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Distinct Domains within Vps35p Mediate the Retrieval of Two Different Cargo Proteins from the Yeast Prevacuolar/Endosomal Compartment

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Resident membrane proteins of the trans-Golgi network (TGN) of *Saccharomyces cerevisiae* are selectively retrieved from a prevacuolar/late endosomal compartment. Proper cycling of the carboxypeptidase Y receptor Vps10p between the TGN and prevacuolar compartment depends on Vps35p, a hydrophilic peripheral membrane protein. In this study we use a temperature-sensitive *vps35* allele to show that loss of Vps35p function rapidly leads to mislocalization of A-ALP, a model TGN membrane protein, to the vacuole. Vps35p is required for the prevacuolar compartment-to-TGN transport of both A-ALP and Vps10p. This was demonstrated by phenotypic analysis of *vps35* mutant strains expressing A-ALP mutants lacking either the retrieval or static retention signals and by an assay for prevacuolar compartment-to-TGN transport. A novel *vps35* allele was identified that was defective for retrieval of A-ALP but functional for retrieval of Vps10p. Moreover, several other *vps35* alleles were identified with the opposite characteristics: they were defective for Vps10p retrieval but near normal for A-ALP localization. These data suggest a model in which distinct structural features within Vps35p are required for associating with the cytosolic domains of each cargo protein during the retrieval process.

INTRODUCTION

The secretory pathway consists of a series of membrane-enclosed compartments that are distinct from each other in terms of morphology, molecular composition, and function. The establishment and maintenance of the unique identity of each organelle depend on the correct localization and retention of its resident proteins (Rothman and Wieland, 1996). One mechanism used by secretory pathway organelles to retain their resident proteins is to prevent entry of the resident proteins into transport vesicles that form from an organelle. A second mechanism that can also be used is the selective retrieval of resident proteins after they have left the organelle with other proteins. For example, the endoplasmic reticulum (ER) retains its resident proteins by a sorting mechanism that ensures that ER-derived vesicles are enriched for certain secretory proteins and depleted of resident proteins (Rexach *et al.*, 1994; Bednarek *et al.*, 1995). Any ER resident proteins that aberrantly enter these vesicles and are delivered to the cis-Golgi are transported back to the ER by selective retrieval machinery (Dean and Pelham, 1990; Gaynor *et al.*, 1994; Townsley *et al.*, 1994).

The mechanism of protein retention in the Golgi apparatus is less clear, although recent studies are beginning to illuminate this process. Several studies of the retention of membrane proteins in the earlier regions of the Golgi support a model in which the Golgi enzymes form complexes too large to enter transport vesicles, thus leading to static retention (Weisz *et al.*, 1993; Nilsson *et al.*, 1994). Since the transmembrane domains of Golgi enzymes have often been found to contain features necessary for proper retention, it has also been proposed that Golgi enzymes achieve residence by partitioning into distinct lipid domains within Golgi stacks (Pelham and Munro, 1993).

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[1] Abbreviations used: *CEN*, yeast centromere; CPY, carboxypeptidase Y; DPAP, dipeptidyl aminopeptidase; ER, endoplasmic reticulum; ORF, open reading frame; TGN, trans-Golgi network.

In contrast to the earlier regions of the Golgi, the localization of membrane proteins to the trans-Golgi network (TGN) appears to be a highly dynamic process. In animal cells, membrane proteins such as TGN38 (Stanely and Howell, 1993), furin (Chapman and Munro, 1994b; Molloy *et al.*, 1994), and the mannose 6-phosphate receptor (Duncan and Kornfeld, 1988) are known to continuously cycle between the TGN, plasma membrane, and endosomes. The sorting signals responsible for trafficking between these compartments reside on the cytosolic domains of these proteins and include aromatic amino acid, dileucine, and acidic cluster-based signals (Kornfeld, 1992; Stanely and Howell, 1993; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhees *et al.*, 1995). Endocytosis of these proteins from the plasma membrane and exit from the TGN appear to be mediated by interactions of their cytosolic domain signals with clathrin/adaptor coat complexes (for a review see Kirchhausen *et al.*, 1997). Retrograde transport from the late endosome to the TGN appears to be vesicle mediated and requires the small GTP-binding protein Rab9 at a postvesicle formation step (Riederer *et al.*, 1994). Although little is known about components of the vesicle coat that act in retrieval of TGN proteins from late endosomes, two recently identified peripheral membrane proteins, PACS-1 (Wan *et al.*, 1998) and TIP47 (Diaz and Pfeffer, 1998), are good candidates for such components. PACS-1 and TIP47 may play roles in cargo sorting at the late endosome because they localize to endosomal structures and bind in a sorting-signal–specific manner to the cytosolic domains of furin and the mannose 6-phosphate receptor, respectively.

Resident membrane proteins of the yeast TGN (sometimes referred to as the late Golgi) are also localized in a dynamic manner. The yeast TGN contains membrane proteins Kex2p, Kex1p, and dipeptidyl aminopeptidase (DPAP) A, which are involved in proteolytic processing of the secreted mating pheromone, α-factor (Fuller *et al.*, 1988). These proteins are known to cycle between the TGN and a prevacuolar endosomal compartment but, in contrast to animal cell TGN proteins, do not appear to visit the cell surface (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992; Bryant and Stevens, 1997). Localization of DPAP A and Kex2p is achieved, in part, by retrieval from the prevacuolar compartment, a process mediated by aromatic amino acid signals in their cytosolic domains (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993; Brickner and Fuller, 1997; Bryant and Stevens, 1997). In DPAP A, this signal consists of a FXFXD motif in which both F residues are absolutely required. Recently, a second signal in the DPAP A cytosolic domain required for reducing its rate of transport from the TGN to the prevacuolar compartment was identified (Bryant and Stevens, 1997).

The yeast TGN is also the site of sorting of vacuolar hydrolases, a process mediated by Vps10p. Vps10p functions as a membrane-bound sorting receptor in the TGN for the vacuolar hydrolase, carboxypeptidase Y (CPY) (Marcusson *et al.*, 1994; Cooper and Stevens, 1996). Analogous to the function of the mannose 6-phosphate receptor of animal cells, Vps10p/CPY complexes at the TGN are thought to enter vesicles that are transported to the prevacuolar compartment. CPY then dissociates from its receptor and is transported via a default pathway to the vacuole, the yeast equivalent of the lysosome. The receptor is then transported back to the TGN via a transport mechanism that is probably also vesicle mediated (Piper *et al.*, 1995; Seaman *et al.*, 1997; Seaman *et al.*, 1998). Vps10p also contains an aromatic-amino-acid–based signal in its cytosolic domain necessary for retrieval (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). Genetic analysis of the molecular machinery for transport between the TGN and prevacuolar compartment indicates that, for the most part, DPAP A, Kex2p, and Vps10p require the same machinery, although some notable exceptions have been observed (Rothman and Stevens, 1986; Robinson *et al.*, 1988; Nothwehr *et al.*, 1996; Voos and Stevens, 1998).

Recent experimental advances in yeast have shed light on the mechanism of retrograde traffic between the prevacuolar endosomal compartment and the TGN. In strains containing mutations in the *VPS29*, *VPS30*, and *VPS35* genes, Vps10p is mislocalized to the vacuole by a pathway dependent upon the prevacuolar compartment t-SNARE Pep12p but independent of late-secretory-pathway functions (Seaman *et al.*, 1997). This observation suggests that these three genes play a role in retrieval of Vps10p. More recently, the products of these three genes, along with the products of three other genes known to be involved in trafficking between the TGN and prevacuolar compartment, Vps5p, Vps17p, and Pep8p/Vps26p, have been shown to form a complex (Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997; Seaman *et al.*, 1998). This complex, termed the "retromer complex," localizes to the cytosolic face of the prevacuolar compartment and vesicles. This group of proteins may therefore act as a coat for retrograde vesicles that originate from the prevacuolar compartment and have a role in cargo-protein sorting. Homologues of the retromer proteins have been identified in higher eukaryotes, including humans, suggesting that they may perform an analogous role in cargo retrieval from an endosomal compartment in these systems. Implicit in the working model of yeast retromer function is that one or more of the retromer proteins binds to the retrieval signals on the cytosolic domains of Vps10p, DPAP A, and Kex2p and mediates their selective entry into forming vesicles. However, little is known about the nature of such interactions.

Table 1. Plasmids used in this study

Plasmid	Description	Reference
pAH16	pRS314 containing a DNA fragment encoding A-ALP	This study
pSN54	pRS313 containing a DNA fragment encoding A-ALP	Nothwehr et al. (1993)
pSN55	pRS316 containing a DNA fragment encoding A-ALP	Nothwehr et al. (1993)
pAH49	pSN55 with codons 2-11 of the A-ALP construct deleted	This study
pSN100	pSN55 with the Phe85 and Phe87 codons of A-ALP mutated to Ala	Nothwehr et al. (1993)
pLS13	*VPS35* gene in pRS316	This study
pLS12	*vps35-101* allele in pRS316	This study
pPB7-9.7	*vps35-103* allele in pRS316	This study
pPB7-10.1	*vps35-104* allele in pRS316	This study
pPB7-42.8	*vps35-105* allele in pRS316	This study
pPB7-108.5	*vps35-107* allele in pRS316	This study
pPB7-010.4	*vps35-108* allele in pRS316	This study
pHY5	pRS316 containing the *VPS27* gene under *GAL1* promoter control	T. H. Stevens
pPB6	*vps35*Δ::*URA3* allele in Bluescript KS$^+$	This study
pGPY55	*vps35*Δ::*HIS3* allele in a *CEN* plasmid	Paravicini et al. (1992)

In this study we have investigated the role of Vps35p in localization of a model TGN-membrane protein, A-ALP, and Vps10p. Using a variety of experimental approaches, we show that Vps35p is required for retrieval of both proteins from the prevacuolar compartment. Surprisingly, mutant alleles of *VPS35* were found that exhibited specific defects in either DPAP A (A-ALP) or Vps10p retrieval. Thus, distinct structural features within Vps35p are necessary for retrieval of each protein, an observation suggesting that Vps35p has a direct role in cargo sorting.

MATERIALS AND METHODS

Materials

Restriction enzymes and other enzymes used in subcloning procedures were obtained from New England Biolabs (Beverly, MA), United States Biochemical (Cleveland, OH), or Promega (Madison, WI). [^{35}S]express label and [α-^{35}S]dATP were purchased from New England Nuclear (Boston, MA). Oxalyticase was obtained from Enzogenetics (Corvallis, OR). All secondary antibodies used for immunofluorescence experiments were from Jackson ImmunoResearch Labs. (West Grove, PA). Other reagents were obtained from Sigma Chemical (St. Louis, MO) or as indicated.

Genetic and Nucleic Acid Manipulations

Most of the plasmids and all yeast strains used in this study are indicated in Tables 1 and 2, respectively. A centromeric (*CEN*), *TRP1*-based plasmid containing the A-ALP construct was generated

Table 2. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
SNY36-9A	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 pho8*Δ*::ADE2*	Nothwehr et al. (1995)
SNY17	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::LEU2*	Nothwehr et al. (1995)
SNY17-190	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::LEU2 vps35-101*	This study
SNY38	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::ADE2 vps1*Δ*::LEU2*	Nothwehr et al. (1995)
SNY79	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::LEU2 vps35*Δ*::HIS3*	This study
SNY94	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 pho8*Δ*::ADE2 end3-ts*	This study
SNY96-9D	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::ADE2 vps1*Δ*::LEU2 end3-ts*	This study
LSY6-2A	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 pho8::ste13-pho8 vps35*Δ*::HIS3*	This study
LSY9-5B	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::ADE2 end3-ts vps35*Δ*::HIS3*	This study
PBY1	*MAT*α *ra3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 lys2-801 pho8*Δ*::LEU2 pep4*Δ*::TRP1 vps35*Δ*::HIS3*	This study
PBY3	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 pho8*Δ*::ADE2 vps35*Δ*::URA3*	This study
PBY4	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 pho8*Δ*::ADE2 vps35-109 (ts)*	This study
PBY15	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 suc2-*Δ*9 pho8*Δ*::ADE2 pep4-*Δ*H3 vps35*Δ*::HIS3*	This study
W303-1A	*MAT***a** *ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100*	R. Rothstein
AHY65	*MAT***a** *ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pho8*Δ*::ADE2 pep4-*Δ*H3 vps27*Δ*::LEU2*	This study
AHY68	*MAT***a** *ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pho8*Δ*::ADE2 pep4-*Δ*H3 vps27*Δ*::LEU2 vps35*Δ*::HIS3*	This study
AHY69	*MAT***a** *ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 pho8*Δ*::ADE2 pep4-*Δ*H3 vps27*Δ*::LEU2 vps35-101*	This study

All strains are derived from SNY17 or SNY36-9A with the exception of AHY65, AHY68, and AHY69, which are derived from W303-1A.

by subcloning the 2.37-kilobase pair (kbp) *Eag*I–*Eco*RI fragment from pSN55 into the *Eag*I/*Eco*RI sites of pRS314, resulting in pAH16. Deletion of codons 2–11 of the *STE13* gene in plasmid pCJR71 (Roberts *et al.*, 1992) was performed (Kunkel *et al.*, 1987) to generate pAH48. To incorporate the deletion into the context of the *STE13–PHO8* fusion construct that expresses the A-ALP fusion protein, an *Eag*I–*Bgl*II fragment from pAH48 was swapped for the corresponding *Eag*I–*Bgl*II fragment present in pSN55, resulting in plasmid pAH49.

A construct designed to integrate the *STE13–PHO8* fusion at the *PHO8* locus was made by subcloning the 2.37-kbp *Eag*I–*Eco*RI fragment from pSN55 into the *Eag*I/*Eco*RI sites of pRS306 (Sikorski and Hieter, 1989). The resulting plasmid, pSN282, was digested with *Sac*II, blunt-ended with Klenow enzyme and dNTPs, digested with *Eag*I, and then ligated to a 1.4-kbp *Sma*I–*Eag*I fragment from the 5′-untranslated region of the *STE13* gene, resulting in pAH17. pAH17 was digested with *Sac*I, blunt-ended with Klenow enzyme and dNTPs, and was ligated to a blunt-ended 0.83-kbp *Eco*RI–*Hin*dIII fragment from pSN92 (containing the 5′-region of the *PHO8* gene), resulting in plasmid pSN288. The *STE13–PHO8* construct was integrated into the *PHO8* locus by transforming yeast strains with pSN288 digested with *Sac*I and selecting for Ura^+ prototrophs. Ura^+ clones were then grown on media containing 5-fluoroorotic acid (Boeke *et al.*, 1984) to select for clones that looped out wild-type *PHO8*.

A *vps35Δ::URA3* gene replacement construct was made by subcloning the 1.1-kbp *Sma*I fragment from pJJ242 (Jones and Prakash, 1990) containing *URA3* into the *Sna*BI sites of pLS9, a plasmid consisting of the 3.9-kbp *Msc*I fragment containing *VPS35* inserted into the *Sma*I site of Bluescript KS^+. The resulting plasmid, pPB6, was digested with *Bse*RI/*Eco*NI, transformed into yeast strain SNY36–9A, and Ura^+ prototrophs were screened for the Vps^- phenotype, resulting in strain PBY3. Likewise, to generate yeast strains carrying the *vps35Δ::HIS3* allele, plasmid pGPY55 (Paravicini *et al.*, 1992) was digested with *Nci*I/*Xba*I, the 4.5-kbp fragment was purified and transformed into yeast, and His^+ Vps^- transformants were identified.

To construct a *CEN-VPS35* plasmid, a 3.9-kbp *Msc*I fragment containing the *VPS35* gene was obtained from p35–1, a YCp50-based plasmid isolated from a yeast-genomic library (Rose *et al.*, 1987). This fragment was subcloned into the *Sac*I/*Kpn*I sites of pRS316 that had been made blunt by Klenow DNA polymerase, resulting in pLS13. The *vps35–101* allele was cloned by gap-repairing plasmid p35–1 digested with *Sna*BI/*Afl*II in yeast strain SNY17–190 (Table 2), resulting in plasmid pLS11. The 3.9-kbp *Msc*I fragment from pLS11 was subcloned into pRS316 (using the same approach used for pLS13), resulting in plasmid pLS12.

Random Mutagenesis of the VPS35 Gene

The *VPS35* gene was subjected to random PCR mutagenesis using an in vivo gap-repair method. Using a *VPS35*-containing plasmid as a template, a 3.272-kbp PCR fragment was amplified under mutagenic PCR conditions (Cadwell and Joyce, 1992). This fragment corresponded to positions −177 to +3095 of the *VPS35* gene, where the first nucleotide of the open reading frame (ORF) is defined as the +1 nucleotide. The PCR fragment was cotransformed into yeast strain LSY6–2A, along with linearized pLS13 plasmid DNA that had been digested with *Bse*RI/*Eco*NI, so that most of the *VPS35* ORF was removed. Yeast transformants containing circular plasmids generated via homologous recombination were selected on minimal media lacking uracil. A total of 14,000 transformants were screened for defects in CPY sorting using a colony-blotting assay (Roberts *et al.*, 1991) and for A-ALP retention defects using a plate-activity assay (Nothwehr *et al.*, 1996). Plasmids were isolated from mutants that exhibited either the A-ALP retention phenotype or the CPY secretion phenotype (but not both), and the linkage of the phenotype to the plasmid was verified. Mutants were screened separately for temperature-sensitive *vps35* alleles by assessing CPY secretion at 22 and 35°C. Plasmid p11G-2 containing the *vps35–109* allele was isolated from one of the temperature-sensitive mutants.

To replace the *VPS35* allele with the temperature-sensitive *vps35–109* allele in yeast, the insert from p11G-2 was released by digestion with *Bse*RI/*Eco*NI, gel purified, and cotransformed with pRS315 (Sikorski and Hieter, 1989) into strain PBY3. Leu^+ transformants in which the mutant *vps35* allele from the plasmid insert replaced the *vps35Δ::URA3* allele were identified by growth on 5-fluoroorotic acid. The presence of the *vps35–109* allele was confirmed by analyzing CPY secretion at the nonpermissive and permissive temperatures, resulting in yeast strain PBY4. The introduction of the *vps35–101* allele into yeast, resulting in strain AHY69, was achieved using a similar approach.

Mutations causing the CPY secretion or A-ALP retention phenotypes were initially mapped to intervals within the *VPS35* ORF contained within the pLS13-derived mutant plasmids. This was achieved by swapping of restriction fragments with non-mutagenized pLS13 and analyzing the phenotypes of the resulting plasmids when transformed into a *vps35Δ* yeast strain. The region within the interval containing the critical mutation was then DNA sequenced and compared with the wild-type *VPS35* sequence. If an interval contained more than one mutation, site-directed mutagenesis was used to introduce each single mutation into pLS13, and the phenotypic effects of each mutation were assessed, thus leading to identification of a single-residue change responsible for the phenotype.

Radiolabeling, Immunoprecipitation, and Subcellular Fractionation

The procedure for immunoprecipitation of CPY was performed using a rabbit antibody against CPY as described previously (Vater *et al.*, 1992). Likewise, immunoprecipitation of A-ALP was performed using previously generated rabbit anti-ALP serum (Nothwehr *et al.*, 1996) and a procedure previously described (Nothwehr *et al.*, 1993). Radioactively labeled proteins were quantified from gels using a Phosphorimager system (Fuji Photo Film). Half-times of processing were determined by linear-regression analysis followed by plotting the percentage of total and processed protein as a function of time.

Subcellular fractionation was carried out by incubating 5 OD_{600} U of cells grown in minimal media lacking methionine in 1 ml of 0.1 M Tris, pH 9.4, 10 mM DTT for 10 min at room temperature. The cells were pelleted and spheroplasted in a 1 ml solution containing 20 mM Tris, pH 7.5, minimal media lacking methionine, 1 M sorbitol, and 25 μg/ml oxalyticase at 30°C. The spheroplasts were pelleted, resuspended in 1 ml minimal media lacking methionine and containing 1 M sorbitol, and pulsed at 30°C by adding 200 μCi of [^{35}S]express label. After a 30-min pulse, a 45-min chase was initiated by adding 250 μl of a solution containing 25 mM methionine and 5 mM cysteine. The labeled spheroplasts were harvested and lysed by incubating in 1 ml of 25 mM sodium phosphate, pH 7.4, 200 mM mannitol, 1 mM EDTA on ice for 20 min. Unlysed cells were removed by centrifugation at 450 × *g* for 5 min. The 450 × *g* supernatant was then centrifuged at 13,000 × *g* for 15 min to generate pellet (P13) and supernatant (S13) fractions. The S13 was then centrifuged at 150,000 × *g* for 60 min to generate P150 and S150 fractions. The S150 fractions were trichloroacetic acid precipitated and pellets were washed with acetone. The S150 trichoroacetic acid pellets, as well as the P13 and P150 membrane pellets, were resuspended in 100 μl of 8 M urea, 5% SDS, and were heated at 65°C for 10 min. Immunoprecipitation of Vps35p from 20 μl of each fraction was carried out as previously described (Nothwehr *et al.*, 1993) using 1.2 μl of anti-Vps35p rabbit serum (a generous gift from Scott Emr).

Fluorescence Microscopy

The procedures for preparation of fixed spheroplasted yeast cells, attachment to microscope slides, and costaining of the A-ALP fusion

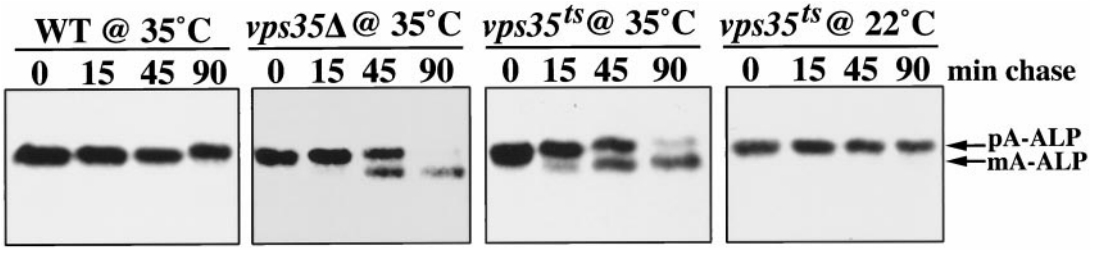

Figure 1. A-ALP is processed rapidly in a *vps35-ts* strain after shifting to the nonpermissive temperature. Wild-type (SNY36–9A), *vps35*Δ (SNY79), and *vps35-ts* (PBY4) yeast strains carrying a *CEN* plasmid directing expression of A-ALP (pSN55) were analyzed. The strains were grown for several doublings at 22°C, preincubated at 35°C for 10 min, and then pulsed with [^{35}S]methionine/cysteine for 10 min before unlabeled amino acids were added to initiate the chase. At the indicated times A-ALP was immunoprecipitated from the cultures and analyzed by SDS-PAGE and fluorography. The positions of unprocessed pro-A-ALP (pA-ALP) and mature A-ALP (mA-ALP) are indicated.

protein and Vma2p using an anti-ALP polyclonal antibody and anti-Vma2p monoclonal antibody 13D11-B2 (Molecular Probes, Eugene, OR) were previously described (Roberts *et al.*, 1991; Nothwehr *et al.*, 1995).

For induction of *VPS27* in yeast strains via the *GAL1* promoter before analysis by fluorescence microscopy, strains were propagated overnight in minimal media containing 2% (wt/vol) raffinose. Log-phase cultures were then adjusted to 2% galactose and, after 0 and 90 min, aliquots of 10 ml were removed and fixed by addition to 1.2 ml of 37% formaldehyde.

To simultaneously detect Vps10p and A-ALP, fixed cells on slides were incubated with the following solutions followed by extensive washing using 5 mg/ml BSA in PBS after every step: 1) a 1:30 dilution of rabbit anti-Vps10 (a generous gift of T.H. Stevens) and a 1:3 dilution of mouse anti-ALP 1D3 monoclonal supernatant (Nothwehr *et al.*, 1996); 2) 1:500 dilution of biotin-conjugated donkey anti-rabbit IgG (H+L); and 3) a 1:500 dilution of FITC–streptavidin and a 1:2000 dilution of Texas Red-conjugated goat anti-mouse IgG (H+L). To simultaneously detect Vps10p and Vma2p, the following incubations were used: 1) a 1:30 dilution of rabbit anti-Vps10 and a 1:10 dilution of mouse anti-Vma2p; 2) a 1:500 dilution of biotin-conjugated goat anti-rabbit IgG (H+L); and 3) 1:500 dilutions of FITC–streptavidin and of Texas Red-conjugated goat anti-mouse IgG (H+L).

Yeast cells were photographed using an Olympus BX-60 fluorescence microscope (Olympus, Lake Success, NY). Film negatives were digitized using a Umax Powerlook III scanner and adjusted using Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA).

RESULTS

Vps35p Has a Direct Role in Retention of a TGN-Membrane Protein

A-ALP is a model TGN-membrane protein consisting of a fusion between the N-terminal cytosolic domain of DPAP A and the transmembrane and lumenal domains of alkaline phosphatase (ALP). A failure to retain A-ALP in the TGN results in its delivery to the vacuole where its C-terminal propeptide is removed by a vacuolar protease (Klionsky and Emr, 1989; Nothwehr *et al.*, 1993). A-ALP processing can be detected either by a mobility change on SDS-PAGE gels or as an increase in enzymatic activity. This is because Golgi-localized pro-A-ALP is inactive, and vacuole-localized mature A-ALP is active. Thus, the processing/activity of A-ALP serves as a convenient assay for TGN retention.

We previously reported a genetic screen for *grd* mutants that exhibit vacuolar processing of A-ALP (Nothwehr *et al.*, 1996). Mutants from the *grd9/vps35* complementation group exhibited a very strong A-ALP processing phenotype and also secreted unprocessed α-factor, suggesting a defect in retention of Kex2p as well. In addition, *vps35* null mutants aberrantly secrete most of their newly synthesized CPY (Paravicini *et al.*, 1992).

The retention defect in *vps35* mutants could reflect a direct role for Vps35p in some aspect of A-ALP retention or could be an indirect consequence of the prolonged absence of Vps35p in *vps35* null mutants. To distinguish between these possibilities, we analyzed Golgi-retention phenotypes after rapid inactivation of Vps35p expressed from a temperature-sensitive allele.

A library of *vps35*Δ strains containing centromeric (*CEN*) plasmids with randomly mutagenized *VPS35* inserts was screened for mutants that exhibited temperature-sensitive defects. One such allele, *vps35–109* (hereafter referred to as *vps35-ts*), exhibited normal TGN retention and CPY sorting at 22°C but severe defects in these processes at 35°C. The rate of appearance of the A-ALP–processing defect was assessed after shifting *vps35-ts* cells from 22 to 35°C. Figure 1 shows the results of an experiment in which wild-type, *vps35*Δ, and *vps35-ts* cells were propagated overnight at 22°C, preincubated at 35°C for 10 min, pulse labeled for 10 min, chased for the indicated times, and subjected to immunoprecipitation of A-ALP. Whereas little or no processing is observed in the wild-type strain even after a 90-min chase, the *vps35*Δ strain exhibits a processing half-time of ~50–60 min. A severe processing phenotype was also observed in the *vps35-ts* strain at 35°C that was virtually indistinguishable from that of the null strain. However, at the permissive temperature of 22°C, the *vps35-ts* strain exhibits no detectable processing during the time course. The observation that A-ALP retention is rapidly lost after inactivating Vps35p suggests that Vps35p performs a function intimately involved in the mechanism of TGN localization.

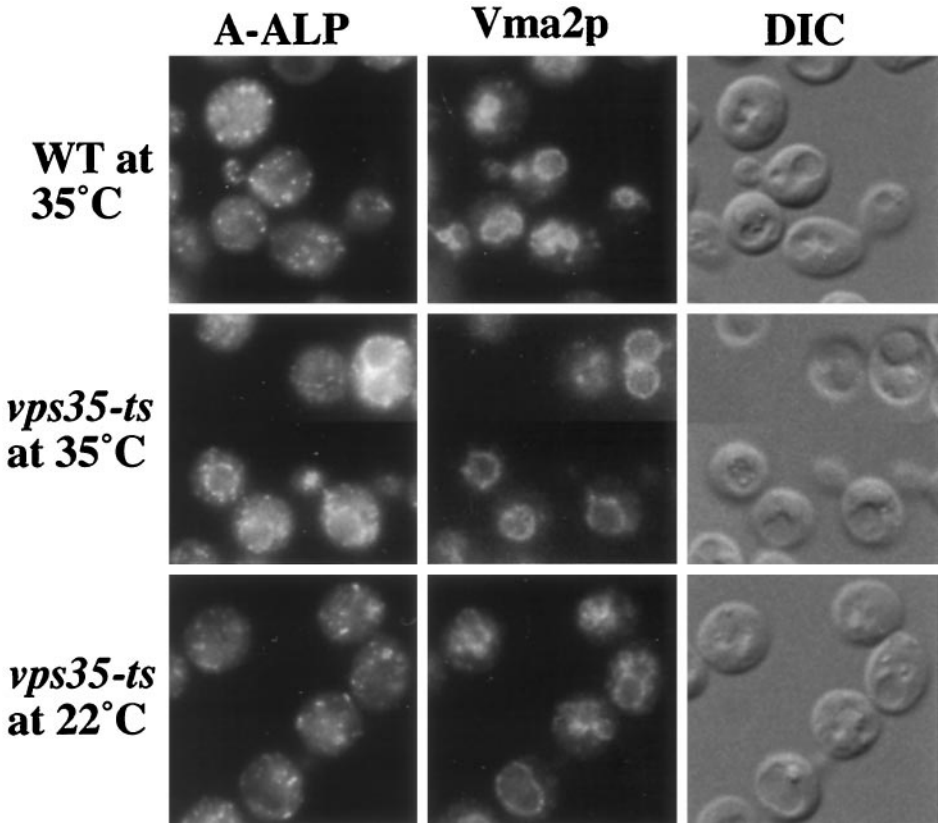

Figure 2. A-ALP is mislocalized to the vacuole in *vps35-ts* mutant cells. Wild-type (SNY17) and *vps35-ts* (PBY4) strains carrying a *CEN* plasmid directing expression of A-ALP (pSN55) were propagated for several doublings at 22°C before being shifted to 35°C for 40 min or incubated at 22°C for 40 min (as indicated). The cells were then fixed, spheroplasted, and costained with antibodies against ALP and Vma2p. After subsequent treatment with fluorochrome-conjugated secondary antibodies, the cells were viewed by DIC optics and by epifluorescence through filters specific for FITC and Texas Red.

Immunofluorescence microscopy was used to address whether A-ALP is indeed mislocalized to the vacuole in *vps35* mutants. The localization of A-ALP and Vma2p, a vacuolar membrane marker, in wild-type and *vps35-ts* cells was analyzed at the permissive and nonpermissive temperatures (Figure 2). In wild-type cells, A-ALP exhibits a nonvacuolar, punctate staining pattern characteristic of the yeast Golgi (Redding *et al.*, 1991; Nothwehr *et al.*, 1993). However, in *vps35-ts* cells incubated at the nonpermissive temperature for 40 min, most of the A-ALP colocalizes with the Vma2p marker, indicating that A-ALP has been mislocalized to the vacuole. The remaining A-ALP that is localized to nonvacuolar structures in these cells could still be retained in the TGN or could be in the prevacuolar compartment awaiting retrieval. Consistent with the pulse–chase experiment in Figure 1, at 22°C A-ALP expressed in the *vps35-ts* strain appears to be localized normally to the Golgi apparatus.

A-ALP Reaches the Vacuole in vps35Δ Cells Independent of the Endocytic Pathway

The observation that a loss of Vps35p function caused A-ALP to be mislocalized to the vacuole suggested that Vps35p could act at the level of the TGN or it could act in retrieval from the prevacuolar compartment. In the latter case, A-ALP in *vps35* mutants would be expected to be directly transported from the TGN to the prevacuolar compartment and then on to the vacuole. However, if Vps35p acts at the TGN, it is possible that, in the absence of Vps35p function, A-ALP may be initially mislocalized to the plasma membrane and then travel to the vacuole via the endocytic pathway, as has been observed in *vps1* mutants (Nothwehr *et al.*, 1995).

To distinguish between these possibilities, we asked whether the transport of A-ALP to the vacuole in *vps35* mutant cells was dependent on the *END3* gene that encodes a protein necessary for the internalization step of endocytosis (Bénédetti *et al.*, 1994). Immunoprecipitation of A-ALP from a *vps35Δ end3-ts* strain incubated at the nonpermissive temperature for the *end3-ts* allele (37°C) shows that, after a 60-min chase, A-ALP is processed with kinetics similar to that of the *vps35Δ* single mutant (Figure 3). As expected, under these conditions no processing is observed in the wild-type and *end3-ts* single-mutant strains. A-ALP in a *vps1Δ* mutant is ~50% processed after 60 min, but this processing is blocked if the *end3-ts* allele is introduced, consistent with prior observations (Nothwehr *et al.*, 1995). Because A-ALP is known to depend on the early-endocytic pathway for delivery to the vacuole in

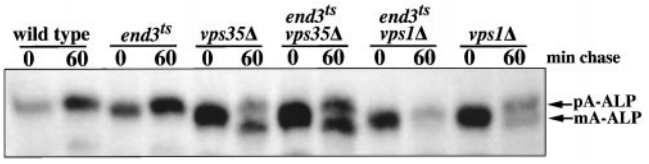

Figure 3. The delivery of A-ALP to the vacuole in *vps35*Δ cells does not require the early endocytic pathway. Wild-type (SNY36–9A), *end3-ts* (SNY94), *vps35*Δ (SNY79), *end3-ts vps35*Δ (LSY9–5B), *vps1*Δ (SNY38), and *end3-ts vps1*Δ (SNY96–9D) cells carrying a plasmid expressing A-ALP (pSN55) were analyzed. Each strain was propagated at 22°C for several doublings before being preincubated at 37°C for 10 min, and then pulsed with [^{35}S]methionine/cysteine for 10 min and chased by the addition of unlabeled amino acids. At the indicated times A-ALP was immunoprecipitated from the cultures and analyzed by SDS-PAGE and fluorography. The positions of unprocessed pro-A-ALP (pA-ALP) and mature A-ALP (mA-ALP) are indicated.

vps1 cells, this control experiment shows that under these conditions the endocytic pathway is blocked in the strains carrying the *end3-ts* allele. These data are consistent with the view that, in the absence of Vps35p function, A-ALP is transported from the TGN to the prevacuolar compartment and then to the vacuole.

VPS35 Is Involved in Retrieval of A-ALP from the Prevacuolar Compartment but Not in Slowing Exit from the TGN

The observation that A-ALP is not transported to the vacuole via the plasma membrane in *vps35* mutant cells is consistent with a role for Vps35p in retrieval of A-ALP from the prevacuolar compartment. If this is the case, a strain containing both a mutation in A-ALP that specifically prohibits retrieval and a *vps35*Δ mutation should have no stronger a retention defect than strains containing either mutation alone. To test this prediction, we analyzed the processing kinetics of A-ALP and a retrieval-defective A-ALP mutant, A(F85A; F87A)-ALP (Nothwehr *et al.*, 1993; Bryant and Stevens, 1997), wild-type and *vps35*Δ strains (Figure 4). Consistent with a role for Vps35p in retrieval, the processing rate of A(F85A; F87A)-ALP in *vps35*Δ cells was no more severe than processing of A(F85A; F87A)-ALP in a wild-type strain or A-ALP in a *vps35*Δ strain and was, in fact, slightly less severe (compare lanes 16–18 with lanes 13–15 and 4–6).

Similarly, if Vps35p is involved in retrieval, then a strain containing both a mutation in A-ALP that specifically prohibits static retention and a *vps35*Δ mutation should exhibit a much more dramatic A-ALP retention defect than strains containing either mutation alone. We indeed observed that the processing rate of static retention-defective A(Δ2–11)-ALP (Bryant and Stevens, 1997) in a *vps35*Δ strain was much more rapid (half-time of 15–20 min) than in a wild-type strain that expressed A(Δ2–11)-ALP (half-time of >180 min) or a *vps35*Δ strain that expressed A-ALP (half-time of ~40 min; compare lanes 10–12 with lanes 4–6 and 7–9). Taken together, these double-mutant analyses indicate that Vps35p is involved in retrieval of A-ALP from the prevacuolar compartment but is not required for static retention.

To extend these results we used a recently developed assay (Bryant and Stevens, 1997) to simultaneously analyze retrieval of both A-ALP and Vps10p from the prevacuolar compartment by indirect-immunofluorescence microscopy. Mutations in the *VPS27* gene are known to cause both a block in the prevacuolar compartment to TGN-retrieval pathway and in the anterograde pathway between the prevacuolar compartment and the vacuole (Piper *et al.*, 1995). Thus, in *vps27* mutant cells, proteins such as A-ALP and Vps10p that normally cycle between the TGN and the prevacuolar compartment are trapped in an exag-

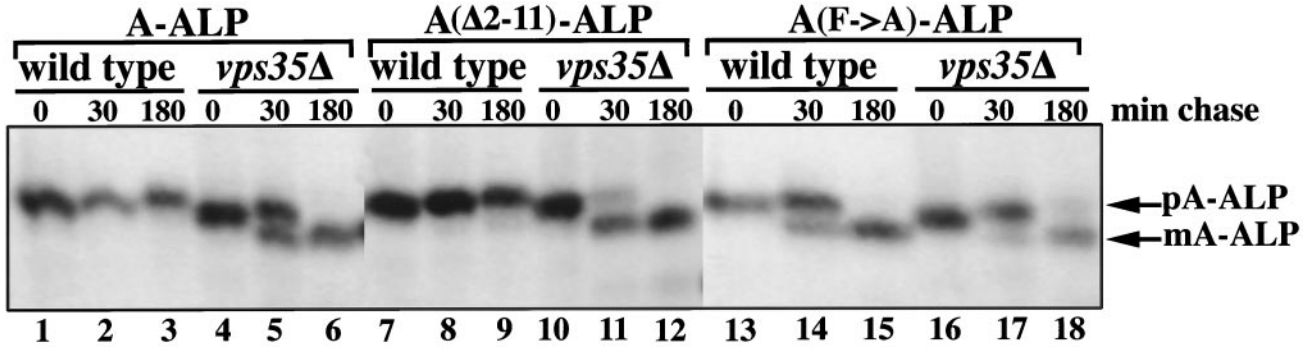

Figure 4. Phenotypes resulting from a loss of Vps35p function combined with a retrieval-defective, or static retention-defective, A-ALP mutant indicate a role for Vps35p in retrieval. Wild-type (SNY17) and *vps35*Δ (SNY79) strains carrying plasmids expressing A-ALP (pSN55; lanes 1–6), A(Δ2–11)-ALP (pAH49; lanes 7–12), or A(F85A; F87A)-ALP (pSN100; lanes 13–18) were propagated at 30°C, radioactively pulsed for 10 min and chased, and at the indicated times wild-type or mutant A-ALP was immunoprecipitated. Samples were analyzed using SDS-PAGE and fluorography.

gerated prevacuolar compartment. Rapid induction of synthesis of Vps27p in a *vps27*Δ strain, using a plasmid containing the *VPS27* gene under control of the galactose-inducible *GAL1* promoter, allows retrieval of Vps10p and A-ALP from the prevacuolar compartment back to the TGN after 90 min of induction (Voos and Stevens, 1998). We therefore tested whether loss of Vps35p function would prevent the retrieval observed in a *vps27*Δ strain after induction of Vps27p synthesis.

Before induction of Vps27p synthesis (0 min time point), A-ALP and Vps10p are localized to one to two prominent, nonvacuolar structures per cell for both the *vps27*Δ *VPS35* and *vps27*Δ *vps35*Δ strains (Figure 5), typical of the staining pattern for these antigens in *vps27* mutants (Raymond *et al.*, 1992; Piper *et al.*, 1995). After 90 min of induction, both A-ALP and Vps10p in the *vps27*Δ *VPS35* strain exhibit a punctate staining pattern, indicating that they have redistributed back to the TGN. We observed a less-than-complete overlap of the staining patterns of A-ALP and Vps10p, perhaps because of the fact that Vps10p cycles between the prevacuolar compartment and TGN much more frequently than A-ALP (Bryant and Stevens, 1997). This would suggest that a much greater fraction of Vps10p might be localized to the prevacuolar compartment in the steady state as compared with A-ALP. In contrast, in the strain lacking Vps35p function (*vps27*Δ *vps35*Δ), both A-ALP and Vps10p exhibited vacuolar membrane staining after 90 min of Vps27p induction (Figure 5; compare staining patterns with the crater-like appearance of vacuoles observed under differential–interference contrast [DIC] optics). These data indicate that, upon induction of Vps27p synthesis, retrieval of A-ALP and Vps10p was blocked because of the absence of Vps35p function, and instead they were transported from the prevacuolar compartment to the vacuole by default. In summary, these experiments indicate that Vps35p performs a function essential for retrieval of both A-ALP and Vps10p from the prevacuolar compartment.

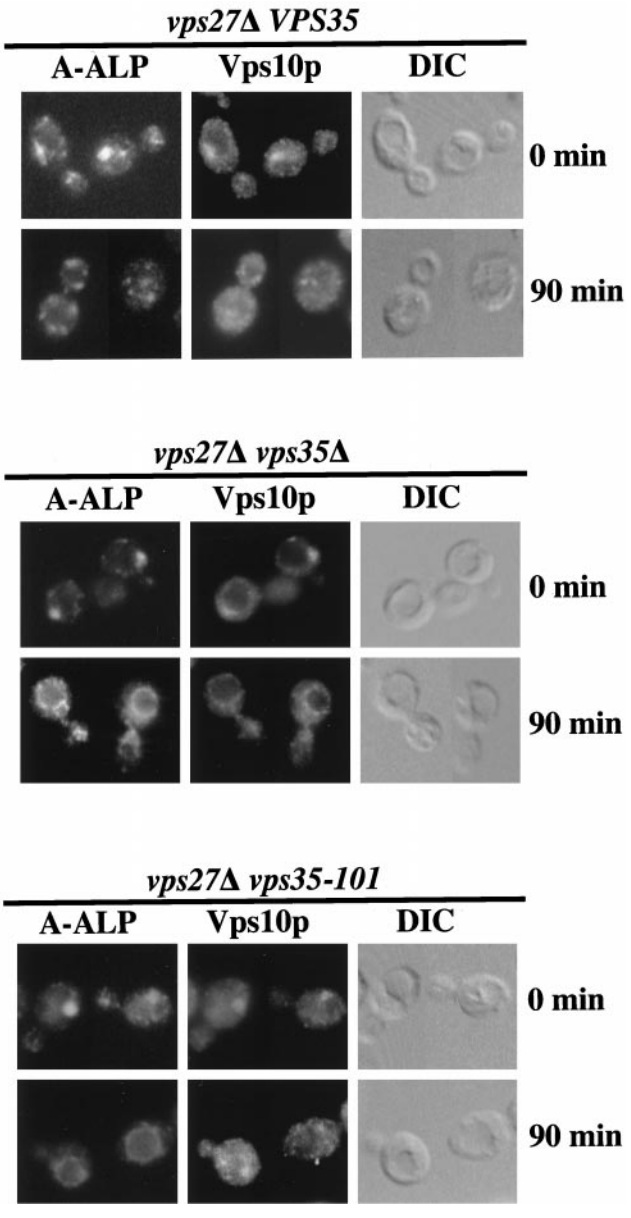

Figure 5. Vps35p is required for retrieval of A-ALP and Vps10p from the prevacuolar compartment to the Golgi. *vps27*Δ (AHY65), *vps27*Δ *vps35*Δ (AHY68), and *vps27*Δ *vps35–101* (AHY69) strains containing *CEN* plasmids directing expression of A-ALP (pAH16) and containing the *VPS27* gene under *GAL1* promoter control (pHY5) were propagated in media containing raffinose as a carbon source. At the 0-min time point the cultures were adjusted to 2% galactose to induce expression of *VPS27*. After 0 and 90 min after galactose addition, cells were fixed, spheroplasted, and costained with antibodies against ALP and Vps10p. After subsequent treatment with fluorochrome-conjugated secondary antibodies, the cells were viewed by DIC optics and by epifluorescence through filters specific for FITC and Texas Red.

Distinct Domains in Vps35p Are Involved in Regulating Retrieval of A-ALP and Vps10p

In addition to the *grd* mutants reported to comprise 18 complementation groups (Nothwehr *et al.*, 1996), we have recently characterized additional *grd* mutants. One of these mutant strains (SNY17–190) exhibited a substantial A-ALP-processing phenotype but showed no CPY-missorting defect. Genetic analysis revealed that the mutation responsible for the phenotype was contained within the *GRD9/VPS35* gene. This was surprising because *vps35* null mutants are severely defective for both CPY sorting (Paravicini *et al.*, 1992) and retention of A-ALP (Figure 1). The discovery of a *vps35* allele defective for A-ALP retention but fully functional for CPY sorting suggests that Vps35p may contain a structural feature necessary for retention of A-ALP distinct from a structural feature necessary for proper trafficking of the CPY receptor, Vps10p.

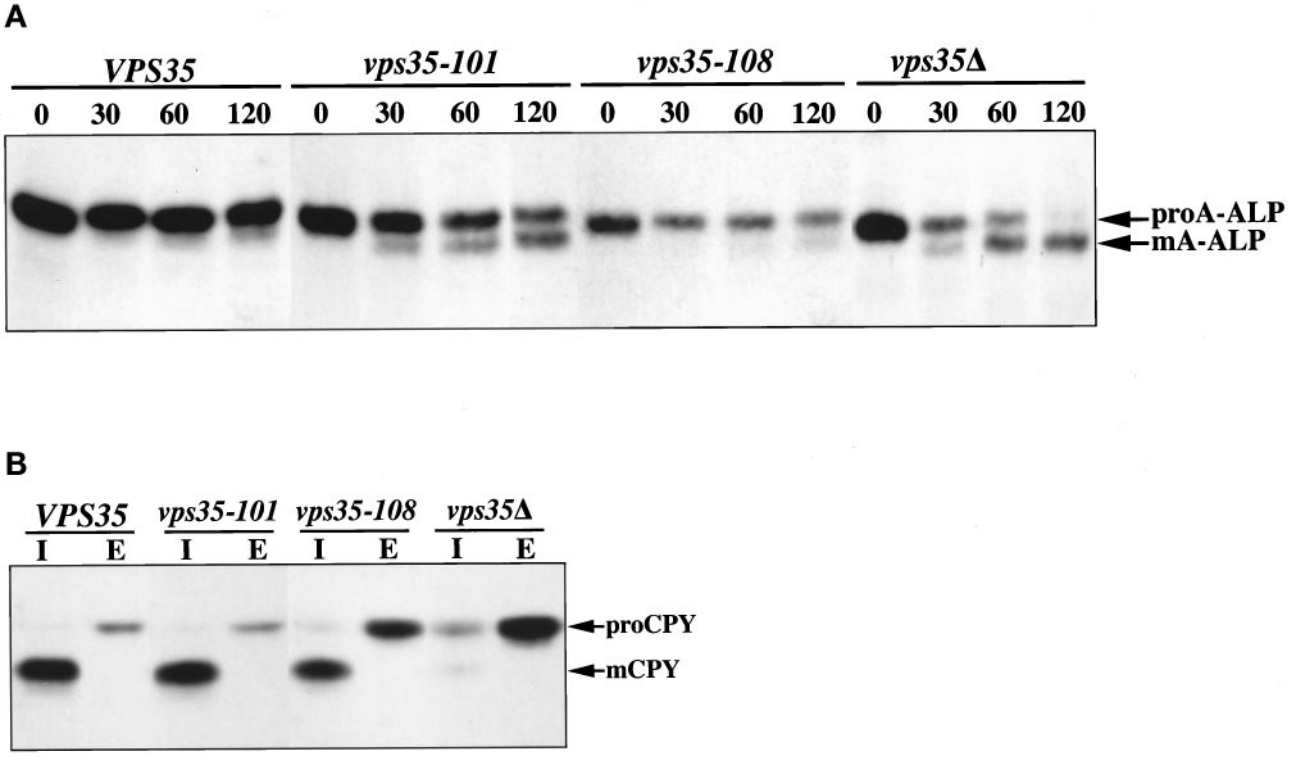

Figure 6. The *vps35–101* and *vps35–108* alleles exhibit specific defects in A-ALP and Vps10p trafficking, respectively. The *VPS35*, *vps35–101*, *vps35–108*, and *vps35*Δ yeast strains correspond to the following strain/plasmid combinations, respectively: SNY79/pSN54/pLS13, SNY79/pSN54/pLS12, SNY79/pSN54/pPB7–010.4, and PBY3/pSN54 (please refer to Tables 1 and 2 for strain and plasmid information). (A) The strains were pulsed for 10 min, chased for the indicated times, and subjected to A-ALP immunoprecipitation as detailed in the legend of Figure 4. (B) The strains were pulsed with [^{35}S]methionine/cysteine for 10 min, and then chased for 45 min with unlabeled amino acids. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions and analyzed by SDS-PAGE and fluorography. The positions of precursor (proCPY) and mature (mCPY) forms of CPY are indicated.

The identification of additional *vps35* alleles with cargo-specific defects would further support the idea that Vps35p contains distinct structural features specific for retrieval of different cargo proteins. Mapping the corresponding mutations would provide insight about structure–function relationships. With these goals in mind, we screened a library of *vps35*Δ strains carrying *CEN* plasmids with randomly mutagenized *VPS35* inserts. Mutants were tested for A-ALP retention defects using an ALP plate activity assay (Chapman and Munro, 1994a; Nothwehr *et al.*, 1996) and for CPY secretion. No additional alleles defective for A-ALP retention and normal for CPY sorting were identified, but five new alleles were identified that exhibited CPY-sorting defects and little or no A-ALP–retention defect.

Yeast strains carrying the six new *VPS35* alleles were pulsed with [^{35}S]methionine/cysteine and chased for various time points, and A-ALP was immunoprecipitated to allow determination of the half-time of A-ALP processing. In addition, CPY was separately immunoprecipitated from the media and from the cells after a 45-min chase to determine the severity of CPY-sorting defects. Representative data for the *vps35–101* and *vps35–108* alleles are shown in Figure 6. The *vps35–101* allele exhibits a pronounced A-ALP retention defect (Figure 6A) with a processing half-time of 115 min. CPY is correctly sorted to the vacuole in the *vps35–101* strain, as indicated by the vast majority of CPY being found in the intracellular fraction in the mature form, similar to the results seen with the wild-type *VPS35* strain (Figure 6B). In contrast, the *vps35–108* allele exhibits near-normal retention of A-ALP with only a very minor amount of processing detected after a 120-min chase and an overall half-time exceeding 180 min (Figure 6A and Table 3). A substantial portion of the CPY in the *vps35–108* strain was aberrantly secreted into the extracellular fraction as Golgi-modified pro-CPY (Figure 6B). Thus, these two alleles exhibit a high level of specificity toward the trafficking of the two cargo proteins, A-ALP and the CPY receptor Vps10p.

Experiments such as those shown in Figure 6 were also performed on the remaining four *vps35* alleles,

Table 3. Phenotypes of *VPS35* alleles and nature of the responsible mutations

Allele	A-ALP processing half-time (min)	% CPY secretion	Responsible mutation
VPS35	>180	18	Not applicable
*vps35*Δ	65	74	Deletion of ORF
vps35-101	115	16	$D_{123}N$
vps35-103	>180	50	$L_{568}P$
vps35-104	>180	50	247-498
vps35-105	>180	32	$D_{528}G$
vps35-107	>180	40	733-935 (stop codon)
vps35-108	>180	41	$L_{563}M$

The half-time of A-ALP processing and the percent CPY secretion were determined from *vps35*Δ strains carrying the indicated *VPS35* alleles on *CEN* plasmids (Table 1) as described in the legend to Figure 6 and in MATERIALS AND METHODS. The data shown for the percent CPY secretion is an average of data from two independent experiments. The values from each experiment for each strain differed by <5% secretion. Mapping of the mutations responsible for the phenotype of each allele was performed as described in MATERIALS AND METHODS. For the *vps35-104* and *vps35-107* alleles the mutation was narrowed down to the indicated region given in terms of codon number.

and the quantified results for all the alleles are shown in Table 3. The alleles fall into two groups: those having a specific defect in A-ALP retention (*vps35–101*) and those specifically defective for CPY sorting (*vps35–103, 104, 105, 107,* and *108*). Although the *vps35–101* allele has a somewhat less severe A-ALP retention defect than the *vps35*Δ allele, it exhibits the cleanest separation of the two phenotypes because it is indistinguishable from the *VPS35* strain for CPY sorting.

Additional experiments were performed to determine whether the phenotypic assays reflect an inability to retrieve either A-ALP or Vps10p from the prevacuolar compartment. Consistent with the CPY-sorting data in Figure 6B, the *vps35–101* strain exhibited a normal Golgi-like punctate staining pattern for Vps10p, whereas the *vps35*Δ and *vsp35–108* strains exhibited a predominantly vacuolar membrane-staining pattern for Vps10p (Figure 7; compare the staining patterns of the vacuolar membrane marker Vma2p with Vps10p). Since the data in Figure 5 demonstrate that Vps35p is required for retrieval of Vps10p, the data in Figure 7 strongly suggest that the *vps35–108* allele is defective for retrieval of Vps10p.

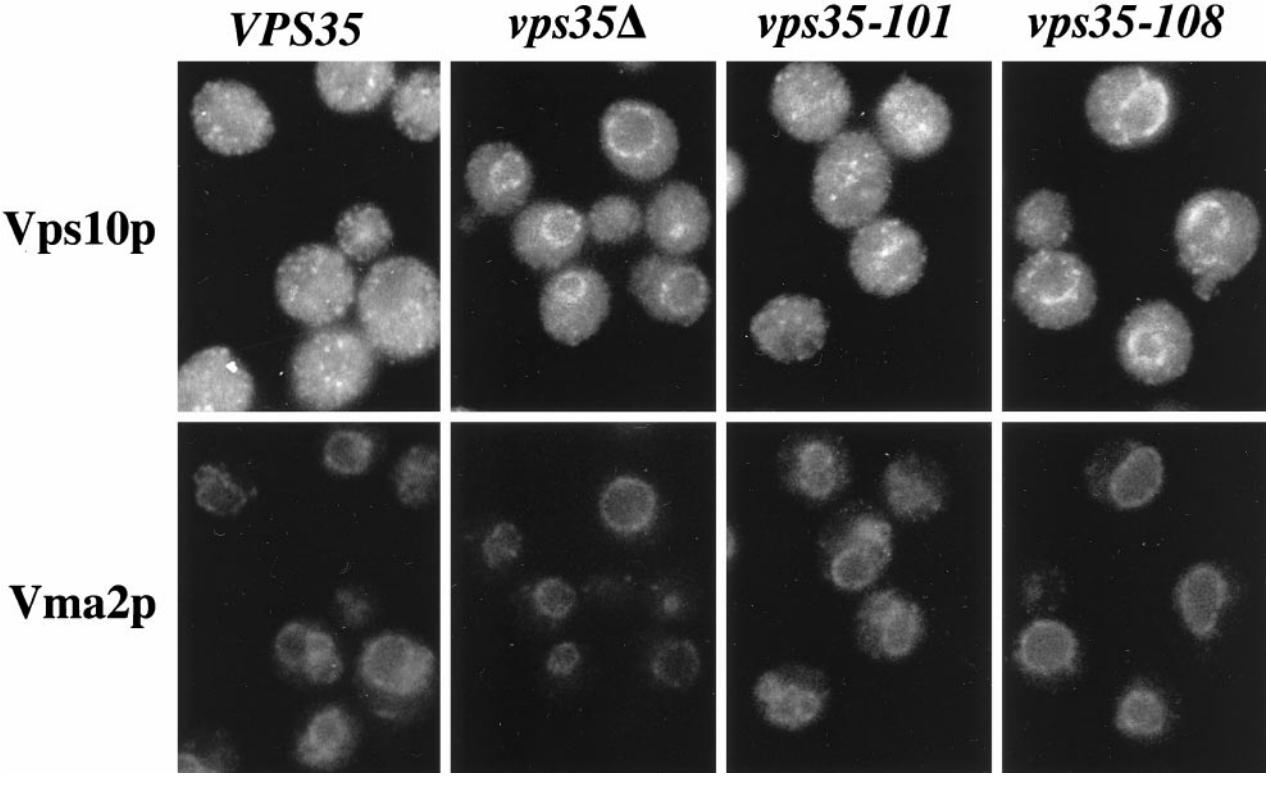

Figure 7. Vps10p is properly localized in a *vps35–101* strain but is mislocalized to the vacuole in a *vps35–108* strain. *VPS35* (LSY6–2A/pLS13), *vps35*Δ (LSY6–2A), *vps35–101* (LSY6–2A/pLS12), and *vps35–108* (LSY6–2A/pPB7–010.4) strains were fixed, spheroplasted, and costained with antibodies against Vps10p and Vma2p. After subsequent treatment with fluorochrome-conjugated secondary antibodies, the cells were viewed by DIC optics and by epifluorescence through filters specific for FITC and Texas Red.

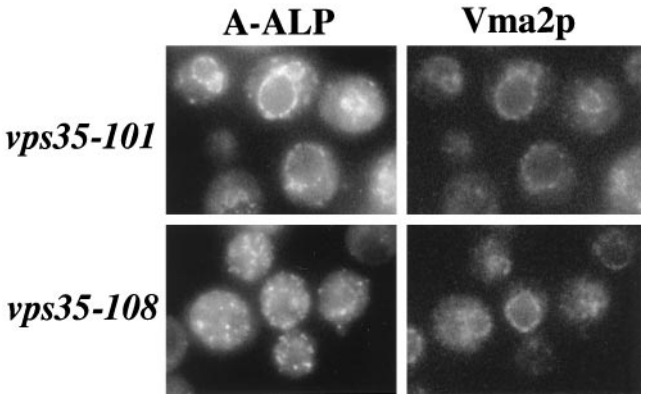

Figure 8. A-ALP is mislocalized to the vacuole in a *vps35–101* strain but exhibits Golgi staining in a *vps35–108* strain. *vps35–101* (SNY79/pLS12/pAH16) and *vps35–108* (SNY79/pPB7–010.4/pAH16) strains were fixed, spheroplasted, and costained with antibodies against A-ALP and Vma2p. After treatment with fluorochrome-conjugated secondary antibodies, the cells were viewed by DIC optics and by epifluorescence through filters specific for FITC and Texas Red.

Moreover, A-ALP predominantly exhibited a punctate, Golgi-like staining pattern in *vps35–108* cells, whereas A-ALP was clearly localized to the vacuole in *vps35–101* cells (Figure 8). These results further emphasize the A-ALP retention defect in *vps35–101* cells and near-normal A-ALP trafficking in *vps35–108* cells.

To test whether a *vps35–101* strain is functional for retrieval of Vps10p and defective for A-ALP retrieval in a more direct manner, we analyzed a *vps35–101* strain for its ability to retrieve these proteins in the *VPS27* induction assay. After induction of *VPS27* for 90 min, A-ALP has clearly been transported from the prevacuolar compartment to the vacuole, whereas Vps10p has instead been retrieved to punctate structures consistent with Golgi staining (Figure 5). Thus, the *vps35–101* allele is defective for retrieval of A-ALP but not Vps10p.

Membrane Association of the Mutant Vps35 Proteins

If Vps35p associates with membranes as a member of the protein complex including cargo proteins such as Vps10p and A-ALP, it is possible that Vps35p mutants that fail to retrieve either Vps10p or A-ALP may exhibit altered membrane association relative to wild-type Vps35p. We therefore carried out subcellular fractionation by pulse labeling spheroplasts with [^{35}S]methionine/cysteine for 30 min and chasing with unlabeled amino acids for 45 min. Lysates were centrifuged at 13,000 $\times$ *g* to generate a pellet (P13) and supernatant (S13). The S13 fraction was then centrifuged at 150,000 $\times$ *g* to generate a P150 fraction and a supernatant (S150) fraction containing soluble proteins. Immunoprecipitation of Vps35p and other marker proteins was then carried out from protein extracts made from the P13, P150, and S150 fractions. Given the fractionation of various markers in similar types of subcellular fractionation schemes performed previously (Marcusson *et al.*, 1994; Nothwehr and Hindes, 1997), the P13 would be expected to contain vacuoles, ER, and plasma membrane, whereas the P150 would contain Golgi, endosomes, and vesicles. Accordingly, we observed under these conditions that ~80% of the vacuolar membrane marker Vph1p fractionated in the P13, whereas ~90% of the Golgi marker DPAP A fractionated in the P150 (our unpublished data).

In the *vps35*Δ strain PBY1 carrying a *CEN* plasmid containing wild-type *VPS35,* most of the Vps35p (65%) was found in the P150 with 30% in the soluble S150 fraction (Figure 9). These results are similar to previously published fractionation data for Vps35p (Paravicini *et al.*, 1992). Overall, the proteins expressed from the *vps35* mutant alleles exhibited membrane association that was quite similar to wild-type Vps35p, with a few subtle differences. For example, a small-but-reproducible increase in the amount of Vps35p found in the P13 fraction was observed in the *vps35–103* and

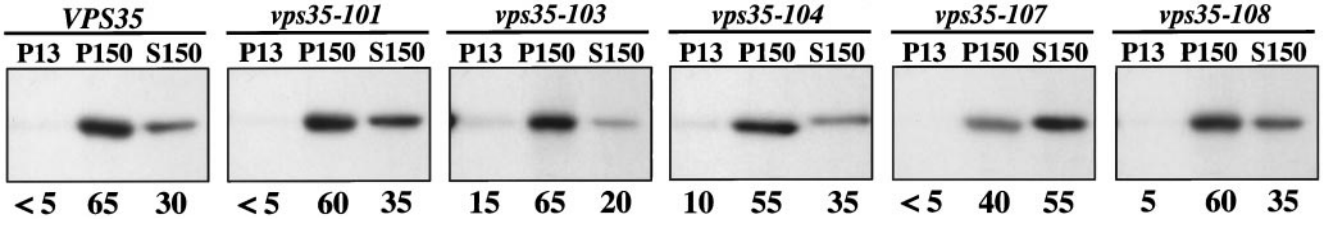

Figure 9. Membrane association of wild-type and mutant Vps35 proteins. Spheroplasts from a *vps35*Δ strain (PBY1) carrying plasmids encoding either the wild-type (pLS13), *vps35–101* (pLS12), *vps35–103* (pPB7–9.7), *vps35–104* (pPB7–10.1), *vps35–107* (pPB7–108.5), or *vps35–108* (pPB7–010.4) alleles were pulsed for 45 min with [^{35}S]methionine/cysteine and chased for 15 min with unlabeled amino acids. The spheroplasts were then lysed, and lysates were centrifuged at 13,000 $\times$ *g* to generate a pellet fraction (P13). The supernatant was then centrifuged at 150,000 $\times$ *g* to generate pellet (P150) and supernatant (S150) fractions. Wild-type and mutant Vps35p were immunoprecipitated from each fraction and analyzed by SDS-PAGE and fluorography. The relative percentage of Vps35p present in each fraction as determined by Phosphorimager analysis is indicated below each panel.

vps35–104 strains compared with wild type. Vps35p was previously shown to fractionate in the P13 fraction containing vacuolar membranes under conditions in which Vps10p was mislocalized to the vacuole (Seaman *et al.*, 1997). Interestingly, the *vps35–103* and *vps35–104* alleles show the most severe CPY-sorting defect (Table 3) and would be expected to have the most severe mislocalization of Vps10p to the vacuole. In addition, one of the alleles, *vps35–107*, encodes Vps35p with somewhat reduced membrane association. Nevertheless, the general trend that mutant Vps35 proteins exhibit near-normal membrane association is consistent with the view that Vps35p membrane association does not depend solely on interaction with the cytosolic domains of cargo proteins.

Mapping of Mutations Responsible for Cargo-specific Phenotypes

Homology searches using the *S. cerevisiae* Vps35p sequence have identified protein sequences in *Caenorhabditis elegans, Mus musculus*, and *Homo sapiens* that exhibit a high degree of similarity to the yeast sequence. Comparison of the sequences allowed division of the 937-residue yeast Vps35p sequence into three domains based on extent of identity (Seaman *et al.*, 1997). Domain I lies between residues 1 and 352 and exhibits the most conservation (43–46% identity), whereas domains II and III lie between 353 and 660 and 661 and 937 and exhibit 20–22% and 23–35% homology, respectively.

Mapping of the mutations responsible for the phenotype in the mutant alleles was carried out to provide information regarding the position of cargo-specific motifs within the Vps35p polypeptide sequence (Table 3). The A-ALP–retrieval defect of the *vps35–101* allele was caused by a point mutation that changed the Asp codon at position 123 to Asn. Interestingly, Asp123 lies within the most highly conserved region of domain I and is itself conserved between *S. cerevisiae* Vps35p and *S. pombe*, *M. musculus,* and *C. elegans* homologues (Figure 10), consistent with this region being necessary for a function common to all four of the proteins. Three of the five alleles specific for Vps10p-retrieval defects have been mapped to single residue changes, whereas the others have been mapped to intervals of ≤251 amino acids. In contrast to the *vps35–101* allele, all five of the Vps10p-specific alleles roughly map within the C-terminal two-thirds of the ORF. The mutations responsible for the phenotype in the *vps35–103*, *vps35–105*, and *vps35–108* alleles map to residues in regions that exhibit little conservation with the homologues from other organisms (Table 3 and our unpublished data), suggesting that these regions may mediate a function specific to yeast Vps35p. Likewise, the other two alleles map to intervals that generally exhibit weak similarity among the

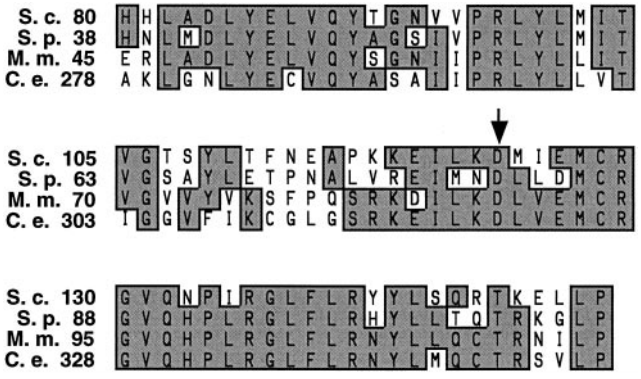

Figure 10. The mutation responsible for the *vps35–101* phenotype eliminates a conserved Asp residue. A comparison of the most conserved region of the *S. cerevisiae* Vps35p (S.c.) sequence with its *S. pombe* (S.p.), *M. musculus* (M.m.), and *C. elegans* (C.e.) homologues is shown. The numbers refer to the amino acid residue number of each sequence. The shaded boxes indicate the most highly conserved residues. The arrowhead indicates the conserved D123 residue that was mutated to N in the vps35–101 allele.

Vps35p homologues. Taken together, these data indicate that 1) a conserved structural motif within domain I of the protein is necessary for A-ALP retrieval but is expendable for Vps10p retrieval and 2) one or more motifs in domains II/III are specifically necessary for efficient Vps10p retrieval.

DISCUSSION

Several membrane proteins are transported from the TGN to the prevacuolar/endosomal compartment of yeast. A subset of these, such as Vps10p and DPAP A, are then retrieved back from the prevacuolar compartment to the TGN, whereas others, such as carboxypeptidase S (Cowles *et al.*, 1997), are transported by default from the prevacuolar compartment to the vacuole. These results imply the existence of a cargo-sorting machinery at the prevacuolar compartment. In this article we characterize the role of Vps35p in retrieval of two cargo proteins, A-ALP and Vps10p. The data strongly suggest that Vps35p plays a role at the prevacuolar compartment in selection of cargo to be transported to the TGN via a retrograde pathway.

A Role for Vps35p in Retrieval of Both A-ALP and Vps10p from the Prevacuolar Compartment

Previous work has supported the proposal that Vps35p is involved in retrieval of Vps10p from the prevacuolar compartment. A loss of Vps35p function caused Vps10p to be mislocalized to the vacuolar membrane in a manner independent of late-secretory-pathway functions (Seaman *et al.*, 1997). However, the delivery of Vps10p to the vacuole in *vps35* mutants was reduced by mutations in *PEP12*, which encodes a

late endosomal, prevacuolar compartment t-SNARE (Becherer *et al.*, 1996).

Our present data provide additional evidence that Vps35p is required for retrieval of Vps10p, a protein that frequently cycles between the TGN and prevacuolar compartment as part of its role in CPY sorting. In addition, we present the first direct evidence that Vps35p is required for retrieval of a resident TGN enzyme (A-ALP). Inactivation of Vps35p using a rapid-onset, temperature-sensitive mutant showed that the A-ALP-retention defect was exhibited very rapidly after shifting to the nonpermissive temperature. This result argues against an indirect role for Vps35p in retention of A-ALP. A-ALP was transported to the vacuole in *vps35*Δ and *vps35-ts* mutant cells with a half-time of ~60 min, which is about the same rate as transport of an A-ALP mutant lacking its retrieval signal (Nothwehr *et al.*, 1993). In contrast to its transport in *vps1* mutant cells, A-ALP is transported to the vacuole in *vps35* cells in a manner independent of the early endocytic pathway, consistent with a TGN-to-prevacuolar compartment-to-vacuole route.

A-ALP employs two independent mechanisms to maintain localization to the TGN: a mechanism for reducing its rate of exit from the TGN and a retrieval mechanism from the prevacuolar compartment (Bryant and Stevens, 1997). We took advantage of A-ALP mutants specifically defective in either static retention or retrieval to determine which mechanism depends on Vps35p function. By assessing the phenotypes of combining the A-ALP mutations with the *vps35*Δ mutation, only the retrieval mechanism that utilizes the FXFXD signal on A-ALP was shown to depend on Vps35p function. Thus, Vps35p and the FXFXD signal must act at the same step to mediate retrieval from the prevacuolar compartment.

An immunofluorescence microscopy assay capable of assessing transport of membrane proteins out of an exaggerated prevacuolar compartment was used to further assess the role of Vps35p in trafficking of Vps10p and A-ALP. Both proteins were retrieved in cells expressing wild-type Vps35p, but A-ALP and Vps10p were transported to the vacuole by default in cells lacking Vps35p. The inability of Vps10p and A-ALP to be retrieved in this assay provides additional evidence that Vps35p is involved in the retrieval pathway.

Although Vps10p and resident TGN enzymes, such as DPAP A (A-ALP) and Kex2p, are generally thought to be retrieved from the same late endosomal, prevacuolar compartment, it has recently been suggested that this may not be the case. Holthuis *et al.* (1998) recently characterized two new t-SNARE family members, Tlg1p and Tlg2p, that appear to be localized to a putative early endosome and the TGN, respectively. In *tlg1*Δ and *tlg2*Δ mutants, Kex2p and DPAP A are destabilized in a manner dependent on vacuolar proteases. In addition, both Tlg1p and Tlg2p interact with Vti1p, a v-SNARE thought to target TGN-derived vesicles to the recycling compartment as well as to mediate other vesicle-targeting events in the endomembrane system (Fischer von Mollard *et al.*, 1997). Little or no CPY-sorting defects were observed in *tlg1*Δ and *tlg2*Δ mutants (Holthuis *et al.*, 1998). This observation, taken together with the above mentioned data for Tlg1p, led to the proposal that Kex2p and DPAP A cycle between the TGN and a Tlg1p-containing early endosome, whereas Vps10p cycles to and from the prevacuolar endosome (Holthuis *et al.*, 1998). However, an assignment of Tlg1p localization to the early endosome must be taken as tenuous since in yeast this compartment is not well characterized, and no membrane protein markers are available.

Other studies are more consistent with the idea that DPAP A (A-ALP), Kex2p, and Vps10p are all recycled from the same prevacuolar compartment. For example, Vps10p, Kex2p, and A-ALP have been shown to be trapped in an exaggerated prevacuolar endosome in class E *vps* mutants (Raymond *et al.*, 1992; Cereghino *et al.*, 1995; Piper *et al.*, 1995), strongly suggesting that they are transported to and from this compartment in wild-type cells. In addition, a more recent analysis of CPY sorting using an immunoprecipitation strategy indicated that *tlg2*Δ cells missort 20% of their CPY (Abeliovich *et al.*, 1998) contrary to previous results (Holthuis *et al.*, 1998). This raises the question of whether Tlg1p may also be involved in CPY sorting and, by extension, trafficking of Vps10p. Clearly, additional characterization of Tlg1p and Tlg2p should help resolve these issues.

vps35 Mutant Alleles Exhibit Cargo-specific Defects in Retrieval

Because a complete loss of Vps35p function results in strong defects in retrieval of both Vps10p and A-ALP, we were surprised to find an allele, *vps35–101*, that has a cargo-specific defect. This allele exhibited entirely-normal CPY sorting and Vps10p retrieval but had a substantial defect in retrieval of A-ALP. Random mutagenesis of *VPS35* led to identification of several other alleles that exhibited the opposite type of phenotype: a defect in Vps10p retrieval and near-normal function for A-ALP retrieval.

If Vps35p were to function in the retrieval pathway downstream of the cargo-sorting step, then all mutations that alter its activity would be expected to have the same effect on different cargo proteins. The discovery of cargo-specific alleles is more consistent with Vps35p having a direct role in sorting of the cargo proteins. We propose that Vps35p may directly interact with the cytosolic domains of DPAP A and Vps10p. The discovery of cargo-specific alleles would reflect the existence of distinct structural features in

Vps35p necessary for interaction with certain cargo proteins but expendable for others. The mapping data suggest that an N-terminal domain in Vps35p is specifically involved with DPAP A interaction, whereas Vps10p-specific structural features reside in the C-terminal two-thirds of the protein.

Previous data also support the idea that Vps35p associates with the cytosolic domains of cargo proteins retrieved from the prevacuolar compartment. Seaman *et al.* (1997) localized both Vps10p and Vps35p by subcellular fractionation in wild-type and *vps29* mutant cells. In wild-type cells, Vps10p and Vps35p fractionated with endosomal and Golgi membranes, but in *vps29* cells both proteins were present in a fraction containing vacuolar membranes. These data led to the suggestion that in the absence of Vps29p function, retrieval of Vps10p was blocked, and a complex containing Vps10p and Vps35p was mislocalized to the vacuolar membrane (Seaman *et al.*, 1997).

An alternative, but more complex, model to explain the existence of the cargo-specific alleles would propose that distinct protein complexes associate with the cytosolic domains of each cargo protein. The complexes would be involved in sorting of the cargo proteins into newly forming vesicles at the prevacuolar compartment. Each protein complex would have subunits in common such as Vps35p and Vps29p and other subunits not shared between the complexes. The existence of cargo-specific Vps35p alleles could then be explained by the use of distinct structural features that mediate association with each complex, the nature of which would vary somewhat between cargo proteins. In a related model, Vps35p could act as a scaffold that interacts with different cargo-recognizing proteins such as Grd19p (see below). Such an interaction with Vps35p would mediate binding of a given cargo-recognizing protein with the cytosolic domain of the appropriate cargo protein. In this model, distinct domains in Vps35p could be used for interaction with different cargo-recognizing proteins. The recent characterization of Grd19p (Voos and Stevens, 1998), a PX domain-containing protein, is consistent with the idea that the protein machinery that associates with the cytosolic domains of Vps10p and DPAP A is not identical. Grd19p has been shown to exhibit a very mild defect in Vps10p retrieval while exhibiting a strong defect in A-ALP retrieval. Grd19p has been observed to interact with the cytosolic domain of DPAP A under certain in vitro conditions, although it is not known whether the two proteins interact in vivo. Under the same in vitro conditions, no interaction of Grd19p with Kex2p or Vps10p was observed. Thus Grd19p may represent a component of the sorting machinery specific to DPAP A.

New insight into the vesicle formation step of prevacuolar compartment-to-TGN retrieval has been gained by characterization of a protein complex termed the "retromer" (Seaman *et al.*, 1998). A subcomplex containing Pep8p, Vps29p, and Vps35p was found to associate with another subcomplex containing Vps5p and Vps17p at the prevacuolar compartment membrane. Based primarily on cell fractionation and immunoelectron microscopy data, the complete retromer complex has been proposed to function as a vesicle coat and have a role in cargo selection. Interestingly, *pep8*, *vps29*, and *vps35* mutants exhibit little or no vacuolar morphology defect, whereas *vps5* and *vps17* exhibit highly fragmented vacuoles (Raymond *et al.*, 1992; Köhrer and Emr, 1993; Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997). Based primarily on this observation and on the severity of vacuolar hydrolase missorting, it has been proposed that Vps5p and Vps17p are part of general machinery for retrieval. Our results with Vps35p are thus consistent with the proposal that the Pep8p/Vps29p/Vps35p subcomplex is involved in sorting of certain cargo, whereas Vps5p/Vps17p serves a structural role in vesicle formation (Seaman *et al.*, 1998).

In accordance with the role of vesicle coats in other transport steps (Rothman and Wieland, 1996; Schmid, 1997), the coat present on retrograde vesicles originating from the yeast prevacuolar compartment would probably be shed shortly after vesicle budding and before fusion with the TGN. Thus, the association between Vps35p and the cytosolic domains of cargo proteins would be transient in nature and relatively unstable. Indeed, our efforts at detecting interactions between Vps35p and the cytosolic domains of cargo proteins using biochemical approaches have been unsuccessful to date. However, novel alleles, such as *vps35–101*, that have subtle structural alterations resulting in cargo-specific defects may provide tools for detecting such interactions by using a genetic approach.

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