Golgi and Vacuolar Membrane Proteins Reach the Vacuole in vpsl Mutant Yeast Cells via the Plasma Membrane

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Abstract. The Vpsl protein of Saccharomyces cerevisiae is an 80-kD GTPase associated with the Golgi apparatus. Vpslp appears to play a direct role in the retention of late Golgi membrane proteins, which are mislocalized to the vacuolar membrane in its absence. The pathway by which late Golgi and vacuolar membrane proteins reach the vacuole in vpslΔ mutants was investigated by analyzing transport of these proteins in vpslΔ cells that also contained temperature sensitive mutations in either the SEC4 or END4 genes, which are required for a late step in secretion and the internalization step of endocytosis, respectively. Not only was vacuolar transport of a Golgi membrane protein blocked in the vpslΔ sec4-ts and vpslΔ end4-ts double mutant cells at the non-permissive temperature but vacuolar delivery of the vacuolar membrane protein, alkaline phosphatase was also blocked in these cells. Moreover, both proteins expressed in the vpslΔ end4-ts cells at the elevated temperature could be detected on the plasma membrane by a protease digestion assay indicating that these proteins are transported to the vacuole via the plasma membrane in vpsl mutant cells.

These data strongly suggest that a loss of Vpsl function causes all membrane traffic departing from the late Golgi normally destined for the prevacuolar compartment to instead be diverted to the plasma membrane. We propose a model in which Vpslp is required for formation of vesicles from the late Golgi apparatus that carry vacuolar and Golgi membrane proteins bound for the prevacuolar compartment.

NEWLY synthesized soluble proteins that are destined to be delivered to the lysosome of animal cells, or lysosome-like vacuole of yeast, traverse the early parts of the secretory pathway together with secretory proteins. However, in a late Golgi compartment (trans-Golgi network) the pathways followed by secretory and lysosomal proteins diverge. This process is carried out by a protein sorting apparatus that recognizes targeting signals on soluble lysosomal proteins and directs them into a pathway eventually leading to the lysosome (reviewed by Kornfeld and Mellman, 1989; Klionsky et al., 1990; Raymond et al., 1992b).

Genetic approaches in the yeast Saccharomyces cerevisiae have identified over 50 genes required for a soluble vacuolar hydrolase, carboxypeptidase Y (CPY), to reach the vacuole (Klionsky et al., 1990; Pryer et al., 1992; Raymond et al., 1992a,b). Lesions in any one of these genes cause CPY to be aberrantly secreted. Recently one of these genes, VPS10 (Van Dyck et al., 1992; Marcusson et al., 1994), was found to encode a membrane glycoprotein present in Golgi or post-Golgi membranes that could be cross-linked to wild-type but not sorting-defective mutant forms of CPY indicating that Vpsl0p is the receptor for CPY (Marcusson et al., 1994). Therefore, the sorting of vacuolar hydrolases in yeast appears to occur via their recognition by a membrane bound receptor in the Golgi apparatus in a manner analogous to mammalian cells where lysosomal protein sorting is mediated by the mannose 6-phosphate receptor (Kornfeld, 1992). The manner by which the receptor/ligand complexes are packaged into transport vesicles that enter the pathway to the vacuole and the events that trigger formation of those vesicles are not yet understood.

The product of another gene required for delivery of CPY to the vacuole, VPS1, is a member of a family of high molecular weight GTPases that are involved in diverse cellular processes (Rothman et al., 1990). Two members of this family involved in vesicular transport are mammalian dynamin (Shpetner and Vallee, 1989; Obar et al., 1990) and its Drosophila melanogaster homologue, shibire (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). A high degree of sequence identity is shared between the amino-terminal GTP-binding regions of these proteins (66% between Vpslp and dynamin) but much less similarity is seen in their carboxy-terminal regions. A temperature sensitive allele of the shibire gene rapidly and reversibly causes a depletion of
brane protein responsible for processing of the c~-factor pre-
coated pit constriction and coated vesicle budding (Damke
Shibire et al., 1993; Herskovic et al., 1993). More recently,
analysis of HeLa cells expressing dominant-negative dynamin alleles indicates that dynamin is required for
coated pit construction and coated vesicle budding (Damke
et al., 1994).

VPS1 is a non-essential gene encoding a 80-kD protein that
is peripherally associated with Golgi membranes (Rothman
et al., 1990). Yeast carrying a temperature sensitive allele of
VPS1 secrete CPY rapidly after shifting to the elevated
temperature suggesting a direct role in CPY sorting (Vater
et al., 1992). Recently, vpsl mutants were found in a screen for
mutants exhibiting a defect in the maturation of the al-
factor mating pheromones (Wilsbach and Payne, 1993a). Upon
further examination the primary defect was shown to be a
loss of retention of the Kex2p endoprotease, a Golgi mem-
brane protein responsible for processing of the a-factor
precursor. In vpsl mutant cells the half-time of turnover of
Kex2p was greatly reduced and degradation of Kex2p in vpsl
mutant cells was shown to be dependent on protease A, a
vaccuuolase encoded by the PEP4 gene. These results strongly
suggested that in vpsl mutants Kex2p was mislocalized
to the vacuole where it was degraded. In contrast, in
mutants lacking the clathrin heavy chain (chcl), which is
also required for retention of Golgi membrane proteins,
much of the Kex2p and DPAP A were found on the plasma
membrane (Seeger and Payne, 1992a).

The structural similarity of Vpslp to dynamin suggests the
possibility that Vpslp may carry out an analogous function
(vesicle formation from a donor compartment) at a mem-
brane transport step necessary for both CPY sorting and
Golgi membrane protein retention in yeast. In this scenario,
the CPY sorting defect could be explained by a defect in the
trafficking of the CPY receptor that, in analogy with the
mannose 6-phosphate receptor (Kornfeld, 1992), may cycle
between the late Golgi and a post-Golgi/prevacuolar com-
partment. Resident late Golgi membrane proteins in yeast
such as Kex2p and DPAP A may also undergo a similar
cycling pattern as part of their retention mechanism (Wilsbach
and Payne, 1993a; Nothwehr and Stevens, 1994). In prin-
ciple Vpslp could either carry out its function at the level of
the late Golgi or at the point of retrieval from a post-Golgi
compartment. If Vpslp is solely required for retrieval, Golgi
membrane proteins in vpsl mutants should be transported
directly from the late Golgi to the vacuole without passing
through the plasma membrane. We report here that in vpsl
mutant cells vacuolar and Golgi membrane proteins reach
the vacuole only after transport to the plasma membrane and
dendyctic uptake. These results are consistent with a model
where Vpslp is involved in formation of vesicles from the
Golgi apparatus that are targeted to the prevacuolar compart-
ment and contain vacuolar hydrolases as well as Golgi and
vacuolar membrane proteins.

Materials and Methods
Plasmids, Strains, and Materials
Plasmids used in this study are listed in Table I. pSN110 was constructed
by inserting a 3.5-kbp KpnI-EcoRI fragment from pAL144 (a gift from Y.
Kaneko, Osaka University, Osaka, Japan) containing the PH08 gene into
pRS306 (Sikorski and Hieter, 1989). A 0.45-kbp XhoI fragment from
within the open reading frame of PH08 was removed from pSN110 giving
rise to pSN111. The pho8A::LEU2 construct (pGP10) was made by first subclon-
ing the 3.0-kbp EcoRI fragment from pAL119 (Kaneko et al., 1985) into
EcoRI-digested pGEM2 (Promega Corp., Madison, WI) then replacing the
XhoI-BglII fragment from the insert with an XhoI-BglII fragment contain-
ing the LEU2 gene. pSN239 was constructed by digesting pGP10 with XhoI
and BglII, blunting with Klenow enzyme, and ligating with a 2-kbp
BglII-BglII (blunt ended) fragment containing the ADE2 gene. pPL02010
was constructed by first subcloning the 4.6-kbp SacI-Sall fragment contain-
ing the PEP4 gene into pRS306, then removing the 1.3-kbp HindIII frag-
ment contained within the SacI-Sall insert. pCAV40 was constructed by
subcloning the Xbal-Spel insert from a hydroxylamine-mutagenized isolate
of pCKR19 containing the vpsl-100 allele (Vater et al., 1992) into the
Xbal/Spel sites of a pRS306 derivative lacking the EcoRI site. A construct
for integrating the sec4-8 allele (pLC4-8) was constructed by subcloning the
SacI-BamHI fragment from pNB159 (Salminen and Novick, 1987) into the
SacI/BamHI sites of pRS306.

Epitope tagging of KEX2 was performed by introducing a BamHI site at
the stop codon of the KEX2 open reading frame contained within plasmid
pSN215 (KEX2 in pBluescript KS+) resulting in plasmid pSN216. A BglII
fragment encoding of three repeated copies of the 9-amino acid epitope
(YPYDVPDYA) from influenza virus hemagglutinin protein HAI (Wilson
et al., 1984) was then inserted into the BamHI site of plasmid pSN216
resulting in pSN217. The KEX2::HA allele was then isolated from pSN217
as a Sall-Eagl fragment and subcloned into the SalI/Eagl sites of a URA3-
based, centromere (CEN)-containing plasmid (pRS316; Sikorski and
Hieter, 1989) resulting in pSN218. The KEX2::HA allele born on plasmid
pSN218 was observed to fully complement a kex2A mutation for processing
of pro-a-factor (data not shown).

Yeast strains used in this study are listed in Table II. The PH08 gene of
SNY28 was disrupted to give rise to SNY55 by transforming pSN111 lin-
erized with SalI, selecting for Ura + colonies, then selecting for Ura-
loop-outs on media containing 5-fluoroorotic acid. Western blot analysis
was used to identify Ura- loop-outs lacking Pho8p. The PEP4 gene
of SNY55 was disrupted giving rise to SNY56 by transforming with
pPLO2010 linearized with EcoRI, selecting for Ura- colonies, then selecting for Ura-
loop-outs on media containing 5-fluoroorotic acid. The APNE plate assay
(Wolf and Fink, 1975) was used to identify pep4 mutant colonies. The
pvs8A::LEU2 mutation was introduced by transforming yeast cells with

| Table I. Plasmids Used in This Study |
|-----------------------------------|
| Plasmid | Description | Source or reference |
| pCAV40 | vpsI-100 allele in pRS306 | This study |
| pCKR7A | pks8A::HA in pUC12 | Rothman et al. (1990) |
| pPP10 | pho8A::LEU2 in pGEM2 | Raymond et al. (1990) |
| pLC4-8 | sec4-8 allele in RS306 | This study |
| pPLO2010 | pep4-38 allele in pRS306 | This study |
| pSN239 | STE13-PH08 gene fusion in pRS316 | Nothwehr et al. (1993) |
| pSN29 | STE13-PH08 gene fusion in pRS316 | This study |
| pSN11 | pho8A::ADE2 allele in pGEM2 | This study |
| pSN218 | KEX2::HA allele in pRS316 | This study |

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Table II. Yeast Strains Used in This Study

| Strain     | Genotype                        | Source or reference   |
|------------|---------------------------------|-----------------------|
| SNCY25     | MATa ura3-52 leu2-3,112 his4-519 ade6 | This study            |
| SNCY55     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX | This study            |
| SNCY56     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX pep4-ΔH3 | This study            |
| SNCY57     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1Δ::LEU2 | This study            |
| SNCY58     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1Δ::LEU2 | This study            |
| LCY22      | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps3Δ::LEU2 | This study            |
| SNCY4      | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1-100 | This study            |
| SNCY24     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1Δ::LEU2 | This study            |
| SNCY31     | MATa ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX sec4-8 vps1Δ::LEU2 | This study            |
| RH144-3D   | MATa ura3 leu2 his4 bar1-1       | H. Riezman            |
| RH268-1C   | MATa ura3 leu2 his4 bar1-1 end4-1 | H. Riezman            |
| LCY14      | MATa ura3 leu2 his4 bar1-1 vps1Δ::LEU2 | This study            |
| LCY16      | MATa ura3 leu2 his4 bar1-1 end4-1 vps8-ΔX | This study            |
| LCY19      | MATa ura3 leu2 his4 bar1-1 end4-1 vps1Δ::LEU2 | This study            |
| SNCY44     | MATa/c ura3/ura3-52 leu2/leu2-3,112 his4/4 his4-519 ADE6/ade6 bar1-1/1 BARI end4-1/END4 pho8-ΔX pho8-ΔX VPS1/vps1Δ::LEU2 | This study            |
| SNCY44-2C  | MATa ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX vps1Δ::LEU2 | This study            |
| SNCY44-13B | MATa ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX ade6 end4-1 | This study            |
| SNCY44-1A  | MATa ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX end4-1 vps1Δ::LEU2 | This study            |
| SEY6210    | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 | Robinson et al. (1988) |
| SEY6211    | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-901 ade2-101 suc2-Δ9 | Robinson et al. (1988) |
| SNCY17     | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-901 ade2-101 suc2-Δ9 vps9Δ::ADE2 | This study            |
| SNCY18     | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-901ade2-101 suc2-Δ9 vps9Δ::ADE2 | This study            |
| SNCY36     | MATa/c ura3-52/ura3-52 leu2-3,112 his3-Δ200/his3-Δ200 trp1-901/trp1-Δ901 lys2-801 ade2-101/101 ADE2/ade2 suc2-Δ9/suc2-Δ9 vps9Δ::ADE2/ade2 pho8Δ::ADE2/ade2 pho8A::ADE2 | This study            |
| SNCY36-9A  | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 | This study            |
| SNCY36-9B  | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8A::ADE2 lys2-801 | This study            |
| SNCY38     | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 lys2-801 vps1Δ::LEU2 | This study            |
| SNCY46     | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 ade6 end4Δ::URA3 | This study            |
| SNCY53     | MATa/c ura3-52/ura3-52 leu2-3,112 his3-Δ200/his3-Δ200/trp1-901/trp1-Δ901 lys2-801/LYS2 ade2-101/101 ADE2 suc2-Δ9/suc2-Δ9 vps9Δ::ADE2/ade2 pho8Δ::ADE2/ade2 pho8A::ADE2/VPS1 | This study            |
| SNCY53     | MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 ade6 end4Δ::URA3 | This study            |

The sec4-8, end4-1, and vps1-100 mutations are temperature sensitive mutant alleles and throughout the text will be referred to as sec4-ts, end4-ts, and vps1-ts, respectively. The following strains are congenic with SNCY28: SNCY55, SNCY56, SNCY57, SNCY58, LCY22, SNCY4, SNCY24, and SNCY31. LCY14 is congenic with RH144-3D. LCY16 and LCY19 are congenic with RH268-1C. SNCY44 resulted from a cross of LCY16 with SNCY57. SNCY44-2C, SNCY44-13B, and SNCY44-1A are spores that arose from sporulation of SNCY44. SNCY17 is congenic with SEY6210 whereas SNCY18 is congenic with SEY6211. SNCY36 was formed by mating SNCY17 with SNCY18. Sporulation of SNCY36 gave rise to SNCY36-9B and SNCY36-9A which were sister spores from the same tetrad. SNCY38 is congenic with SNCY36-9B and SNCY46 is congenic with SNCY36-9A. Diploid SNCY53 was generated by crossing SNCY38 with SNCY46.

the Sec1-Xbal fragment from pCKR3A and selecting from Leu" colonies. The pho8Δ::ADE2 mutations were introduced by transforming digests of pGP10 (cut with Stul/BamH1) and pSN239 (digested with EcoRI/Sall) and selecting for Leu+ or Ade+ colonies, respectively. The vps1Δ::LEU2 construct was integrated by transforming pCKR70A digested with BamH1 and selecting for Leu+ colonies. A yeast strain harboring the end4Δ::URA3 mutation was generated by transforming pG51 digested with EcoRI and selecting for Ura+ colonies. To introduce the vps1-100 mutation, strains were transformed with pCAV30 linearized with EcoRI and Ura+ colonies selected. Ura+ loop-outs (selected on 5-fluoroorotic acid media) that exhibited a temperature sensitive Vps- phenotype were chosen. To introduce the sec4-8 (sec4-ts) mutation, plC4-8 was digested with Xbal, transformed to obtain Ura+ colonies, and Ura+ loop-outs were subsequently selected on 5-fluoroorotic acid media. Authentic sec4-ts strains were identified based on their temperature sensitive growth phenotype.

The preparation of fixed, spheroplasted yeast cells and attachment to slides was carried out essentially as described by Roberts et al. (1991) with the following modifications. Spheroplasts of the fixed cells was carried out by incubation in 1.2 M sorbitol, 50 mM potassium phosphate, pH 7.3, 1 mM MgCl2, and 20 μg/ml oxalyticase for 30 min at 30°C. The fixed, spheroplasted cells were treated with 1.5% SDS, 1.2 M sorbitol for 2 min. Co-staining of the A-ALP fusion protein and the 60-kD subunit of the proton-translocating vacuolar ATPase was carried out as described previously (Nothwehr et al., 1993). For detection of HA epitope-tagged Kex2p, fixed spheroplasts were incubated with the following solutions followed by extensive washing with 5 mg/ml BSA in PBS after each step: (a) 1:250 dilution of mouse anti-HA monoclonal antibody 12CA5 (Babco Inc., Berkeley, CA); (b) 1:500 dilution of FITC-conjugated streptavidin.

Radiolabeling and Immunoprecipitation

Several hours prior to labeling, yeast strains were grown at 30°C (or 21-22°C for temperature sensitive strains) in selective synthetic media lacking methionine and cysteine. Cells were then harvested and resuspended in fresh media at a concentration of 1 OD600 (~107 cells) per ml. The cells were pulsed by adding NEN 35S-express label and chased by adding 50 μg/ml of methionine and cysteine. 0.5 ODs of cells were harvested per time point and spheroplasted (Stevens et al., 1986). The spheroplasts were lysed by incubation in 1% SDS, 8 M urea, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin at 100°C for 5 min. The samples were then diluted

Nothwehr et al. Membrane Traffic in vps1 Mutant Cells
Figure 1. Analysis of PEP4-dependent processing of A-ALP in vps mutants. Wild type (SNY55), vps1A (SNY-57), vps3A (LCY22), and vps1A pep4 (SNY58) cells carrying pSN55 (CEN-A-ALP) were 35S-labeled for 10 min and then chased by adding 50 μg/ml cysteine and methionine. At the indicated times the cells were spheroplasted, lysed, and extracts immunoprecipitated with a polyclonal antibody against ALP followed by SDS-PAGE and fluorography to assess the conversion of proA-ALP (pA-ALP) to mature A-ALP (mA-ALP).

on ice to 1 ml to give the following final concentrations: 10 mM Tris, pH 8.0, 0.1% Triton X-100, 2 mM EDTA, 0.05% SDS, 40 mM urea, and 0.5% IgGsorb. After preadsorbing to IgGsorb for 15 min the samples were centrifuged and an affinity purified rabbit anti-ALP antibody was added to the supernatant followed by a 1-h incubation on ice. Immune complexes were precipitated by addition of IgGsorb to 0.5% followed by a 1-h incubation on ice. The precipitates were subjected to three washes with a solution containing 10 mM Tris, pH 8.0, 0.1% SDS, 0.1% Triton X-100, and 2 mM EDTA. The samples were then analyzed by SDS-PAGE and fluorography as described (Stevens et al., 1986). Gels were quantified using the Radioanalytic Imaging System (AMBIS Inc., San Diego, CA).

Assay For Plasma Membrane Localization

The pronase assay procedure used to determine plasma membrane localization was modified from that of Davis et al. (1993). Following labeling of cells with NEM 35S-express label (described above), 0.5 ml of labeled culture (0.3 OD units) was added to 0.5 ml of ice-cold media containing 20 mM KF and 20 mM NaN3. The samples were held on ice for 20 min, pelleted, and the cells were washed once with pronase buffer (1.4 M sorbitol, 2 mM MgCl2, 0.5% β-mercataptoethanol, and 25 mM Tris, pH 7.5). The cell pellet was resuspended with 100 μl of pronase buffer containing 4 mg/ml pronase and digestion was allowed to proceed for 20 min on ice. Mock reactions were set up identically except no pronase was added. The reaction was stopped with the addition of two volumes ice-cold pronase wash buffer (1.4 M sorbitol, 2 mM EDTA, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 25 mM Tris, pH 7.5). The cells were then pelleted and washed twice with ice-cold pronase wash buffer before being subject to lysis in 1% SDS, 8 M urea, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin at 100°C for 5 min. The cells were then subjected to glass bead lysis and the resulting extract subjected to immunoprecipitation using a rabbit anti-ALP antibody as described above. The same extracts were then immunoprecipitated a second time using a rabbit antibody against phosphoglycerol kinase (PGK; Rothman et al., 1986; Stevens et al., 1986).

Results

Late Golgi Membrane Proteins Are Mislocalized To The Vacuole In vps1 Mutant Cells

A fusion protein consisting of the cytoplasmic domain of the late Golgi membrane protein dipeptidyl aminopeptidase (DPAP) A fused to the transmembrane and lumenal domains of alkaline phosphatase (ALP) is very efficiently localized and retained within the late Golgi apparatus (Nothwehr et al., 1993). Mutant versions of this A-ALP fusion protein lacking a cytoplasmic retention signal are mislocalized to the vacuole where the propeptide is removed in a manner dependent on the vacuolar protease, protease A, the product of the PEP4 gene. This event is detectable by a mobility change on SDS-PAGE (Nothwehr et al., 1993).

The vps mutants were originally identified as mutants that failed to properly sort the vacuolar hydrolase CPY to the vacuole (Klionsky et al., 1990; Raymond et al., 1992b). Instead, these mutants secrete CPY in a Golgi-modified form, p2CPY, that lacks vacuolar proteolytic modification. Using A-ALP processing as a sensitive assay system for measuring Golgi retention, the vps mutants were screened for Golgi retention defects with the idea that some overlap may exist between functions required for sorting of CPY to the vacuole and retention of late Golgi membrane proteins. vps1A mutant yeast strains were found to be severely defective in Golgi retention (Fig. 1). A-ALP expressed in vps1A mutant cells is processed with a half-time of roughly 60 min whereas little if any processing is seen in wild type cells. This processing rate is similar to that seen for A-ALP mutants lacking an intact Golgi retention signal (Nothwehr et al., 1993). In contrast, most other vps mutants exhibited little or no defect in retention of A-ALP. For example vps3A mutant cells, which secrete ~90% of newly synthesized CPY (Raymond et al., 1990), exhibit little if any processing of A-ALP even after 180 min of chase (Fig. 1). The processing of A-ALP in vps1A mutant cells is dependent on protease A (Fig. 1), suggesting that A-ALP is delivered to the vacuole in the absence of Vps1p function.

To determine whether Golgi membrane proteins are indeed mislocalized to the vacuole in vps1 mutants, the localization of A-ALP and another late Golgi membrane protein, Kex2p, were determined in wild type and vps1A mutants using indirect immunofluorescence microscopy. A fully functional epitope tagged version of Kex2p (see Materials and Methods) and A-ALP expressed in wild type cells exhibited cytoplasmic, punctate staining patterns (Fig. 2, B and H) as previously reported (Redding et al., 1991; Nothwehr et al., 1993). However, in the vps1A cells a comparison of the staining patterns for A-ALP with that of the 60-kD subunit of the vacuolar proton-translocating ATPase indicates that a considerable fraction of A-ALP is localized to...
the vacuolar membrane (Fig. 2, E and F). In the majority of vps1Δ cells Kex2p is also substantially mislocalized to the vacuolar membrane as shown by a comparison of its staining pattern with the corresponding Nomarski image (Fig. 2, I and J). The remainder of the A-ALP and Kex2p staining is restricted to a large number of small cytoplasmic punctate structures. The number and small size of these structures as compared to late Golgi compartments in wild type cells suggests that they could be compartments transiently occupied by A-ALP and Kex2p en route to the vacuole.

**Loss of Vpslp Function Rapidly Leads To A Defect In Golgi Membrane Protein Retention**

The defect in Golgi membrane protein retention observed in cells lacking VPS1 could reflect a direct role for Vpslp in this process. Alternatively, a loss of retention could be an in-

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**Figure 2.** Indirect immunofluorescence microscopy of A-ALP and Kex2p in vps1Δ cells. In A–F, wild type and vps1Δ mutant cells (SNY56 and SNY58, respectively) carrying pSN55 (CEN-A-ALP) were fixed, spheroplasted, and co-stained with a rabbit antibody against ALP (B and E) and a mouse antibody against the 60-kD vacuolar proton-translocating ATPase subunit (V-ATPase, C and F). In G–J, SNY56 and SNY58 carrying pSN218 (CEN-KEX2::HA) were treated as described above and stained with a mouse anti-HA antibody (H and J). The cells were viewed by Nomarski optics (A, D, G, and J) and by epifluorescence through filter sets specific for fluorescein (B, E, H, and J) and Texas red (C and F) fluorescence.

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**Figure 3.** Onset of the Golgi retention defect in vps1-ts mutant cells. In A, cultures of wild type (SNY55) or vps1-ts mutant (SNY4) cells carrying pSN55 (CEN-A-ALP) were propagated at 21°C, split, and one portion shifted to 35°C for 5 min while the other portion remained at 21°C. The cultures were immediately 35S-labeled for 10 min and chased for the indicated times at their respective temperatures before A-ALP was immunoprecipitated (see legend to Fig. 1). In B, the cultures from A at the 0 and 90 min chase times were divided into intracellular (I) and extracellular (E) fractions that subsequently were immunoprecipitated with anti–CPY antibody. The washed immunoprecipitates were run on SDS-PAGE and subjected to fluorography. The positions of the p2, p1, and mature (m) forms of CPY are indicated.
direct consequence of prolonged absence of Vpslp in vpsl null mutants. In order to distinguish between these possibilities yeast cells carrying a temperature sensitive allele of VPS1 that causes secretion of CPY at 35°C but not at 21°C (Vater et al., 1992) were analyzed to determine how quickly Golgi membrane protein retention is lost after a loss of Vpslp function.

In the experiment shown in Fig. 3, wild-type and vpsl-ts cells expressing A-ALP were propagated at the permissive temperature of 21°C, and then preincubated at either 35°C or 21°C for 5 min, and were then radioactively labeled and chased at 35°C or 21°C, respectively. The vpsl-ts cells incubated at 21°C exhibited very little A-ALP processing (Fig. 3 A) or CPY secretion (Fig. 3 B) as judged by comparison with the wild-type cells. However, at 35°C the vpsl-ts mutant exhibited strong defects in both A-ALP Golgi retention and CPY sorting. The defect observed for the vpsl-ts cells at 35°C was similar to that seen for vpsl Δ cells at 30°C (Fig. 1). In addition, the A-ALP processing kinetics of the vpsl-ts mutant at the elevated temperature resembled the kinetics of A-ALP retention-defective mutants expressed in wild-type cells (Nothwehr et al., 1993). The observation that Golgi membrane protein retention is rapidly lost after losing Vpslp function is consistent with the idea that Vpslp performs a function that is intimately involved in the mechanism of late Golgi membrane protein retention.

SEC4 Is Required For Vacuolar Delivery Of Both Golgi And Vacuolar Membrane Proteins in vpsl Mutant Cells

To understand more clearly the role of Vpslp in retention of Golgi membrane proteins, experiments were conducted to determine the pathway by which late Golgi membrane proteins are delivered to the vacuole in vpsl mutant cells. Two general pathways are conceivable: (a) "direct" Golgi to vacuole delivery along the same pathway normally taken by vacuolar membrane proteins such as ALP or DPAP B (Klionsky and Emr, 1989; Roberts et al., 1989); or (b) initial mislocalization to the plasma membrane followed by delivery to the vacuole via the endocytic pathway.

The initial experiment carried out to distinguish between these possibilities was to address whether Golgi membrane proteins are able to reach the vacuole in vpsl mutant cells after imposition of a block in the late secretory pathway. For this purpose we made a double mutant containing both the vpsl and the temperature sensitive sec4-ts mutations, the latter of which prevents fusion of secretory vesicles with the plasma membrane at the nonpermissive temperature (Salminen and Novick, 1987; Pryer et al., 1992). Fig. 4 A shows that A-ALP failed to reach the vacuole and be processed to the mature form in a vpsl Δ sec4-ts double mutant at the restrictive temperature of 35°C. However, A-ALP processing did occur in the vpsl Δ single mutant at 35°C and also in the vpsl Δ sec4-ts double mutant at 22°C (data not shown), the permissive temperature for the sec4-ts mutation. As expected the sec4-ts mutation alone had no effect on Golgi retention of A-ALP as evidenced by the lack of processing seen in Fig. 4 A.

Surprisingly, we found that vacuolar processing of the vacuolar membrane protein, ALP, was also dramatically inhibited in the vpsl Δ sec4-ts double mutant at the nonpermissive temperature suggesting that ALP was not efficiently reaching the vacuole under these conditions (Fig. 4 B). The sec4-ts mutation alone had no effect on delivery of ALP to the vacuole (Fig. 4 B) consistent with other studies demonstrating that vacuolar membrane proteins are transported directly from the Golgi to the vacuole in wild-type cells (Klionsky and Emr, 1989; Roberts et al., 1989). ALP processing in the double mutant at the permissive temperature of 22°C resembled the vpsl Δ single mutant (data not shown). The results of Fig. 4 suggest that both resident Golgi membrane proteins and vacuolar membrane proteins are initially mislocalized to the plasma membrane before being transported to the vacuole in vpsl Δ cells.

The end4-ts Mutation Traps Golgi and Vacuolar Membrane Proteins At the Plasma Membrane In vpsl Mutant Cells

The results of Fig. 4 are consistent with the idea that A-ALP and ALP may be reaching the vacuole by way of the plasma membrane in vpsl mutant cells. To further test the model that ALP and A-ALP pass through the plasma membrane in vpsl mutants we tested whether the endocytic pathway was required for delivery of ALP and A-ALP to the vacuole in vpsl mutants. For these purposes the vpsl Δ mutation was combined with a temperature sensitive mutant allele of the END4 gene. Mutations in the END4 gene have been shown to

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**Figure 4.** Trafficking of A-ALP and ALP in vpsl Δ sec4-ts double mutant cells. Wild type (SNY55), vpsl Δ (SNY57), sec4-ts (SNY24), and vpsl Δ sec4-ts (SNY31) cells carrying either pSN55 (A) or pSN92 (B) were grown at 22°C. The cultures were then shifted to 35°C for 10 min before labeling with 35S for 15 min and chasing with 50 μg/ml cysteine and methionine. At 0 and 60 min chase times, extracts were prepared, A-ALP (A) or ALP (B) was immunoprecipitated (see legend to Fig. 1), and the immunoprecipitates were separated on SDS-PAGE and subjected to fluorography. The positions of the pro and mature forms of A-ALP and ALP are indicated (pA-ALP, mA-ALP, pALP, mALP).
specifically block the internalization step of endocytosis (Raths et al., 1993). If ALP and A-ALP depend on endocytosis for delivery to the vacuole, these proteins should be blocked at the cell surface in the double mutant at the restrictive temperature and therefore not be processed by vacuolar proteases. To test this directly, the cells were 35S-labeled, chased for 0 and 60 min, and then a non-specific protease, pronase, was added to the intact yeast cells under conditions where only the periplasmic space (extracellular surface of the plasma membrane) of the cells was accessible to the protease.

Fig. 5A shows an experiment in which ALP has been immunoprecipitated from an end4-ts vpslA double mutant and control strains that had been 35S-labeled, chased at the non-permissive temperature (36°C), and subjected to exogenously added pronase for 20 min on ice (see Materials and Methods). Analysis of ALP processing indicates that ALP was fully processed in the wild type and end4-ts strains after 60 min and showed a delay in processing in the vpslA strain. We have consistently observed a delay in ALP processing in both vpslΔ and vpsl-ts mutants although the magnitude of the delay is more apparent at temperatures above 35°C (Fig. 4 and 5 and data not shown). Interestingly, much of the unprocessed ALP present after a 60-min chase was degraded by the exogenously added protease demonstrating that the processing delay was due at least in part to the passage of ALP through the cell surface. Little if any processing of ALP was observed in the end4-ts vpslA double mutant even after 60 min indicating that End4p is required for ALP to reach the vacuole in vpslA mutant cells. Importantly, the unprocessed ALP that accumulated in the double mutant after 60 min was almost completely degraded by pronase whereas the processed ALP in the other strains was completely pronase resistant as expected. These data indicate that newly synthesized ALP accumulated at the cell surface in end4-ts vpslΔ mutant cells.

Several lines of evidence support the validity of this technique for analyzing the localization of proteins at the plasma membrane. In the vpslΔ sample chased for 60 min (Fig. 5A) where unprocessed ALP was pronase sensitive, the processed species of ALP which had been transported to the vacuole was protected. However, if 0.2% Triton X-100 was included in the pronase reaction, processed ALP was completely degraded (Fig. 5B) demonstrating that the processed
species of ALP was capable of being degraded by pronase but only if membrane integrity was compromised. Treatment of the cells with 0.2% Triton X-100 in the absence of pronase had a negligible effect on the recovery of ALP. For all samples analyzed in Fig. 5, the cytoplasmic protein phosphoglycerol kinase (PGK) was immunoprecipitated from cell extracts after the immunoprecipitation of ALP. Like ALP, PGK was protected from pronase digestion unless 0.2% Triton X-100 was added to the pronase digestion whereas the addition of detergent alone had little effect on its recovery. Given its sensitivity to pronase, PGK served as a useful internal control in the experiments shown in Figs. 5 A and 6 to confirm that pronase activity was excluded from the cytoplasm.

Similar results were found for trafficking of A-ALP in the end4-ts vpslA double mutant (Fig. 6). First, the delivery of A-ALP to the vacuole observed in vpslA mutant cells as indicated by processing was not observed in the end4-ts vpslA double mutant. Secondly, unprocessed A-ALP present at the 60-min time point in the double mutant was almost completely degraded by pronase. Taken together, the results of Figs. 5 and 6 indicate that both late Golgi and vacuolar membrane proteins reach the vacuole from the plasma membrane via the endocytic pathway in vpslA mutant cells.

A Complete Loss of Vpslp and End4p Functions Is Lethal

The above results are compatible with the idea that in vpsl mutant cells all membrane traffic originating from the Golgi that is destined for vacuolar delivery passes through the plasma membrane. If this is the case, loss of endocytic functions in vpsl mutant cells would be expected to have a major effect on the growth rate of the cells. Fig. 7 shows the growth of a end4-ts vpslA double mutant strain as a function of temperature compared to control strains. Growth of the double mutant was much more sensitive to increases in temperature than was either single mutant. In addition, at lower temperatures a dramatic decrease in growth rate was observed for the double mutant as compared to the vpslA or end4-ts single mutants. For example, at 23°C the doubling time of the end4-ts vpslA double mutant was 7.3 h, whereas the doubling times were 2.9 h for each single mutant, and 2.1 h for wild-type cells.

To investigate the synthetic growth defect between vpsl and end4 mutations further, the fate of cells completely disrupted for both genes was investigated. SNY53, a diploid strain heterozygous for both VPS1 (VPS1/vpslA::LEU2) and END4 (END4/end4A::URA3), was sporulated and tetrads dissected. The tetrads fell into three classes: (a) 1/16 contained four viable spores (2 Leu+ Ura-, 2 Leu- Ura+); (b)
The data presented in this paper indicate that Vpslp, a 80-kD GTPase, performs a function at the Golgi apparatus necessary for proper membrane protein trafficking and for proper sorting of CPY to the vacuole. In the absence of Vpslp both a vacuolar membrane protein, ALP, and a Golgi membrane protein, A-ALP, are delivered to the vacuole by way of the plasma membrane. These data taken together with previous studies demonstrating that >90% of newly synthesized soluble vacuolar proteins are secreted in vpsl mutant cells suggests that all membrane traffic normally routed from the late Golgi to the vacuole is diverted to the plasma membrane. These data taken together with previous data contained in this paper indicate that the endocytic pathway becomes essential in a vpsl mutant background, in which vacuolar membrane traffic is routed through the cell surface.

**Discussion**

The data presented in this paper indicate that Vpslp, a 80-kD GTPase, performs a function at the Golgi apparatus necessary for proper membrane protein trafficking and for proper sorting of CPY to the vacuole. In the absence of Vpslp both a vacuolar membrane protein, ALP, and a Golgi membrane protein, A-ALP, are delivered to the vacuole by way of the plasma membrane. In wild type cells (A) Vpslp and Chclp are required for all membrane traffic from the late Golgi to the vacuolar compartment and their possible involvement in the retrieval step is noted by question marks. The requirement of Sec4p for membrane traffic from the late Golgi to the plasma membrane and of End4p and Chclp for endocytosis are also indicated. Because chcl-ts cells exhibit only a slowing of endocytosis (Tan et al., 1993), Chclp is in parenthesis. In vpslA mutant cells (B), all membrane traffic from the late Golgi to the prevacuolar compartment is blocked and the delivery of integral membrane proteins to the vacuole occurs via the plasma membrane.

The thick and thin arrows represent pathways that are taken and not taken by late Golgi membrane proteins, respectively. In wild type cells (A) Vpslp and Chclp are required for all membrane traffic from the late Golgi to the prevacuolar compartment and their possible involvement in the retrieval step is noted by question marks. The requirement of Sec4p for membrane traffic from the late Golgi to the plasma membrane and of End4p and Chclp for endocytosis are also indicated. Because chcl-ts cells exhibit only a slowing of endocytosis (Tan et al., 1993), Chclp is in parenthesis. In vpslA mutant cells (B), all membrane traffic from the late Golgi to the prevacuolar compartment is blocked and the delivery of integral membrane proteins to the vacuole occurs via the plasma membrane.

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These data help to reconcile the dual role of Vpslp in both trafficking of membrane proteins and CPY sorting. The recently identified receptor for CPY, Vpslp (Van Dyck et al., 1992; Marcusson et al., 1994), is a Golgi localized membrane protein with a large lumenal domain (Marcusson et al., 1994). This receptor is thought to bind CPY in the late Golgi and then enter vesicles bound for a prevacuolar compartment. CPY would then dissociate from the receptor at the prevacuolar compartment and the receptor retrieved back to the Golgi for another round of sorting. Given the effect of the vpslA mutation on ALP and A-ALP, it is likely that Vpslp is also mislocalized to the cell surface in vpsl mutants thereby preventing sorting of CPY to the vacuole. Interestingly, Vpslp is mislocalized to the vacuolar membrane in vpsl mutant cells (Nothwehr, S. F., and T. H. Stevens, unpublished results) as is seen for A-ALP and Kex2p. Experiments are currently underway to determine whether Vpslp is sensitive to surface protease digestion in vpsl end4-ts double mutant cells at the non-permissive temperature.

Models have previously been put forward to explain the role and site of action of Vpslp in membrane trafficking events. In one model, Vpslp has been proposed to carry out a role in retrieval of Golgi membrane proteins from a prevacuolar (late endosomal) compartment (Wilsbach and Payne, 1993a). This model was based on the idea that Golgi retention occurs via retrieval from a prevacuolar compartment (discussed below) and was supported by the observation that Kex2p not retained in the Golgi in vpsl mutant cells was degraded in a manner dependent on vacuolar proteases.

### Table III. Synthetic Lethality of vpslΔ::LEU2 and end4Δ::URA3 Mutations

| Phenotype | Leu+ Ura+ | Leu+ Ura+ | Leu+ Ura+ | Leu+ Ura+ | Total |
|-----------|-----------|-----------|-----------|-----------|-------|
| Genotype  | VPS1      | VPS1Δ     | vpslΔ     | vpslΔ     |       |
|           | END4      | END4Δ     | end4Δ     | end4Δ     |       |
| Number observed | 19        | 13        | 13        | 0         | 45    |
| Predicted* | 16        | 16        | 16        | 0         | 48    |
| Predicted† | 16        | 16        | 16        | 16        | 64    |

The diploid strain SNY53 was sporulated and tetrads dissected onto YEPD media. Growth of colonies was allowed to proceed at 23°C for 5 d at which time the nutritional requirements of the haploid colonies arising from the viable spores were determined. Shown is the number of spores obtained from dissection of diploid SNY53 (see Table III A) having each of four possible genotypes.

* The predicted distribution of the spores into the four possible genotypes if the vpslΔ and end4Δ mutations are unlinked and exhibit synthetic lethality.
† The predicted distribution of the spores between the four genotypes if the vpslΔ and end4Δ mutations do not show synthetic lethality.

11/16 contained three viable spores (1 Leu+ Ura+, 1 Leu+ Ura+, 1 inviable); and (c) 4/16 contained two viable spores (2 Leu+ Ura-, two inviable). This observation suggested that the two mutations segregated independently and a combination of both mutations caused lethality. Subsequent analysis of the spores that gave rise to viable haploid strains (Table III) also indicated that spores that received both the vpslΔ and end4Δ mutations were not viable. In all cases, the inviable spores were observed to germinate and undergo two to four cell divisions before completely ceasing growth. The synthetic lethality of the end4Δ and vpslΔ mutations taken together with other data contained in this paper indicate that the endocytic pathway becomes essential in a vpslΔ background, in which vacuolar membrane traffic is routed through the cell surface.
However, the data in this paper support a model (Fig. 8) in which Vpslp acts at the level of the late Golgi apparatus to promote vesicle traffic to the vacuole. In this model, Vpslp carries out a function at the Golgi similar to that of mammalian dynamin in endocytosis (Herskovits et al., 1993; van der Bliek et al., 1993), i.e., vesicle budding (Damke et al., 1994). According to this model, Vpslp-dependent, Golgi-derived vesicles would contain late Golgi membrane proteins (such as Kex2p, Kex1p, and DPAP A), vacuolar membrane proteins (such as ALP) and soluble vacuolar hydrolases bound to their receptors (such as CPY bound to Vps10p). Thus, Vpslp function would be required for both "signal-mediated" (CPY) and "default" membrane transport (ALP) from the late Golgi compartment to the prevacuole and eventually the vacuole. Late Golgi membrane proteins delivered in a Vpslp-dependent transport step to the prevacuole would be transported back to the Golgi apparatus in a retrieval step requiring the aromatic amino acid-based retention signals in their cytoplasmic domains (Nothwehr and Stevens, 1994). At the present time it is not possible to rule out the involvement of Vpslp in the prevacuole to late Golgi retrieval step as well.

The emerging picture for the role of Vpslp in membrane trafficking is very reminiscent of that of the yeast clathrin heavy chain, Chclp. Chclp has also been proposed to function at the late Golgi since significant amounts of both Kex2p and DPAP A were rapidly transported to the plasma membrane in cells containing a temperature sensitive allele of the CHC1 gene (Seeger and Payne, 1992a). Given the well characterized role of clathrin in vesicle formation from both the plasma membrane and trans-Golgi network in animal cells (Pearse and Robinson, 1990), an intriguing possibility is that in yeast Chclp and Vpslp act together in the formation and budding of vesicles from the late Golgi that are bound for the prevacuolar compartment. In accordance with this idea, vpsl-ts and chcl-ts mutations exhibit a strong synthetic growth defect (Nothwehr, S. F., and T. H. Stevens, unpublished results), and each mutation results in secretion of newly synthesized vacuolar hydrolases (Vater et al., 1992; Seeger and Payne, 1992b).

Although vpsl and chcl mutants both mislocalize Golgi membrane proteins to the cell surface, their phenotypes appear to differ with respect to the amount of these proteins found at the cell surface in the steady state. Our results indicate that only modest steady-state amounts of ALP and A-ALP are present at the plasma membrane in vpsl mutants consistent with the results of Wilsbach and Payne (1993a), who showed that little Kex2p was present at the plasma membrane in vpsl cells. However, in chcl-ts cells shifted to the nonpermissive temperature 70% of Kex2p and 30% of DPAP A were mislocalized to the cell surface, yet newly synthesized ALP continued to undergo vacuolar protease-dependent processing. In light of the results presented in this manuscript, it is entirely possible that the vacuolar membrane protein ALP is delivered to the vacuole via the plasma membrane in chcl-ts yeast cells shifted to the high temperature. Thus, the difference in the behavior of the various membrane proteins under study in vpsl and chcl-ts yeast cells may be that they are all diverted to the plasma membrane in these cells, but each protein exhibits a differential rate of endocytosis in the clathrin defective cells (very slow for Kex2p, intermediate for DPAP A and rapid for ALP). In fact, clathrin-independent endocytosis has been well documented in yeast, since chcl mutant yeast cells endocytose α-factor (Payne et al., 1988) and the plasma membrane receptor Ste3p at near normal rates (Tan et al., 1993). Nevertheless, our results indicate that End4p is required for clearing both A-ALP and ALP from the cell surface in vpsl mutant cells and it is possible that End4p is required for both clathrin-dependent and clathrin-independent pathways of endocytosis.

These results also have implications for our understanding of the mechanism of retention of late Golgi membrane proteins such as DPAP A and Kex2p. These proteins are retained via aromatic residue-containing signals in their cytoplasmic domains (Wilcox et al., 1992; Nothwehr et al., 1993). Mutation of their retention signals causes these proteins to be mislocalized directly to the vacuole without first passing through the cell surface (Roberts et al., 1992; Wilcox et al., 1992). Several lines of evidence argue that a retrieval mechanism is involved in the retention of these proteins in the Golgi membrane (Wilsbach and Payne, 1993b; Nothwehr and Stevens, 1994). Wilcox et al. (1992) have shown that a retention-defective form of Kex2p is depleted from the late Golgi more quickly in a wild-type strain than in a vacuolar protease-deficient mutant strain. These data argue that Kex2p can be retrieved from a vacuolar protease containing compartment such as the prevacuolar compartment. In yeast, the endocytic and vacuolar biogenesis pathways converge at a prevacuolar/late endosomal compartment (Raymond et al., 1992; Vida et al., 1993; Schimmüller and Riezman, 1993). However, in vpsl cells Golgi membrane proteins internalized from the cell surface are delivered to the vacuole rather than recycling back to the late Golgi, suggesting a role for Vpslp function in this retrieval pathway. Whether Vpslp and Chclp have a direct role in the retrieval of Golgi membrane proteins from the prevacuolar compartment must await further investigation.

There is growing evidence that there is a static component as well as a retrieval component to Golgi membrane protein retention in yeast. Retention-defective forms of A-ALP (Nothwehr et al., 1993) and Kex2p (Wilcox et al., 1992) do not reach the vacuole nearly as rapidly as vacuolar proteins such as ALP, consistent with the model that static and retrieval mechanisms are both operational. The presence of a static component in Golgi retention of A-ALP would also be consistent with the slower kinetics of delivery of A-ALP to the vacuole as compared to ALP in vpsl mutant cells.

While our model for Vpslp function proposes a single class of Golgi-derived vesicle containing all prevacuole-bound cargo, the results of Herman et al. (1991) with vpsl5-ts mutants suggest that ALP and CPY may be partitioned into different vesicles. The observation that vacuolar processing of CPY, but not ALP, is blocked in vpsl5-ts cells could reflect a Vps15p requirement for CPY:receptor complexes but not ALP to be packaged into Golgi-derived vesicles. If there are indeed two classes of Golgi-to-prevacuole vesicles, our results argue that Vpslp function would be required for the formation of both classes. With regard to the possibility that Vpslp may be required for the formation of two classes of Golgi-derived vesicles, it is interesting that the Vpslp homologue, dynamin, may be required for formation of non-coated as well as coated endocytic vesicles in Drosophila (Kosaka and Ikeda, 1983; Kessel et al., 1989; Masur et al., 1990).
In summary, our results demonstrate that vpslp yeast cells vacuolar and Golgi membrane proteins reach the vacuole only after transport to the plasma membrane and endocytic uptake. These results suggest that Vpslp may be part of a cytoplasmic machinery associated with the Golgi apparatus involved in vesicle formation. We are currently screening for other mutants that exhibit vpslp-like phenotypes to identify new gene products that function together with Vpslp at this earliest step in vacuolar protein sorting in the yeast Golgi apparatus.

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