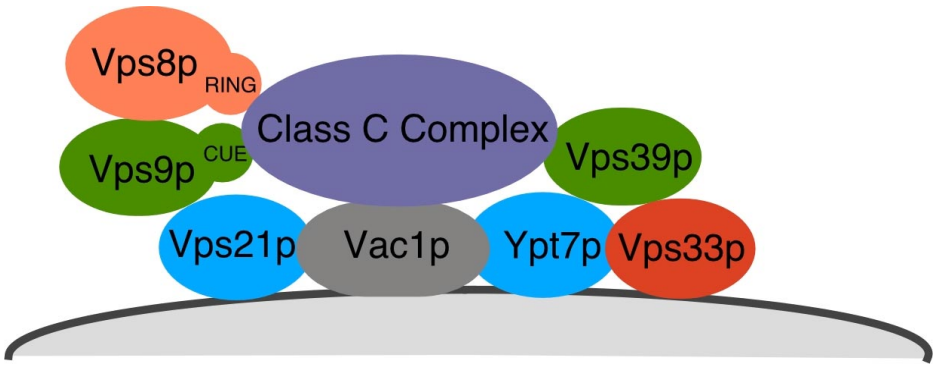

Figure 2 **Vac1p as a nexus for communication between Rab domains on late endosomes.** After fusion of Golgi-derived transport vesicles, the Class C complex and the potentially divalent Rab effector protein Vac1p would drive communication between the Vps21p complex and a Ypt7p complex containing the Sec1p homolog, Vps33p. The respective RING and CUE domains of Vps8p and Vps9p would be required in this process, such that the *vps8ΔRING* and *vps9ΔCUE* mutants display endosome to vacuole trafficking defects.

similar domain composition, Rabenosyn5. Rabenosyn5 links Rab5 and Rab4 complexes in mammalian early endosomes, perhaps allowing for exchange of cargo between Rab domains. Although such domains and communication between Rab complexes have not been described in the yeast endosomal system, the results described above seem to point to Vac1p working in much the same way as Rabenosyn5, connecting the Vps21p and Ypt7p complexes (Figure 2). As Rabenosyn5 binds both Rab4 and Rab5, such a model would predict an as of yet undiscovered interaction between Ypt7p and Vac1p.

How can the apparent effects of the *vps8ΔRING* mutant on endosome/vacuole transport be explained in this context? Extending the model for Vac1p function described above, it is possible that the Vps8p RING is required for communication between the Class D and Class C Rab domains, independent of the role of Vps8p upstream of Vps21p in CPY sorting. This model is consistent with the observation that activated Vps21p is unable to complement the Ste3p sorting phenotype of the *vps8ΔRING* mutant. It will be interesting to see whether the Ste3p phenotype of *vps9ΔCUE* is corrected by *vps21Q66L*. Might Vps9p also be required for something other than activation of Vps21p? Again, how these defects would selectively affect endocytic cargo such as Ste3p is unclear, nor would this model predict the target of the putative Vsp8p E3 ubiquitin ligase activity.

The sorting defects in the *vps8ΔRING* and *vps9ΔCUE* mutants suggest that these domains are more immediately involved in endosome to vacuole traffic, and less important for Golgi to endosome traffic. Only transit of the endocytic cargo Ste3p is affected, and not

the biosynthetic cargoes CPY and CPS. The Ste3p sorting defect is not a result of defective incorporation into MVBs, however, as delivery of CPS also requires this mechanism. This implies that there is separation of endocytic and biosynthetic cargo, even through the last stages of transit to the vacuole. This idea could be tested by visualizing various cargo trapped in late endosomal compartments. Temperature sensitive Class C mutants would be expected to accumulate cargo in the late endosome upon shifting to the restrictive temperature. The distribution of fluorescence-labeled cargo under these conditions would be very informative. Would they accumulate in distinct late endosomes, completely separated until mixing in the vacuole?

A more thorough structure/function analysis of Vps8p is in order. Vps41p is thought to participate with the Class C complex in fusion of Golgi-derived vesicles at the vacuole (Price et al. 2000). It has also been reported that it is required for formation of these vesicles in conjunction with the AP-3 adapter complex (Rehling et al. 1999). Similar to Vps8p, Vps41p carries two WD40 repeats near its N-terminus and contains one clathrin heavy chain repeat, though it lacks a C-terminal RING domain. It should be noted that the human homologue has an isoform that does have an H2 RING finger, placing it firmly in the same family of proteins as Vps8p, Pep3p, and Pep5p. Mutational analysis reveals that the WD40 repeats are required for association of Vps41 with the AP-3 complex. WD40 repeats are not well conserved at the sequence level, but usually adopt the β-propeller conformation found at the N-terminus of the clathrin heavy chain, which interacts with AP-1 and AP-2 complexes. Furthermore, a single clathrin heavy chain

repeat, which is present in Vps8p as well as Pep3p, and Pep5p, is thought to mediate the formation of Vps41p into homo-oligomers (Darsow et al. 2001). On this basis, it has been suggested that such oligomers act as a vesicle coat that is linked to membranes through association with the AP complexes. Proteins that play a structural role in membrane traffic might be expected to be fairly abundant, however. Vps8p, Vps41p and the Class C proteins, however, are present in only very small quantities, indicating that they most likely fulfil a catalytic rather than a structural function.

It will be important, however, to determine the contribution of its WD40 and clathrin heavy chain repeats in Golgi to endosome and endosome to vacuole membrane traffic. Vps8p, Vps41p, Pep5p, and Pep3p appear to comprise a family of proteins with similar domain structure that participate in transport to the vacuole/lysosome in all eukaryotes. Understanding the function of this family of proteins will solve basic mechanistic questions about conserved processes in membrane traffic.

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