

Golgi and Vacuolar Membrane Proteins Reach the Vacuole in *vps1* Mutant Yeast Cells via the Plasma Membrane

Steven F. Nothwehr, Elizabeth Conibear, and Tom H. Stevens

Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

Abstract. The Vps1 protein of *Saccharomyces cerevisiae* is an 80-kD GTPase associated with the Golgi apparatus. Vps1p 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 *vps1*Δ mutants was investigated by analyzing transport of these proteins in *vps1*Δ 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 *vps1*Δ *sec4-ts* and *vps1*Δ *end4-ts* double mutant cells at the non-permissive temperature but vacuolar delivery of the vacuolar membrane pro-

tein, alkaline phosphatase was also blocked in these cells. Moreover, both proteins expressed in the *vps1*Δ *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 *vps1* mutant cells.

These data strongly suggest that a loss of Vps1p 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 Vps1p 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)¹, 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 Vps10p 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 Bliik and Meyerowitz, 1991). A high degree of sequence identity is shared between the amino-terminal GTP-binding regions of these proteins (66% between Vps1p 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

Address all correspondence to T. H. Stevens, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403. Ph.: (503) 346-5884. Fax: (503) 346-4854.

1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; *CEN*, yeast centromere; DPAP, dipeptidyl aminopeptidase; PGK, phosphoglycerol kinase.

synaptic vesicles at the non-permissive temperature causing paralysis in adult flies (Poodry and Edgar, 1979). A more extensive morphological examination of various tissues from *shi^{ts}* flies and derived cell lines revealed that the *Shibire* gene product is required for endocytosis but not secretion in a variety of cell types (Kosaka and Ikeda, 1983; Tsuruhara et al., 1990). Interestingly, mammalian dynamin has also been shown to be essential for receptor-mediated endocytosis (van der Bliek et al., 1993; Herskovits et al., 1993). More recently, analysis of HeLa cells expressing dominant-negative dynamin alleles indicates that dynamin is required for coated pit constriction 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, *vps1* mutants were found in a screen for mutants exhibiting a defect in the maturation of the α -factor mating pheromone (Wilsbach and Payne, 1993a). Upon further examination the primary defect was shown to be a loss of retention of the Kex2p endoprotease, a Golgi membrane protein responsible for processing of the α -factor precursor. In *vps1* mutant cells the half-time of turnover of Kex2p was greatly reduced and degradation of Kex2p in *vps1* mutant cells was shown to be dependent on protease A, a vacuolar protease encoded by the *PEP4* gene. These results strongly suggested that in *vps1* mutants Kex2p was mislocalized to the vacuole where it was degraded. In contrast, in mutants lacking the clathrin heavy chain (*chc1* Δ), 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 Vps1p to dynamin suggests the possibility that Vps1p may carry out an analogous function (vesicle formation from a donor compartment) at a membrane 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 compartment. 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, 1993b; Nothwehr and Stevens, 1994). In principle Vps1p 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 Vps1p is solely required for retrieval, Golgi membrane proteins in *vps1* mutants should be transported directly from the late Golgi to the vacuole without passing through the plasma membrane. We report here that in *vps1* mutant cells vacuolar and Golgi membrane proteins reach the vacuole only after transport to the plasma membrane and endocytic uptake. These results are consistent with a model where Vps1p is involved in formation of vesicles from the Golgi apparatus that are targeted to the prevacuolar compartment 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 *PHO8* gene into pRS306 (Sikorski and Hieter, 1989). A 0.45-kbp XhoI fragment from within the open reading frame of *PHO8* was removed from pSN110 giving rise to pSN111. The *pho8* Δ ::*LEU2* construct (pGP10) was made by first subcloning 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 containing the *LEU2* gene. pSN239 was constructed by digesting pGP10 with XhoI and BglII, blunt ending with Klenow enzyme, and ligating with a 2-kbp BglII-BglII (blunt ended) fragment containing the *ADE2* gene. pPLO2010 was constructed by first subcloning the 4.6-kbp SacI-SalI fragment containing the *PEP4* gene into pRS306, then removing the 1.3-kbp HindIII fragment contained within the SacI-SalI insert. pCAV40 was constructed by subcloning the XbaI-SpeI insert from a hydroxylamine-mutagenized isolate of pCKR19 containing the *vps1-100* allele (Vater et al., 1992) into the XbaI/SpeI 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 HA1 (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 SalI-EagI fragment and subcloned into the SalI/EagI 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 *kex2* Δ mutation for processing of pro- α -factor (data not shown).

Yeast strains used in this study are listed in Table II. The *PHO8* gene of SNY28 was disrupted to give rise to SNY55 by transforming pSN111 linearized 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 *vps1* Δ ::*LEU2* mutation was introduced by transforming yeast cells with

Table I. Plasmids Used in This Study

Plasmid	Description	Source or reference
pCAV40	<i>vps1-100</i> allele in pRS306	This study
pCKR3A	<i>vps1</i> Δ :: <i>LEU2</i> allele in pUC12	Rothman et al. (1990)
pCKR70A	<i>vps3</i> Δ :: <i>LEU2</i> allele in pUC12	Raymond et al., 1990
pGP10	<i>pho8</i> Δ :: <i>LEU2</i> allele in pGEM2	This study
pLC4-8	<i>sec4-8</i> allele in RS306	This study
pPLO2010	<i>pep4</i> Δ <i>H3</i> allele in pRS306	This study
pSG1	<i>end4</i> Δ :: <i>URA3</i> allele-containing plasmid	D. Drubin
pSN55	<i>STE13-PHO8</i> gene fusion in pRS316	Nothwehr et al. (1993)
pSN92	4 kbp <i>PHO8</i> fragment in pRS316	Nothwehr et al. (1993)
pSN111	<i>pho8</i> Δ <i>X</i> allele in pRS306	This study
pSN218	<i>KEX2</i> :: <i>HA</i> allele in pRS316	This study
pSN239	<i>pho8</i> Δ :: <i>ADE2</i> allele in pGEM2	This study

Table II. Yeast Strains Used in This Study

Strain	Genotype	Source or reference
SNY28	<i>MATα ura3-52 leu2-3,112 his4-519 ade6</i>	Hill and Stevens (1994)
SNY55	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX</i>	This study
SNY56	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX pep4-ΔH3</i>	This study
SNY57	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1Δ::LEU2</i>	This study
SNY58	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX pep4-ΔH3 vps1Δ::LEU2</i>	This study
LCY22	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps3Δ::LEU2</i>	This study
SNY4	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX vps1-100</i>	This study
SNY24	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX sec4-8</i>	This study
SNY31	<i>MATα ura3-52 leu2-3,112 his4-519 ade6 pho8-ΔX sec4-8 vps1Δ::LEU2</i>	This study
RH144-3D	<i>MATα ura3 leu2 his4 bar1-1</i>	H. Riezman
RH268-1C	<i>MATα ura3 leu2 his4 bar1-1 end4-1</i>	H. Riezman
LCY14	<i>MATα ura3 leu2 his4 bar1-1 vps1Δ::LEU2</i>	This study
SNY16	<i>MATα ura3 leu2 his4 bar1-1 end4-1 pho8-ΔX</i>	This study
LCY19	<i>MATα ura3 leu2 his4 bar1-1 end4-1 vps1Δ::LEU2</i>	This study
SNY44	<i>MATα/α ura3/ura3-52 leu2/leu2-3,112 his4/his4-519 ADE6/ade6 bar1-1/BAR1 end4-1/END4 pho8-ΔX/pho8-ΔX VPS1/vps1Δ::LEU2</i>	This study
SNY44-2C	<i>MATα ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX vps1Δ::LEU2</i>	This study
SNY44-13B	<i>MATα ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX ade6 end4-1</i>	This study
SNY44-1A	<i>MATα ura3-52 leu2-3,112 his4-519 BAR1 or bar1-1 pho8-ΔX end4-1 vps1Δ::LEU2</i>	This study
SEY6210	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9</i>	Robinson et al. (1988)
SEY6211	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 ade2-101 suc2-Δ9</i>	Robinson et al. (1988)
SNY17	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 pho8Δ::LEU2</i>	This study
SNY18	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 ade2-101 suc2-Δ9 pho8Δ::ADE2</i>	This study
SNY36	<i>MATα/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-Δ200/his3-Δ200 trp1-Δ901/trp1-Δ901 LYS2/lys2-801 ade2-101/ADE2 suc2-Δ9/suc2-Δ9 pho8Δ::ADE2/ pho8Δ::LEU2</i>	This study
SNY36-9A	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 pho8Δ::ADE2</i>	This study
SNY36-9B	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 lys2-801</i>	This study
SNY38	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 lys2-801 vps1Δ::LEU2</i>	This study
SNY46	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 end4Δ::URA3</i>	This study
SNY53	<i>MATα/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-Δ200/his3-Δ200 trp1-Δ901/trp1-Δ901 lys2-801/LYS2 ade2-101/ADE2 suc2-Δ9/suc2-Δ9 pho8Δ::ADE2/pho8Δ::LEU2 END4/end4Δ::URA3 vps1Δ::LEU2/VPS1</i>	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 SNY28: SNY55, SNY56, SNY57, SNY58, LCY22, SNY4, SNY24, and SNY31. LCY14 is congenic with RH144-3D. LCY16 and LCY19 are congenic with RH268-1C. SNY44 resulted from a cross of LCY16 with SNY57. SNY44-2C, SNY44-13B, and SNY44-1A are spores that arose from sporulation of SNY44. SNY17 is congenic with SEY6210 whereas SNY18 is congenic with SEY6211. SNY36 was formed by mating SNY17 with SNY18. Sporulation of SNY36 gave rise to SNY36-9B and SNY36-9A which were sister spores from the same tetrad. SNY38 is congenic with SNY36-9B and SNY46 is congenic with SNY36-9A. Diploid SNY53 was generated by crossing SNY38 with SNY46.

the SacI-XbaI fragment from pCKR3A and selecting from Leu⁺ colonies. The *pho8Δ::LEU2* and *pho8Δ::ADE2* mutations were introduced by transforming digests of pGP10 (cut with StuI/BamHI) and pSN239 (digested with EcoRI/SalI) and selecting for Leu⁺ or Ade⁺ colonies, respectively. The *vps3Δ::LEU2* construct was integrated by transforming pCKR70A digested with BamHI and selecting for Leu⁺ colonies. A yeast strain harboring the *end4Δ::URA3* mutation was generated by transforming pSG1 digested with EcoRI and selecting for Ura⁺ colonies. To introduce the *vps1-100* mutation, strains were transformed with pCAV40 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 XbaI, 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 following reagents were obtained from the indicated sources: ³⁵S-express label was from New England Nuclear (Boston, MA), oxalyticase was from Enzogenetics (Corvallis, OR), all secondary and fluorochrome-conjugated antibodies were from Jackson ImmunoResearch Labs. Inc. (West Grove, PA), and pronase was from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Immunofluorescence Microscopy

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. Spheroplasting 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 biotin-conjugated goat anti-mouse IgG (H+L); and (c) 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

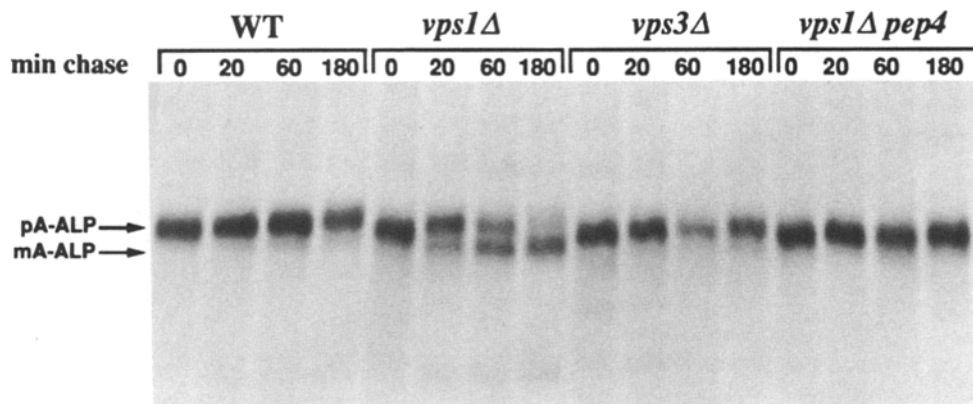


Figure 1. Analysis of *PEP4*-dependent processing of A-ALP in *vps* mutants. Wild type (SNY55), *vps1Δ* (SNY57), *vps3Δ* (LCY22), and *vps1Δ pep4* (SNY58) cells carrying pSN55 (*CEN-A-ALP*) were ^{35}S -labeled for 10 min and then chased by adding 50 $\mu\text{g/ml}$ cysteine and methionine. At the indicated 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% IgG-sorb. After preadsorbing to IgG-sorb 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 IgG-sorb 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 NEN ^{35}S -express label (described above), 0.5 ml of labeled culture (0.5 OD units) was added to 0.5 ml of ice-cold media containing 20 mM KF and 20 mM NaN_3 . 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 MgCl_2 , 0.5% β -mercaptoethanol, 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 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ leupeptin, and 1 $\mu\text{g/ml}$ 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

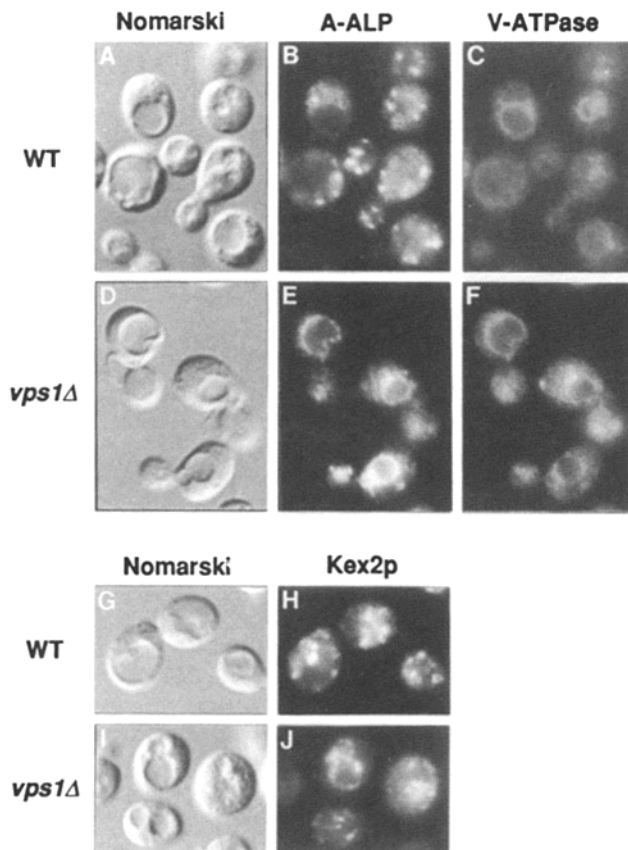
Late Golgi Membrane Proteins Are Mislocalized To The Vacuole In *vps1* Mutant Cells

A fusion protein consisting of the cytoplasmic domain of the late Golgi membrane protein dipeptidyl aminopeptidase (DPAP) A fused to the transmembrane and luminal 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. *vps1Δ* mutant yeast strains were found to be severely defective in Golgi retention (Fig. 1). A-ALP expressed in *vps1Δ* 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 *vps3Δ* 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 *vps1Δ* 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 *vps1Δ* 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 *vps1Δ* 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 *Vps1p* 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 *Vps1p* in this process. Alternatively, a loss of retention could be an in-

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 *I*) and by epifluorescence through filter sets specific for fluorescein (*B*, *E*, *H*, and *J*) and Texas red (*C* and *F*) fluorescence.

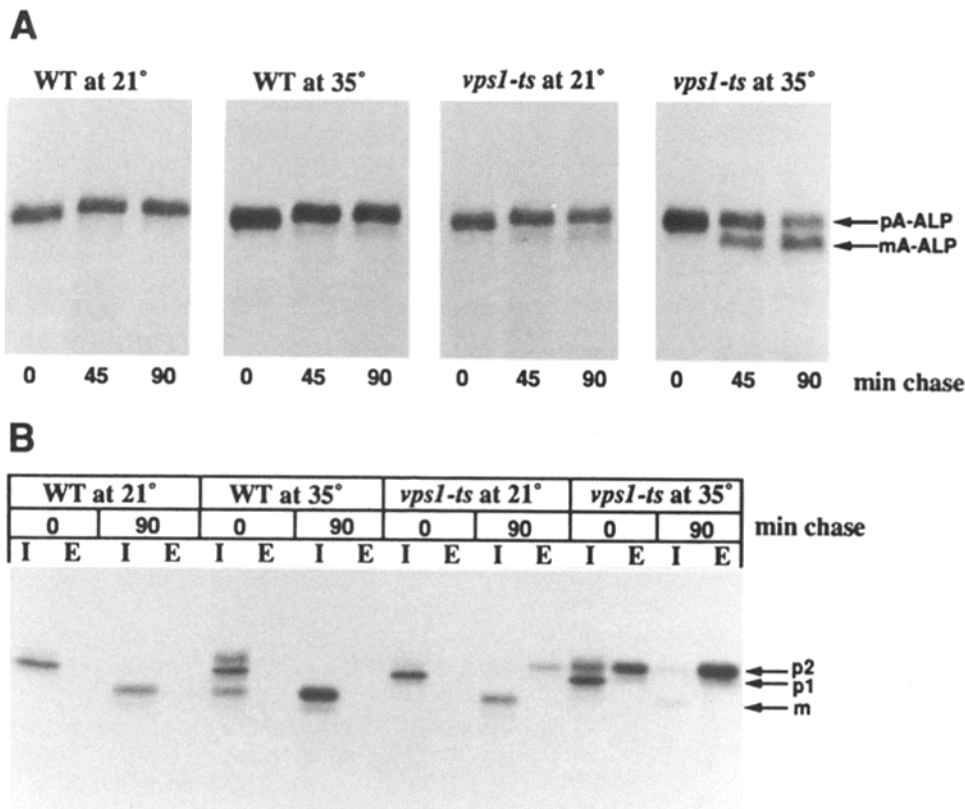


Figure 3. Onset of the Golgi retention defect in *vps1-ts* mutant cells. In *A*, cultures of wild type (SNY55) or *vps1-ts* mutant (SNY4) cells carrying pSN55 (*CEN-A-ALP*) were propagated at 21°C, split, and one portion shifted to 35°C for 5 min while the other portion remained at 21°C. The cultures 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.

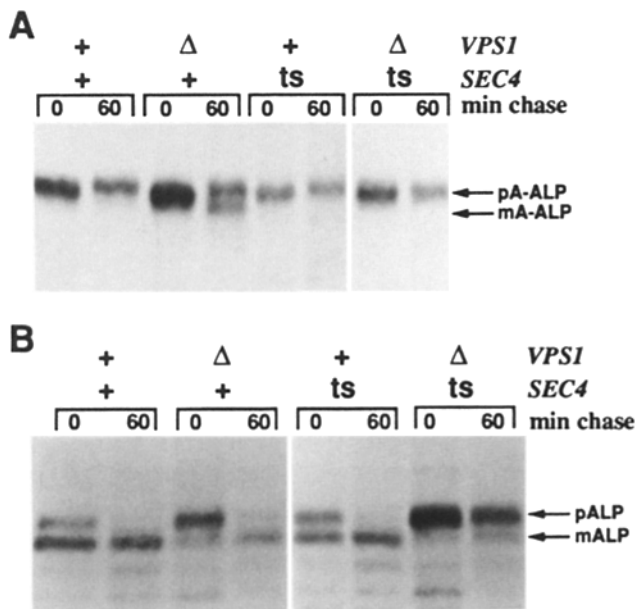


Figure 4. Trafficking of A-ALP and ALP in *vps1Δ sec4-ts* double mutant cells. Wild type (SNY55), *vps1Δ* (SNY57), *sec4-ts* (SNY24), and *vps1Δ sec4-ts* (SNY31) cells carrying either pSN55 (A) or pSN92 (B) were grown at 22°C. The cultures were then shifted to 35°C for 10 min before labeling with ³⁵S 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).

direct consequence of prolonged absence of Vps1p in *vps1* 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° 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 Vps1p function.

In the experiment shown in Fig. 3, wild-type and *vps1-ts* cells expressing A-ALP were propagated at the permissive temperature of 21°C, and then preincubated at either 35° or 21°C for 5 min, and were then radioactively labeled and chased at 35° or 21°C, respectively. The *vps1-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 *vps1-ts* mutant exhibited strong defects in both A-ALP Golgi retention and CPY sorting. The defect observed for the *vps1-ts* cells at 35°C was similar to that seen for *vps1Δ* cells at 30°C (Fig. 1). In addition, the A-ALP processing kinetics of the *vps1-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 Vps1p function is consistent with the idea that Vps1p 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 vps1 Mutant Cells

To understand more clearly the role of Vps1p 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 *vps1* 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 *vps1* mutant cells after imposition of a block in the late secretory pathway. For this purpose we made a double mutant containing both the *vps1Δ* and the temperature sensitive *sec4-ts* mutations, the latter of which prevents fusion of secretory vesicles with the plasma membrane at the nonpermissive temperature (Salmiinen 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 *vps1Δ sec4-ts* double mutant at the restrictive temperature of 35°C. However, A-ALP processing did occur in the *vps1Δ* single mutant at 35°C and also in the *vps1Δ 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 *vps1Δ 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 *vps1Δ* 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 *vps1Δ* cells.

The end4-ts Mutation Traps Golgi and Vacuolar Membrane Proteins At the Plasma Membrane In vps1 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 *vps1* mutant cells. To further test the model that ALP and A-ALP pass through the plasma membrane in *vps1* mutants we tested whether the endocytic pathway was required for delivery of ALP and A-ALP to the vacuole in *vps1* mutants. For these purposes the *vps1Δ* mutation was combined with a temperature sensitive mutant allele of the *END4* gene. Mutations in the *END4* gene have been shown to

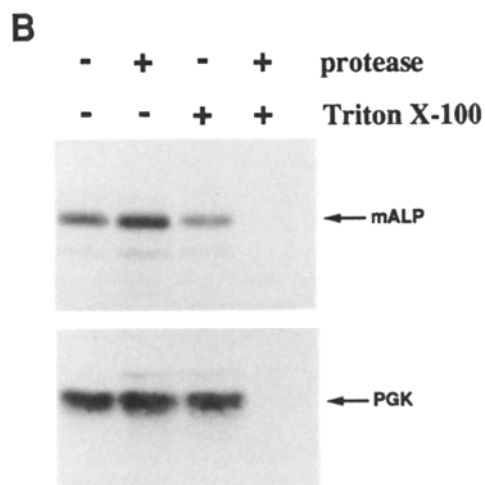
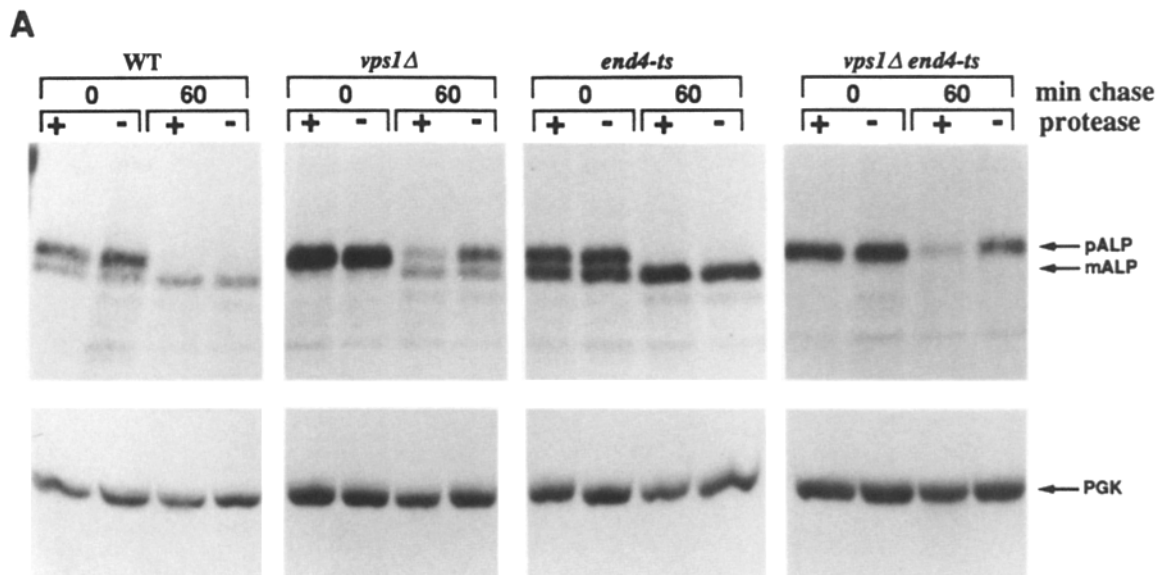


Figure 5. Trafficking of ALP in *vps1Δ end4-ts* mutant cells. (A) Wild type (SNY55), *vps1Δ* (SNY57), *end4-ts* (SNY44-13B), and *vps1Δ end4-ts* (SNY44-1A) cells carrying pSN92 (*CEN-ALP*) were propagated at 22°C and shifted to 36°C for 10 min whereupon the cultures were ³⁵S-labeled for 10 min and chased by the addition of 50 μg/ml cysteine and methionine. After 0 and 60 min of chase the cells were digested with 4 mg/ml pronase (+) or mock digested with no protease (–) for 20 min at 0°C. (B) Wild type (SNY55) cells carrying pSN92 (*CEN-ALP*) were pulsed and chased as described above in A. After 60 min of chase the cells were digested with pronase (as above) in the presence or absence of 0.2% Triton X-100 (as indicated by + or –) or mock digested with no protease in the presence or absence of 0.2% Triton X-100. In both A and B, the cells were then subjected to glass bead lysis and immunoprecipitated with an anti-ALP polyclonal antibody. The same extracts were subsequently immunoprecipitated with an anti-PGK polyclonal antibody. The immunoprecipitates were then analyzed by SDS-PAGE and fluorography.

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. 5 A shows an experiment in which ALP has been immunoprecipitated from an *end4-ts vps1Δ* 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 *vps1Δ* strain. We have consistently observed a delay in ALP processing in both *vps1Δ* and *vps1-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 vps1Δ* double mutant even after 60 min indicating that End4p is required for ALP to reach the vacuole in *vps1Δ* 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 vps1Δ* mutant cells.

Several lines of evidence support the validity of this technique for analyzing the localization of proteins at the plasma membrane. In the *vps1Δ* sample chased for 60 min (Fig. 5 A) 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. 5 B) demonstrating that the processed

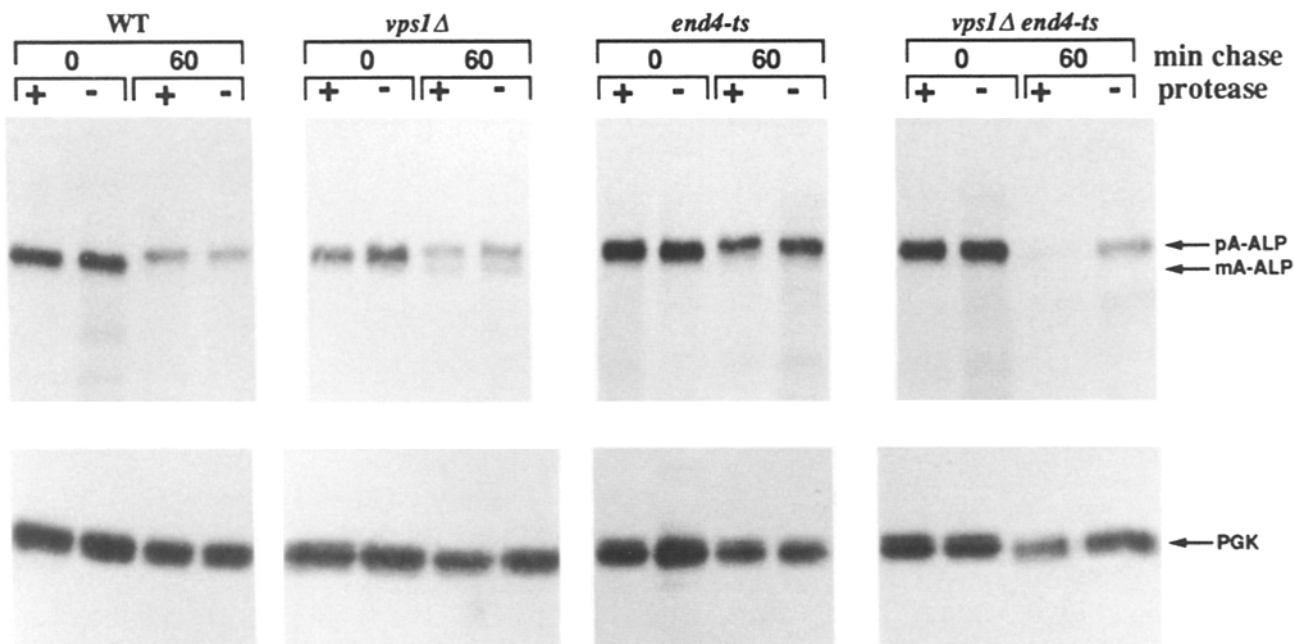


Figure 6. Trafficking of A-ALP in *vps1Δ end4-ts* mutant cells. Wild type (SNY55), *vps1Δ* (SNY57), *end4-ts* (SNY44-13B), and *vps1Δ end4-ts* (SNY44-1A) cells carrying pSN55 (*CEN-A-ALP*) were treated as described in the legend of Fig. 5 A.

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 vps1Δ* double mutant (Fig. 6). First, the delivery of A-ALP to the vacuole observed in *vps1Δ* mutant cells as indicated by processing was not observed in the *end4-ts vps1Δ* 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 *vps1Δ* mutant cells.

A Complete Loss of *Vps1p* and *End4p* Functions Is Lethal

The above results are compatible with the idea that in *vps1* 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 *vps1* 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 vps1Δ* 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 *vps1Δ* or *end4-ts* single mutants. For example, at 23°C the doubling time of the *end4-ts vps1Δ* 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 *vps1* and *end4* mutations further, the fate of cells completely disrupted for both genes was investigated. SNY53, a diploid strain heterozygous for both *VPS1* and *END4* (*VPS1/vps1Δ::LEU2 END4/end4Δ::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)

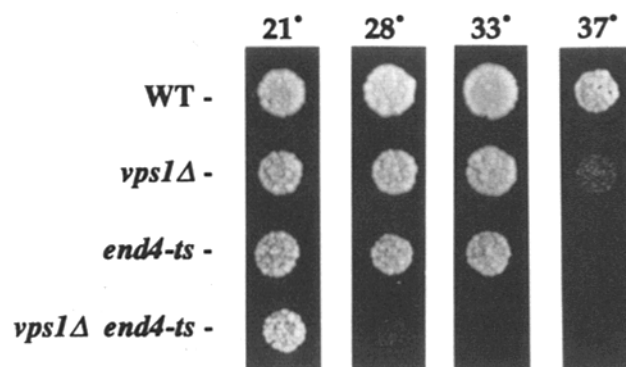


Figure 7. Synthetic growth defect of the *vps1Δ end4-ts* double mutant. Wild type (RH144-3D), *vps1Δ* (LCY14), *end4-ts* (RH269-1C), and *vps1Δ end4-ts* (LCY19) cells were grown for 1 d (28°, 33°, 37°C) or 2 d (21°C) on YEPD plates before being photographed as shown.

Table III. Synthetic Lethality of *vps1Δ::LEU2* and *end4Δ::URA3* Mutations

Phenotype Genotype	Leu ⁻ Ura ⁻ <i>VPS1</i> <i>END4</i>	Leu ⁺ Ura ⁻ <i>vps1Δ</i> <i>END4</i>	Leu ⁻ Ura ⁺ <i>VPS1</i> <i>end4Δ</i>	Leu ⁺ Ura ⁺ <i>vps1Δ</i> <i>end4Δ</i>	Total
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 *vps1Δ* and *end4Δ* mutations are unlinked and exhibit synthetic lethality.

‡ The predicted distribution of the spores between the four genotypes if the *vps1Δ* and *end4Δ* mutations do not show synthetic lethality.

11/16 contained three viable spores (1 Leu⁺ Ura⁻, 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 *vps1Δ* 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 *vps1Δ* mutations taken together with other data contained in this paper indicate that the endocytic pathway becomes essential in a *vps1Δ* background, in which vacuolar membrane traffic is routed through the cell surface.

Discussion

The data presented in this paper indicate that Vps1p, 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 Vps1p 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 *vps1* mutant cells suggests that all membrane traffic normally routed from the late Golgi to the vacuole is diverted to the plasma membrane in *vps1* mutants. Consistent with the idea that the endocytic pathway serves as an essential salvage route to retrieve vacuolar components from the plasma membrane in *vps1* mutant cells, *VPS1* and a gene required for the internalization step of endocytosis, *END4*, exhibit synthetic lethality.

These data help to reconcile the dual role of Vps1p in both trafficking of membrane proteins and CPY sorting. The recently identified receptor for CPY, Vps10p (Van Dyck et al., 1992; Marcusson et al., 1994), is a Golgi localized membrane protein with a large luminal 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

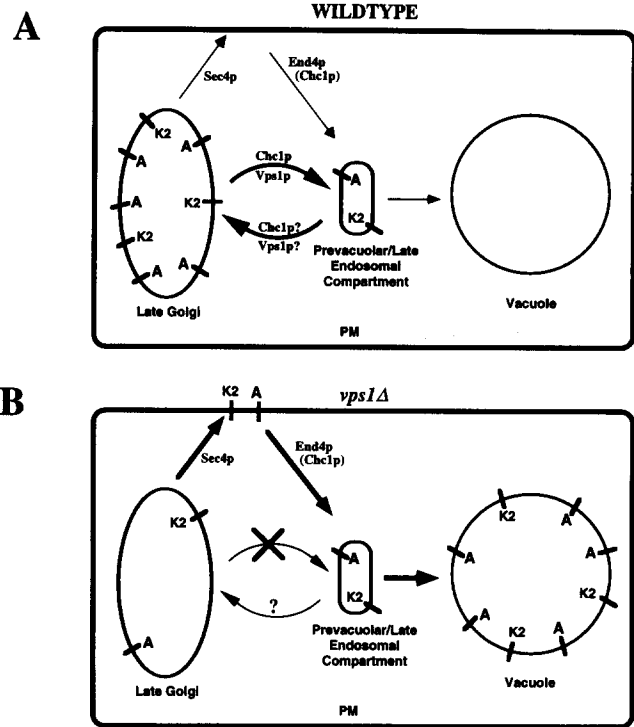


Figure 8. Model for the role of Vps1p in membrane trafficking events. DPAP A and Kex2p are represented by the letters A and K2, respectively, whereas the plasma membrane is represented by PM. The thick and thin arrows represent pathways that are taken and not taken by late Golgi membrane proteins, respectively. In wild type cells (A) Vps1p and Chc1p 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 Chc1p for endocytosis are also indicated. Because *chc1-ts* cells exhibit only a slowing of endocytosis (Tan et al., 1993), Chc1p is in parenthesis. In *vps1Δ* 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.

of the *vps1Δ* mutation on ALP and A-ALP, it is likely that Vps10p is also mislocalized to the cell surface in *vps1* mutants thereby preventing sorting of CPY to the vacuole. Interestingly, Vps10p is mislocalized to the vacuolar membrane in *vps1* 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 Vps10p is sensitive to surface protease digestion in *vps1 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 Vps1p in membrane trafficking events. In one model, Vps1p 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 *vps1* mutant cells was degraded in a manner dependent on vacuolar proteases.

However, the data in this paper support a model (Fig. 8) in which Vps1p acts at the level of the late Golgi apparatus to promote vesicle traffic to the vacuole. In this model, Vps1p 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, Vps1p-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, Vps1p 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 Vps1p-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 Vps1p in the prevacuole to late Golgi retrieval step as well.

The emerging picture for the role of Vps1p 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 (*chcl-ts*) 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 Vps1p 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, *vps1-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 *vps1* 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 *vps1* mutants consistent with the results of Wilsbach and Payne (1993a), who showed that little Kex2p was present at the plasma membrane in *vps1* 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 *vps1* 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 *vps1* 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; Schimmöller and Riezman, 1993). However, in *vps1* 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 Vps1p function in this retrieval pathway. Whether Vps1p 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 *vps1* mutant cells.

While our model for Vps1p function proposes a single class of Golgi-derived vesicle containing all prevacuole-bound cargo, the results of Herman et al. (1991) with *vps15-ts* mutants suggest that ALP and CPY may be packaged into different vesicles. The observation that vacuolar processing of CPY, but not ALP, is blocked in *vps15-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 Vps1p function would be required for the formation of both classes. With regard to the possibility that Vps1p may be required for the formation of two classes of Golgi-derived vesicles, it is interesting that the Vps1p 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 in *vps1* 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 *vps1*-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.

We acknowledge N. Davis for advice on the pronase sensitivity assay and many helpful discussions, D. Drubin and P. Novick for plasmids, H. Riezman for strains and advice, and J. Horecka for tetrad dissection expertise. E. Whitters and A. Cooper provided insightful comments on the manuscript.

This work was supported by an American Cancer Society postdoctoral fellowship to S. F. Nothwehr (PF-3608), an AHA Oregon Affiliate award to E. Conibear, and grants from the National Institutes of Health (GM 32448 and GM38006) to T. H. Stevens.

Received for publication 13 December 1994 and in revised form 10 January 1995.

References

Chen, M. S., R. A. Obar, C. C. Schroeder, T. W. Austin, C. A. Poodry, S. C. Wadsworth, and R. B. Vallee. 1991. Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. *Nature (Lond.)* 351:583-586.

Davis, N. G., J. L. Horecka, and G. F. Sprague. 1993. *Cis*- and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.* 122:53-65.

Danke, H., T. Baba, D. E. Warnock, and S. L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915-934.

Herman, P. K., J. H. Stack, and S. D. Emr. 1991. A genetic and structural analysis of the yeast Vps15 protein kinase: evidence for a direct role of Vps15p in vacuolar protein delivery. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4049-4060.

Herskovits, J. S., C. C. Burgess, R. A. Obar, and R. B. Vallee. 1993. Effects of mutant rat dynamin on endocytosis. *J. Cell Biol.* 122:565-578.

Hill, K. J., and T. H. Stevens. 1994. Vma21p is a Yeast membrane protein retained in the endoplasmic reticulum by a di-lysine motif and is required for the assembly of the vacuolar H⁺-ATPase complex. *Mol. Biol. Cell.* 5:1039-1050.

Kaneko, Y., Y. Tamai, A. Toh-e, and Y. Oshima. 1985. Transcriptional and post-transcriptional control of *PHO8* expression by *PHO* regulatory genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 5:248-252.

Kessel, J., B. Holst, and T. F. Roth. 1989. Membrane intermediates in endocytosis are labile, as shown in a temperature-sensitive mutant. *Proc. Natl. Acad. Sci. USA.* 86:4968-4972.

Klionsky, D. J., and S. D. Emr. 1989. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2241-2250.

Klionsky, D. J., P. K. Herman, and S. D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* 54:266-292.

Kornfeld, S. 1992. Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. *Ann. Rev. Biochem.* 61:307-330.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster*, *shibire*^o. *J. Cell Biol.* 97:499-507.

Marcusson, E. G., B. F. Horazdovsky, J. L. Cereghino, E. Gharakhanian, and S. D. Emr. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. *Cell.* 77:579-586.

Masur, S. K., Y.-T. Kim, and C.-F. Wu. 1990. Reversible inhibition of endocytosis in cultured neurons from the *Drosophila* temperature-sensitive mutant, *shibire*. *J. Neurogenet.* 6:191-206.

Nothwehr, S. F., C. J. Roberts, and T. H. Stevens. 1993. Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues. *J. Cell Biol.* 121:1197-1209.

Nothwehr, S. F., and T. H. Stevens. 1994. Sorting of membrane proteins in the yeast secretory pathway. *J. Biol. Chem.* 269:10185-10188.

Obar, R. A., C. A. Collins, J. A. Hammarback, H. S. Shpetner, and R. B. Vallee. 1990. Molecular cloning of the microtubule-associated mechano-

chemical enzyme dynamin reveals homology with a new family of GTP-binding proteins. *Nature (Lond.)* 347:256-261.

Payne, G. S., D. Baker, E. van Tuinen, and R. Schekman. 1988. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast. *J. Cell Biol.* 106:1453-1461.

Pearse, B. M., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151-171.

Poodry, C. A., and L. Edgar. 1979. Reversible alterations in the neuromuscular junctions of *Drosophila melanogaster* bearing a temperature sensitive mutation, *shibire*. *J. Cell Biol.* 81:520-527.

Pryer, N. K., L. J. Wuestehube, and R. Schekman. 1992. Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* 61:471-516.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55-65.

Raymond, C. K., P. J. O'Hara, G. Eichinger, J. H. Rothman, and T. H. Stevens. 1990. Molecular analysis of the yeast *VPS3* gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. *J. Cell Biol.* 111:877-892.

Raymond, C. K., I. Howald-Stevenson, C. A. Vater, and T. H. Stevens. 1992a. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell.* 3:1389-1402.

Raymond, C. K., C. J. Roberts, K. E. Moore, I. Howald, and T. H. Stevens. 1992b. Biogenesis of the vacuole in *Saccharomyces cerevisiae*. *Int. Rev. Cytol.* 139:59-120.

Redding, K., C. Holcomb, and R. S. Fuller. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 113:527-538.

Roberts, C. J., G. Pohl, J. H. Rothman, and T. H. Stevens. 1989. Structure, biosynthesis, and localization of dipeptidyl aminopeptidase B, an integral membrane glycoprotein of the yeast vacuole. *J. Cell Biol.* 108:1363-1373.

Roberts, C. J., C. K. Raymond, C. T. Yamashiro, and T. H. Stevens. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* 194:644-661.

Roberts, C. J., S. F. Nothwehr, and T. H. Stevens. 1992. Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* 119:69-83.

Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936-4948.

Rothman, J. H., C. P. Hunter, L. A. Valls, and T. H. Stevens. 1986. Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene. *Proc. Natl. Acad. Sci. USA.* 83:3248-3252.

Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara, and T. H. Stevens. 1990. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. *Cell.* 61:1063-1074.

Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527-538.

Schimmöller, F., and H. Riezman. 1993. Involvement of Ypt7p, a small GTPase, in traffic from late endosome to the vacuole in yeast. *J. Cell Sci.* 106:823-830.

Seeger, M., and G. S. Payne. 1992a. Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J. Cell Biol.* 118:531-540.

Seeger, M., and G. S. Payne. 1992b. A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2811-2818.

Shpetner, H. S., and R. B. Vallee. 1989. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell.* 59:421-432.

Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19-27.

Stevens, T. H., J. H. Rothman, G. S. Payne, and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* 102:1551-1557.

Tan, P. K., N. G. Davis, G. F. Sprague, and G. S. Payne. 1993. Clathrin facilitates the internalization of seven transmembrane segment receptors for mating pheromones in yeast. *J. Cell Biol.* 123:1707-1716.

Tsuruhara, T., J. H. Koenig, and K. Ikeda. 1990. Synchronized endocytosis in the oocyte of a temperature-sensitive mutant of *Drosophila melanogaster*. *Cell Tissue Res.* 259:199-207.

van der Blik, A. M., and E. M. Meyerowitz. 1991. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature (Lond.)* 351:411-414.

van der Blik, A. M., T. E. Redelmeier, H. Danke, E. J. Tisdale, E. M. Meyerowitz, and S. L. Schmid. 1993. Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell Biol.* 122:553-563.

Van Dyck, L., B. Purnelle, J. Skala, and A. Goffeau. 1992. An 11.4 kb DNA segment on the left arm of yeast chromosome II carries the carboxypeptidase Y sorting gene *PEP1*, as well as *ACH1*, *FUS3*, and a putative *ARS*. *Yeast.* 8:769-776.

Vater, C. A., C. K. Raymond, K. Ekena, I. Howald-Stevenson, and T. H.

- Stevens. 1992. The *VPS1* protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. *J. Cell Biol.* 119:773-786.
- Vida, T. A., T. R. Graham, and S. D. Emr. 1993. Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment. *J. Cell Biol.* 12:1245-1256.
- Wilcox, C. A., K. Redding, R. Wright, and R. S. Fuller. 1992. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell.* 3:1353-1371.
- Wilsbach, K., and G. S. Payne. 1993a. Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3049-3059.
- Wilsbach, K., and G. S. Payne. 1993b. Dynamic retention of TGN membrane proteins in *Saccharomyces cerevisiae*. *Trends Cell Biol.* 3:426-432.
- Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenon, M. L. Conolly, and R. A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell.* 37:767-778.
- Wolf, D. H., and G. R. Fink. 1975. Proteinase C (carboxypeptidase Y) mutants of yeast. *J. Bacteriol.* 123:1150-1156.