

Vps10p Transport from the *trans*-Golgi Network to the Endosome Is Mediated by Clathrin-coated Vesicles

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A native immunoprecipitation procedure has been used to investigate the role of clathrin-coated vesicles (CCVs) in the transport of vacuolar proteins between the *trans*-Golgi network (TGN) and the prevacuolar/endosome compartments in the yeast *Saccharomyces cerevisiae*. We find that Apl2p, one large subunit of the adaptor protein-1 complex, and Vps10p, the carboxypeptidase Y vacuolar protein receptor, are associated with clathrin molecules. Vps10p packaging in CCVs is reduced in *pep12Δ* and *vps34Δ*, two mutants that block Vps10p transport from the TGN to the endosome. However, Vps10p sorting is independent of Apl2p. Interestingly, a Vps10C_tΔ mutant lacking its C-terminal cytoplasmic domain, the portion of the receptor responsible for carboxypeptidase Y sorting, is also coimmunoprecipitated with clathrin. Our results suggest that CCVs mediate Vps10p transport from the TGN to the endosome independent of direct interactions between Vps10p and clathrin coats. The Vps10p C-terminal domain appears to play a principal role in retrieval of Vps10p from the prevacuolar compartment rather than in sorting from the TGN.

INTRODUCTION

The sorting and delivery of vacuolar hydrolases in the yeast *Saccharomyces cerevisiae* is similar to the transport of lysosomal proteins in mammalian cells (Klionsky and Emr, 1990). Like all proteins entering the secretory pathway, hydrolytic enzymes destined to the vacuole are first translocated from the cytoplasm into the endoplasmic reticulum lumen, and then travel to the Golgi complex by vesicular transport. At the *trans* face of the Golgi complex, vacuolar hydrolases are sorted away from the secretory pathway to be transported to the vacuole via a prevacuolar/late endosome compartment.

In mammalian cells, the mannose 6-phosphate receptor (M6PR) mediates lysosomal enzyme transport from the *trans*-Golgi network (TGN) to the endosome (for review, see Hille-Rehfeld, 1995). Clathrin has been directly linked to the process of M6PR sorting at the TGN. Assembly promoters (triskelions), consisting of three heavy (CHC) and three light

(CLC) chains, polymerize to form a polyhedral lattice creating a vesicle carrying lysosomal hydrolases with a mannose 6-phosphate residue (Schmid, 1997). The ligand–receptor complex is then transported in clathrin-coated vesicles (CCVs) to a prelysosomal/late endosome compartment. After ligand–receptor dissociation, lysosomal hydrolases are delivered to the lysosome, whereas the M6PR is recycled back to the TGN for another round of transport. Previous work showed that M6PR recruitment into CCVs at the TGN is directed by specific signals (Johnson and Kornfeld, 1992). A dileucine and a tyrosine motif are important for the efficient transport of M6PR from the TGN to the prelysosome. These sequences may represent the binding site for the Golgi-specific clathrin adaptor protein-1 (AP-1) complex (Glickman *et al.*, 1989; Honing *et al.*, 1997; Le Borgne and Hoflack, 1997; Klumperman *et al.*, 1998). Tyrosine and dileucine motifs are also present in many cell surface proteins and interact with the μ 2 subunit of the AP-2 complex to mediate internalization into endocytic CCVs (Kirchhausen *et al.*, 1997; Marks *et al.*, 1997; for review, see Bonifacino and Dell’Angelica, 1999; Heilker *et al.*, 1999). The sequence YXX \emptyset (where X is any amino acid and \emptyset is amino acid with a bulky hydrophobic group) represents the consensus signal for interaction with the μ 2 subunit. Recently, a crystal structure revealed that the sorting signal is in an

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Abbreviations used: BSA, bovine serum albumin; CCV, clathrin-coated vesicle; CPY, carboxypeptidase Y; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

extended conformation when bound, and that the tyrosine and Ø residues reside in hydrophobic pockets (Owen and Evans, 1998).

Two Golgi-to-vacuole protein pathways have been identified in *S. cerevisiae*. One pathway was discovered by the isolation of mutants that missort carboxypeptidase Y (CPY), a soluble vacuolar protein (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). This pathway traverses a prevacuolar/endosomal compartment before reaching the vacuole. An alternative pathway has been identified for alkaline phosphatase (ALP) that bypasses the endosomal compartment to reach the vacuole (Cowles *et al.*, 1997a; Piper *et al.*, 1997).

The CPY-sorting receptor was identified as the product of the *VPS10* gene (Marcusson *et al.*, 1994). Although CPY is sorted by recognition of a peptide rather than a carbohydrate signal, Vps10p function is otherwise similar to that of mammalian M6PR in providing a means of CPY delivery to the endosomal compartment. Recycling of Vps10p to the TGN is mediated by a tyrosine-based signal located in its cytoplasmic C-terminal tail. Mutation of this tyrosine leads to a rapid degradation of the receptor in the vacuole and to missorting of CPY (Cereghino *et al.*, 1995; Cooper and Stevens, 1996).

Clathrin probably participates at some point in this sorting because a brief restrictive incubation of a temperature-sensitive mutant (*chc1-521*) results in rapid secretion of CPY (Seeger and Payne, 1992a). However, a backup process has been proposed to compensate the clathrin defect because a longer restrictive incubation of this mutant or the use of other clathrin mutants restores a normal rate of pro-CPY maturation. The role of the AP complexes is less clear. Three distinct AP complexes have been identified in yeast. Each complex contains two large chains (Apl), one medium chain (Apm) and one small chain (Aps). Null alleles of the different AP-1 subunit genes (*APL2*, *APL4*, *APM4*, and *APS1*) display genetic interactions with clathrin missense mutations, consistent with a role for the AP-1-related subunits in some clathrin-dependent pathway(s) (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995; Yeung *et al.*, 1999). The two other complexes, AP-2R and AP-3, do not interact with clathrin (Yeung *et al.*, 1999). AP-2R displays the highest protein sequence similarity to mammalian AP-2. Although the mammalian AP-2 associates with endocytic CCVs, yeast AP-2R mutants are not defective in endocytosis. Thus, the role of yeast AP-2R remains uncertain. AP3 is required for transport of ALP from the TGN to the vacuole and is clathrin-independent (Cowles *et al.*, 1997b; Vowels and Payne, 1998). Surprisingly, disruptions of the four AP-1 subunits, or subunit disruptions that result in cells with no functional heterotetrameric AP complexes, do not affect clathrin formation and protein transport (Huang *et al.*, 1999; Yeung *et al.*, 1999). Thus, either clathrin alone is sufficient to execute roles in coat assembly and cargo selection or other proteins in CCVs are functionally redundant with the APs.

In this study we have developed a procedure to immunoprecipitate CCVs to determine the membrane proteins associated with clathrin molecules. We found that CCVs transport wild-type and sorting-deficient mutant Vps10p from the TGN to the endosome. This suggests that cargo proteins do

not require a specific sorting signal in their cytoplasmic domain to be packaged into CCVs from the TGN.

MATERIALS AND METHODS

Strains, Growth Conditions, and Reagents

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively, and their construction is described below. Yeast cells were grown in synthetic or rich media (Sherman, 1991) at 30°C, or at 24°C as indicated. DNA manipulations in *Escherichia coli* were performed as described (Ausubel *et al.*, 1987–1995). Yeast transformation was accomplished by using standard methods (Ausubel *et al.*, 1987–1995).

Enzymes for the manipulation of DNA were purchased from New England Biolabs (Beverly, MA) or Boehringer-Mannheim Biochemicals (Indianapolis, IN). A plasmid containing *KEX2HA* (Nothwehr *et al.*, 1995) was a gift from S.T. Nothwehr (University of Missouri, Columbia, MO). Monoclonal Chc1p antibody has been published (Lemmon *et al.*, 1988) and antisera against Apl2p, Apl1p, Vps10p, and CPY have been described (Feldheim *et al.*, 1993; Cooper and Stevens, 1996; Yeung *et al.*, 1999). Mouse12CA5 anti-hemagglutinin (HA) antibodies were purchased from Berkeley Antibody (Richmond, CA). Donkey anti-rabbit and sheep anti-mouse secondary antibodies coupled to horseradish peroxidase were obtained from Amersham (Arlington Heights, IL). Zymolyase 100T was obtained from United States Biological (Swampscott, MA). [³⁵S]Promix was purchased from Amersham. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated.

Strains and Plasmid Construction

The pGEX-2TCLC1GST plasmid was constructed by using one oligonucleotide primer containing a *Bam*HI site and a second primer containing an *Eco*RI site corresponding to the upstream and the downstream region of the *CLC1* open reading frame (ORF), respectively. The *CLC1* gene was amplified by PCR and fused into the *Bam*HI and *Eco*RI sites of pGEX-2T containing a glutathione S-transferase (GST) gene. The *CLC1* (ORF/697 bp) PCR product containing a C-terminal 6 HIS encoding sequence was cloned into *Eco*RI and *Xba*I sites of the pBAD24 vector (Guzman *et al.*, 1995), yielding the pBAD24CLC1HIS₆ plasmid. The influenza HA epitope-tagged *VPS10* allele was constructed by first inserting a *Not*I site by PCR just upstream of the *VPS10* ORF stop codon in the pRS315VPS10 plasmid (Piper *et al.*, 1995). A *Not*I fragment containing three copies of the HA epitope (YPYDVPDYA) digested from pGTEP1 plasmid was then ligated into the *VPS10* gene containing a *Not*I site, resulting in plasmid pRS315VPS10HA. The *VPS10HA* fragment from pRS315VPS10HA was subcloned into the *Sac*I and *Sal*I sites of the pRS306 vector. pRS426*ups10G1423stop-HA* was constructed as described for pRS306VPS10HA by inserting three copies of the HA epitope followed by two stop codons just upstream of the glycine 1423 residue of the open reading frame of the *VPS10* gene.

We constructed strains containing a single copy of *KEX2-HA* or *VPS10-HA*. For the integration of *KEX2-HA*, we digested pRS306KEX2-HA with *Bgl*III (which cuts in *KEX2*) and inserted it into EHY202 and LSY93-2A by transformation, resulting in ODY117 and ODY47, respectively. For the integration of *VPS10-HA*, we digested pRS306VPS10-HA with *Afl*II (which cuts in *VPS10*) and introduced it into LSY93-2A, EHY202, LSY93-5B, GPY1783-10A, RSY1306, and PHY102 by transformation, resulting in ODY50, ODY129, ODY55, ODY62, ODY63, and ODY54, respectively.

Antibody Generation

The pGEX-2TGST, pGEX-2TGSTCLC1, and pBAD24CLC1HIS₆ plasmids were propagated by transformation into BL21 *E. coli* cells. Expression of the Clc1p-HIS₆ protein was induced with 0.1% arabinose and purified by using a nickel-nitrilotetraacetic acid agarose

Table 1. Yeast strains used in this study

Strain	Genotype	Source
EHY191	<i>MATa pep4::TRP1 TPI::SUC2::HIS ade2-1 his3-11, 15 leu2-3, 112 trp1-Δ1 ura3-1</i>	E. Harsay, unpublished
LSY93-2A	<i>MATa leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 CLC1</i>	Silveira <i>et al.</i> , 1990
LSY93-5B	<i>MATa leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 clc1::HIS3</i>	Silveira <i>et al.</i> , 1990
EHY202	<i>MATα chc1-521(ts) ura3-52</i>	E. Harsay, unpublished
GPY409	<i>MATa leu2-3, 112 ura3-52 his4 and/or his6 trp1-289 chc1-521(ts) sst1-3 pep::LEU2</i>	Rad <i>et al.</i> , 1995
PHY102	<i>MATa suc2-Δ9 ura3-52 leu2-3 his3-Δ200 trp1Δ911 lys 2-801 vps34Δ::TRP1</i>	Herman and Emr, 1990
GPY1783-10A	<i>MATα ura3-52 leu2-3 his3-200 trp1-901 lys2-801 suc1-9 GAL mel apl2::TRP1</i>	Yeung <i>et al.</i> , 1999
RSY1306	<i>MATα ura3-52 leu2,3-113 his3-Δ200 trp1-Δ901 lys2-801 pep12Δ::HIS3</i>	Becherer <i>et al.</i> , 1996
CCV120	<i>MATα vps45Δ2::HIS3 his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9</i>	Burd <i>et al.</i> , 1997
EHY62	<i>MATα sec6-4 vps10Δ::TRP1 his3-Δ200 leu2-3, 112 lys2-801 trp1-1 ura3-52</i>	E. Harsay, unpublished
ODY117	<i>EHY202::KEX2HA::URA3</i>	This study
ODY47	<i>LSY93-2A::KEX2HA::URA3</i>	This study
ODY50	<i>LSY93-2A::PEP1HA::URA3</i>	This study
ODY129	<i>EHY202::VPS10HA::URA3</i>	This study
ODY56	<i>LSY93-5B::VPS10 HA::URA3</i>	This study
ODY62	<i>GPY1783-10A::VPS10 HA::URA3</i>	This study
ODY63	<i>RSY1306::VPS10 HA::URA3</i>	This study
ODY54	<i>PHY102::VPS10 HA::URA3</i>	This study
ODY80	<i>LSY93-2A/pRS426vps10G1423stop-HA</i>	This study
ODY112	<i>CCY120/pRS426vps10G1423stop-HA</i>	This study
ODY114	<i>RSY1306/pRS426vps10G1423stop-HA</i>	This study
ODY140	<i>GPY1783-10A/pRS426vps10G1423stop-HA</i>	This study
ODY141	<i>EHY62/pRS426vps10G1423stop-HA</i>	This study
ODY142	<i>PHY102/pRS426vps10G1423stop-HA</i>	This study
ODY143	<i>EHY191/pRS426vps10G1423stop-HA</i>	This study
ODY144	<i>GPY409/pRS426vps10G1423stop-HA</i>	This study

column. Expression of the GST and Clc1p-GST proteins was induced by isopropyl β -D-thiogalactopyranoside and then purified by using glutathione-agarose chromatography. The purified GST and Clc1p-GST fusion proteins were injected into rabbits (Northview Pacific Laboratories, Berkeley, CA). GST and Clc1p rabbit antibodies were affinity-purified on GST and Clc1p proteins covalently attached to ReactiGel (6X) carboxymethylated-activated agarose (Pierce Chemical, Rockford, IL), respectively, as described (Chuang and Schekman, 1996).

Native Immunoprecipitation

Cells were grown in YPD at 24°C to an OD₆₀₀ < 1. Forty OD₆₀₀ units of cells were sedimented by centrifugation and washed once with 20 mM cold Na₂S₂O₃. Cells were resuspended and incubated in 0.8 ml (50 OD₆₀₀/ml) of 0.1 M Tris-HCl, pH 9.4, 10 mM dithiothreitol (DTT), 20 mM Na₂S₂O₃ for 10 min at room temperature. Cells were then con-

verted to spheroplasts by treatment with Zymolyase T100 (7.5 μ g/OD) in 0.8 ml of spheroplast buffer (10 mM Tris-HCl, pH 7.5, 0.8 M sorbitol, 20 mM Na₂S₂O₃, 1 mM DTT) for 15 min at 30°C. Spheroplasts were sedimented by centrifugation for 2 min at 3000 \times g and washed once with spheroplast buffer and once with 2-(N-morpholino)ethanesulfonic acid (MES)/0.8 M sorbitol buffer (100 mM MES, pH 6.5, 0.8 M sorbitol, 0.5 mM MgCl₂, 1 mM EGTA, 0.2 mM DTT) at 4°C. Spheroplasts were then hypotonically lysed in 0.4 ml (100 OD₆₀₀/ml) of MES/0.2 M sorbitol buffer (100 mM MES, pH 6.5, 0.2 M sorbitol, 0.5 mM MgCl₂, 1 mM EGTA, 0.2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, pepstatin A [1 μ g/ml], and leupeptin [1 μ g/ml]) at 4°C and homogenized in a glass Dounce homogenizer (1 ml) by using a tight-fitting pestle. The yeast lysate was centrifuged for 30 min, 4°C at 21,000 \times g. The pellet was discarded and the supernatant centrifuged for 60 min at 4°C at 100,000 \times g in a TLS 55 rotor (Beckman Instruments, Palo Alto, CA)

Table 2. Plasmids used in this study

Plasmid	Description	Source
pGEX-2TGSTCLC1	<i>CLC1</i> (ORF) was fused to <i>GST</i> gene of pGEX-2T vector	This study
pBAD24CLC1HIS ₆	<i>CLC1</i> (ORF) containing 6 HIS at the C terminus was cloned into the pBAD24 vector	This study
pRS306KEX2HA	pRS306 containing <i>KEX2</i> gene with three copies of the HA epitope inserted at the C terminus of the ORF	Nothwehr <i>et al.</i> , 1995
pRS306VPS10HA	pRS306 containing <i>VPS10</i> gene with three copies of the HA epitope inserted at the C terminus of the ORF	This study
pRS426vps10HAG1423stop	pRS426 containing <i>VPS10</i> gene with three copies of the HA epitope followed by two stop codons inserted at the codon 1423 of the ORF	This study
pGTEP1	pBluescript II SK (+/-) containing three HA copies	RS strain collection

onto a 200- μ l cushion of 80% Percoll. The supernatant was discarded and the material layered on the Percoll pellet was resuspended in 500 μ l of MES/0.2 M sorbitol buffer and divided into two Eppendorf tubes. Both preparations were diluted to 1 ml with MES/0.2 M sorbitol buffer containing 1% bovine serum albumin (BSA) and incubated with 1.5 μ g of affinity-purified Clc1p or GST antibodies, respectively, for 2 h or overnight at 4°C. The immunoprecipitated complexes were isolated by addition of 30 μ l of protein A-Sepharose (20% in MES/0.2 M sorbitol buffer containing 1% BSA) and incubated for an additional hour at 4°C. Nonadsorbed materials were removed by centrifuging and the immunoabsorbate-bound protein A-Sepharose was resuspended and washed once in 1 ml of MES/0.2 M sorbitol buffer containing 0.1% BSA and three times in 1 ml of MES/0.2 M sorbitol buffer without BSA. The immunoabsorbates were eluted in 20 μ l of Laemmli buffer (Laemmli, 1970) and then heated at 95°C for 5 min. Protein A-Sepharose was removed by centrifugation and the supernatant fractions were resolved on 8% SDS-PAGE gels. Proteins were visualized by immunoblot by using the ECL+ kit (Amersham Pharmacia Biotech, Piscataway, NJ). Protein quantitation was performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) according to manufacturer's recommendations.

Radiolabeling, Immunoprecipitation, and Immunoblot Analysis

Vps10p, CPY, and HA-tagged proteins were immunoprecipitated under denaturing conditions from radiolabeled extracts by using a procedure described previously (Nothwehr *et al.*, 1995; Bryant *et al.*, 1998), with the appropriate polyclonal or monoclonal antibodies.

Enzyme Assays

GDPase assays were performed essentially as described (Abeijon *et al.*, 1989; Yanagisawa *et al.*, 1990). A portion (0.00125% of total) of the high-speed membrane pellet (HSP) fraction and all the anti-GST and anti-Clc1p immunoabsorbates were added to 100 μ l of reaction buffer (20 mM imidazole-HCl, pH 7.4, 2 mM CaCl₂, 0.1% Triton X-100, and 9 mM GDP). Reactions were incubated at 30°C for 15 min and stopped by adding 150 μ l of 2% SDS. GDPase activity was measured based on absorbance at 820 nm.

Invertase activity was assayed by the method of Goldstein and Lampen (1975). An aliquot (0.05% of total) of the HSP fraction and all of the anti-GST and anti-Clc1p immunoabsorbates were added to the enzyme reaction buffer containing 0.1% Triton X-100. Invertase activity was measured based on absorbance at 540 nm.

Blanks were measured in reactions conducted without membrane fractions. Error bars were calculated from two independent experiments

RESULTS

Clathrin Associates with Apl2p in a Membrane Fraction

To isolate yeast CCVs, we developed a procedure with anti-clathrin antibodies for the affinity separation of CCVs from a cell extract. Previously, affinity-purified anti-Clc1p antibody was successfully used to immunoprecipitate the intact clathrin trimeric complex (Pishvaei *et al.*, 1997). We reasoned that a similar approach would allow us to immunoprecipitate intact coated-vesicles from a crude, slowly sedimenting membrane fraction. Whole-cell extracts were prepared by gentle osmotic lysis followed by a differential centrifugation to obtain a HSP fraction. A native immunoprecipitation was then performed by using the HSP fraction combined with affinity-purified antibodies against Clc1p followed by protein A-Sepharose. The resulting immunopre-

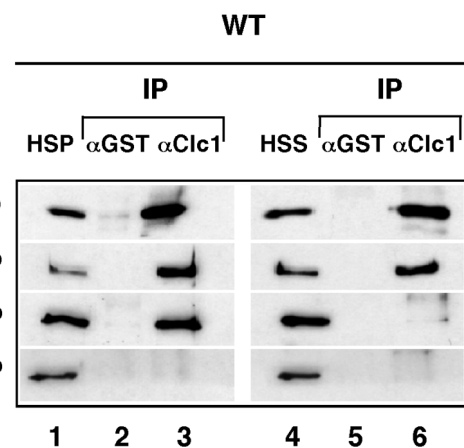


Figure 1. Clathrin interacts with Apl2p. Native immunoprecipitations were performed from a lysate of strain LSY93-2A by using affinity-purified Clc1p and GST antibodies. Lanes 1 and 4 correspond to 1/20 of the HSP and HSS fractions used, respectively, for the immunoprecipitations. Lanes 2 and 3 correspond to the immunoprecipitates of an HSP fraction by using GST and Clc1p antibodies, respectively. Lanes 5 and 6 correspond to the immunoprecipitates from an HSS fraction by using GST and Clc1p antibodies, respectively. The HSP/HSS fractions and the immunoprecipitates were subjected to SDS-PAGE and transferred to a nitrocellulose filter. The nitrocellulose was cut into strips and immunoblotted with Clc1p, Chc1p, Apl2p, and Apl1p antibodies.

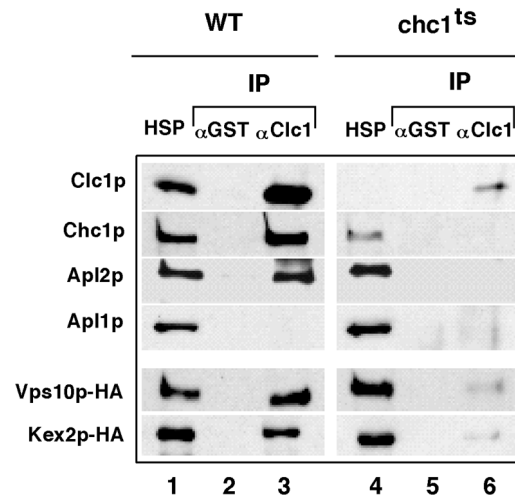
cipitate was analyzed by SDS-PAGE and immunoblotted. Because the AP-1 complex has been shown to interact with clathrin, we first evaluated coimmunoprecipitation of Apl2p, the large subunit of AP-1. We found that 60% of Chc1p and 6% of Apl2p of the total HSP fraction were coprecipitated with Clc1p (Figure 1 and Table 3). As a control, no coprecipitation (<0.5%) was observed when antibodies against GST were used. In contrast, Apl1p, the large subunit of AP-2 was not detected. This result is in good agreement with previous work showing that of the three AP

Table 3. Protein enrichments by immunoprecipitations

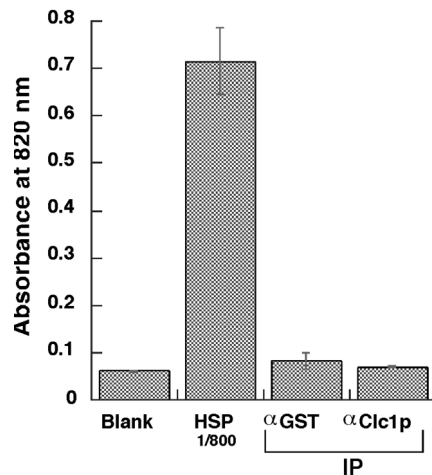
	Protein enrichments (%)			
	WT (%)		<i>chc1</i> (%)	
	α GST	α Clc1p	α GST	α Clc1p
Clc1p	<0.5	>60	ND	ND
Chc1p	<0.5	60	ND	ND
Apl1p	<0.5	<0.5	<0.5	<0.5
Apl2p	<0.5	6	<0.5	<0.5
Vps10p-HA	<0.5	6	<0.5	1.5

Each value represents protein immunoprecipitation with α GST and α Clc1p antibodies, respectively, in percentage of the total HSP fraction with a standard error of \sim 20%. Quantification was performed from three independent immunoprecipitation experiments and determined by using Phosphorimager (Molecular Dynamics). ND, not determined.

A



B. GDPase activity



C. Invertase activity

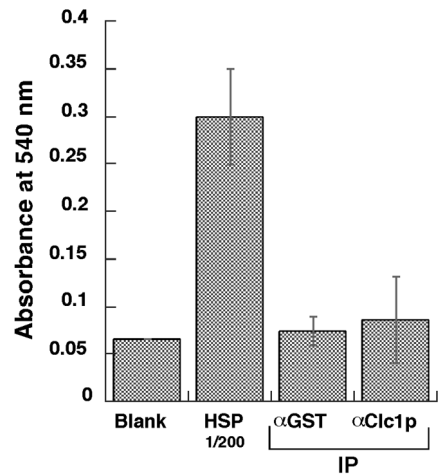


Figure 2. Detection of Vps10p-HA by coimmunoprecipitation with clathrin antibodies. Native immunoprecipitations were performed as in Figure 1. (A) Lanes 1–3 correspond to native immunoprecipitation from WT/ODY50. Lanes 4–6 correspond to native immunoprecipitation from *chc1ts*/ODY129. Native immunoprecipitations for Kex2p-HA enrichment were performed from WT/ODY47 (lane 1–3) and from *chc1ts*/ODY117 (lane 4–6). Kex2p-HA and Vps10p-HA were detected by using monoclonal 12CA5 anti-HA antibody. GDPase (B) and invertase (C) activities were performed on HSP and immunoprecipitation fractions from WT/EHY191.

complexes, only AP-1 interacts with clathrin (Yeung *et al.*, 1999). Clathrin and AP are soluble proteins that are recruited on membrane for the formation of vesicles. Thus, we tested whether Alp2p could be coprecipitated with clathrin from the high-speed soluble (HSS) fraction. As expected, Chc1p was coprecipitated with Clc1p because clathrin is present as soluble triskelions in the cytosol (Pishvaea *et al.*, 1997). In contrast, Apl2p was not detected, suggesting that interaction between clathrin and Apl2p requires additional components from the membrane fraction. It is possible that lipids or/and membrane proteins induce a conformational change in AP-1 to increase its affinity for clathrin.

Vps10p Is Coimmunoprecipitated with Clathrin

To determine whether our immunoisolation provides intact CCVs, we explored the enrichment of specific cargo proteins associated with clathrin. First, we evaluated Kex2p because this Golgi membrane protein, which is required for the maturation of α -factor precursor, is mislocalized to the cell surface in clathrin mutant strains and coelutes with clathrin in CCVs resolved by gel filtration (Payne and Schekman, 1989; Chu *et al.*, 1996). We performed a similar native immunoprecipitation experiment as described in Figure 1. To improve the detection of Kex2p, we used a construct con-

taining a functional version of Kex2p tagged with the influenza HA epitope integrated at the chromosomal *KEX2* locus. We found that Kex2p-HA was coimmunoprecipitated with Clc1p, Chc1p, and Apl2p (Figure 2A).

In the next experiment, we examined the role of clathrin in the sorting of vacuolar proteins. Prior work showed that a rapid inactivation of clathrin results in a severe defect in the sorting of the vacuolar soluble protein CPY (Seeger and Payne, 1992a). The precursor form of CPY (p2CPY) interacts with Vps10p in the Golgi complex and is believed to be transported to the endosome by CCVs (see INTRODUCTION). We examined the association of Vps10p with clathrin and found that 6% of a functional HA-tagged Vps10p was coimmunoprecipitated (Figure 2A and Table 3). To further demonstrate the specificity of cargo detection, we used a *chc1-521* mutant in which the stability of the clathrin trimeric complex is decreased (Pishvaei *et al.*, 1997). We found that clathrin remains cytosolic (no Clc1p in HSP; Figure 2A, lane 4) in a *chc1-521* cell lysate prepared from cells grown at a permissive temperature, conditions where Kex2p and Vps10p trafficking is not affected. In the absence of clathrin in the HSP fraction, Apl2p (<0.5%) was not detected and very little contamination of Kex2p-HA and Vps10p-HA (<1.5%) was observed (Figure 2A and Table 3), confirming a specific protein enrichment.

These results do not exclude the possibility that Kex2-HA and Vps10p-HA are part of clathrin-coated TGN membranes in our native immunoprecipitation. Therefore, we examined the precipitation of proteins that are excluded from CCVs. We used GDPase and invertase as Golgi and secreted protein markers, respectively. Figure 2, B and C, show that no GDPase and invertase activities above background were coimmunoprecipitated with clathrin antibodies, whereas a significant level of both activities was detected in the HSP fraction. Together these results provide reasonable evidence that transport of Kex2p and Vps10p is mediated by CCVs.

Vps10p Is Degraded in the *chc1* Mutant

Previous studies showed that Kex2p is rapidly degraded in a *chc1* mutant (Redding *et al.*, 1996). We examined the stability of Vps10p in a *chc1* mutant by a pulse-chase experiment. Vps10p is an extremely stable protein and no degradation was observed after a chase of 90 min at 37°C in wild-type (WT) cells (Cereghino *et al.*, 1995; Cooper and Stevens, 1996) (Figure 3). However, in the *chc1* mutant, Vps10p was slowly degraded (Figure 3). Degradation was blocked in a protease-deficient *pep4* mutant strain (Figure 3A, lane 9). A similar result was obtained with a *clc1*Δ strain (Figure 4A). Clearly, Kex2p and Vps10p are degraded at distinct rates in the clathrin mutant, possibly reflecting the fact that Vps10p continues to sort CPY by a clathrin-independent process.

Coimmunoprecipitation of Vps10p Is Reduced in *pep12*Δ, and *vps34*Δ, but not in *apl2*Δ Mutants

To further characterize Vps10p transport by CCVs, we examined mutants that affect clathrin or protein transport from the TGN to the endosome. However, to be able to assess coimmunoprecipitation of Vps10p with clathrin, we restricted our analysis to mutants in which Vps10p was not degraded ($t_{1/2} < 1$ h) and was retained in an HSP sediment-

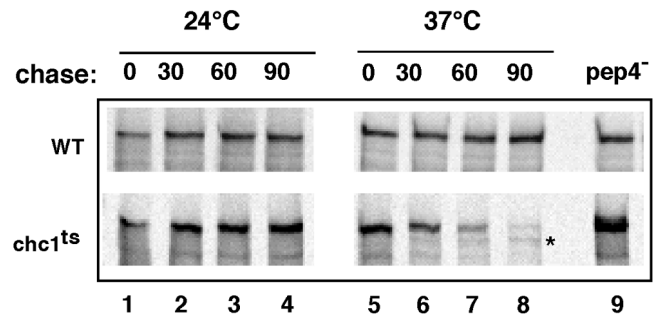


Figure 3. Vps10p is degraded in *chc1ts* cells. WT/LSY93-2A and *chc1ts*/EHY202 strains were radiolabeled for 10 min with [³⁵S]methionine and cysteine directly at 24°C (lanes 1–4) or were incubated for 45 min (lanes 5–9) at 37°C before labeling at 37°C. After the indicated chase times, aliquots of cells were removed and subjected to immunoprecipitation with antibodies to Vps10p. Lane 9 corresponds to Vps10p immunoprecipitation after a 90-min chase at 37°C in a *pep4* background (WT/EHY191 and *chc1ts*/GPY409). The PEP4-dependent cleavage product is indicated (*). Samples were analyzed by SDS-PAGE and autoradiography.

able fraction as in wild-type cells. Apl2p interacts with clathrin, but disruption of *APL2* displays no discernable phenotypes (Rad *et al.*, 1995). In agreement with these results, a pulse-chase analysis showed that Vps10p stability and CPY maturation were not affected in the *apl2*Δ mutant (Figure 4).

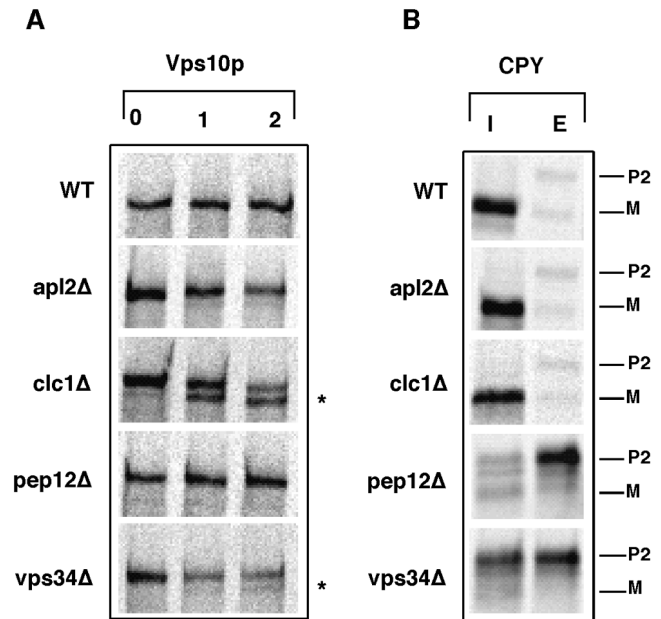


Figure 4. Vps10p stability and CPY maturation in WT, *apl2*Δ, *clc1*Δ, *pep12*Δ, and *vps34*Δ strains. WT/LSY93-2A, *apl2*Δ/GPY1783-10A, *clc1*Δ/LSY93-5B, *pep12*Δ/RSY1306, and *vps34*Δ/PHY102 strains were radiolabeled for 10 min with [³⁵S]methionine and cysteine directly at 30°C. After a chase for the indicated times (0, 1, and 2 h), aliquots of cells were removed and subjected to immunoprecipitation with antibodies to Vps10p (A) or CPY (B). The PEP4-dependent cleavage product is indicated (*).

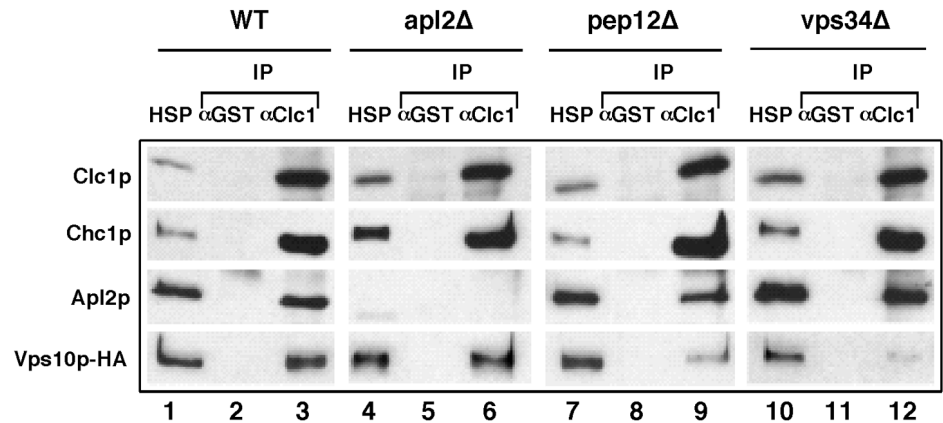


Figure 5. Coimmunoprecipitation of Vps10p-HA with clathrin is reduced in *pep12Δ* and *vps34Δ* but not in *apl2Δ* mutants. Native immunoprecipitations were performed from ODY50 (WT/lane 1–3), ODY62 (*apl2Δ*/lane 4–6), ODY63 (*pep12Δ*/lane 7–9), and ODY54 (*vps34Δ*/lane 10–12) as described in Figure 1.

Pep12p is a t-SNARE component required for the fusion of vesicles with the endosome (Becherer *et al.*, 1996; Burd *et al.*, 1997). Vps10p was stable in *pep12Δ* cells (Figure 4A), most likely because it accumulated in proteolytically inactive vesicles and was protected from degradation, as proposed for other mutants (*vps45* and *vps21*) that also block vesicle fusion with endosome (Bryant *et al.*, 1998; Gerrard *et al.*, 2000). As a result, >90% of CPY is then secreted because Vps10p cannot be recycled back to the TGN for another round of transport of CPY (Figure 4B). A similar result was obtained with *vps34Δ*. Vps34p is a phosphoinositide 3-kinase required for protein trafficking (Schu *et al.*, 1993; Stack and Emr, 1994; Stack *et al.*, 1995). Mutations in *VPS34* block protein transport from the TGN to the endosome. As a consequence, Vps10p fails to return to the TGN, resulting in secretion of CPY (Figure 4B; Herman and Emr, 1990). We found that Vps10p was degraded to some extent (Figure 4A), but still accumulated in an HSP fraction.

We next performed immunoprecipitations as described in Figure 1 from WT, *apl2Δ*, *pep12Δ*, and *vps34Δ* cells. Interestingly, in *apl2Δ*, no decrease of clathrin (Chc1p and Clc1p) in the HSP was observed (Figure 5), indicating that the AP-1 complex is not essential for the recruitment of clathrin onto the membrane. Furthermore, Vps10p-HA was detected with WT efficiency. Thus, Apl2p is totally dispensable for Vps10p transport. In contrast, in *pep12Δ* and *vps34Δ* cells in which CPY sorting from the TGN is blocked, Vps10p-HA enrichment was clearly reduced (Figure 5). This result can be explained by the formation of new CCVs that are depleted of Vps10p because of deficient receptor recycling to the TGN.

Vps10p Does Not Interact with Clathrin via its C-Terminal Sorting Domain

Early studies showed that Vps10p contains a retrieval signal in the C-terminal cytoplasmic domain that is essential for its recycling from the endosome to the TGN (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). A Vps10p mutant lacking the C-terminal cytoplasmic domain (Vps10C_tΔp) is rapidly degraded in the vacuole with a half-time of ~25 min in WT cells. The degradation of Vps10C_tΔp is blocked in *vps45Δ*, an observation that has been explained by entrapment of Vps10C_tΔp inside proteolytically inactive vesicles that are unable to fuse with a post-Golgi/endosome compartment

(Bryant *et al.*, 1998). We examined the fate of Vps10C_tΔp in *pep12Δ* and *vps34Δ*, by using a construct of Vps10p containing an HA epitope in place of its C-terminal cytoplasmic tail (Vps10C_tΔp-HA). From a pulse-chase analysis, we found that Vps10C_tΔp-HA was processed with a $t_{1/2} < 30$ min in WT cells; proteolysis was blocked in *pep12Δ*, *vps45Δ*, and *vps34Δ* mutants (Figure 6). This result supports a model in which both Vps10p-HA and Vps10C_tΔp-HA are transported in the same Golgi-derived vesicles that require Pep12p, Vps45p, and Vps34p to reach the endosome. We next examined whether Vps10C_tΔp-HA was contained in CCVs. To compensate for the lack of recycling of Vps10C_tΔp-HA, we overproduced the protein by using a 2- μ plasmid (pRS426 vector). A degradation product of Vps10C_tΔp-HA was de-

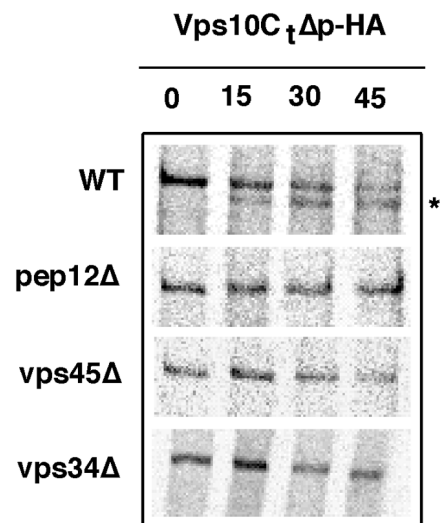


Figure 6. *pep12Δ*, *vps45Δ*, and *vps34Δ* mutants block Vps10C_tΔp-HA degradation. WT/ODY80, *pep12Δ*/ODY114, *vps45Δ*/ODY112, and *vps34Δ*/ODY142 strains containing plasmid encoding Vps10C_tΔp-HA were subjected to pulse-chase immunoprecipitation and analyzed as described in Figure 4. Except that monoclonal 12CA5 anti-HA antibody was used to immunoprecipitate Vps10C_tΔp-HA from the cell extracts. The PEP4-dependent cleavage product is indicated (*).

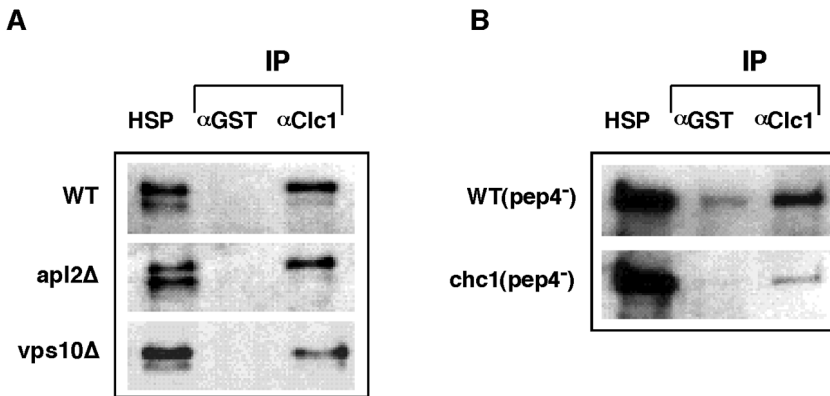


Figure 7. Detection of Vps10CtΔp-HA by coimmunoprecipitation with clathrin antibodies. Native immunoprecipitations were performed as in Figure 1. (A) pRS426*vps10G1423stop-HA* plasmid was introduced into WT (ODY80), *apl2Δ* (ODY140), and *vps10Δ* (ODY141) strains. (B) pRS426*vps10G1423stop-HA* was introduced into WT (ODY143) and *chc1ts* (ODY144) strains. Vps10CtΔp-HA was detected by using monoclonal 12CA5 anti-HA antibody.

tected in the HSP (Figure 7A). However, only the full-length Vps10C_iΔp-HA was coimmunoprecipitated with clathrin. A similar result was obtained by using *pep4* cells in which Vps10C_iΔp-HA is stable (WT, Figure 7B). Coprecipitation was dependent on functional clathrin because little Vps10C_iΔp-HA was detected in Clc1p precipitates from *pep4* cells lacking clathrin heavy chain (*chc1*, Figure 7B). Vps10C_iΔp-HA association with clathrin was not affected in the *apl2Δ* mutant. Detection of plasmid-encoded Vps10C_iΔp-HA in CCVs isolated from cells missing the chromosomal *VPS10* locus (*vps10*, Figure 7A) rules out the possibility that Vps10C_iΔp-HA is recruited into CCVs by interaction with endogenous full-length Vps10p. Together, these data indicate that CCVs mediate anterograde transport of proteins from the TGN to the endosome and that the cytoplasmic C-terminal tail of Vps10p is not essential for its recruitment into CCVs.

DISCUSSION

Clathrin-coated vesicles are required for sorting and retention of proteins in a late-Golgi compartment in yeast. In clathrin mutants, Golgi membrane proteins such as the dipeptidyl-aminopeptidase A (DPAP A) and Kex2p, two endoproteases required for the maturation of α -factor precursor, are mislocalized to the cell surface (Seeger and Payne, 1992b). These results suggested that Golgi proteins may accompany anterograde cargo proteins in CCVs leaving a late Golgi membrane, and then be efficiently recycled from the endosome by virtue of a retrieval mechanism. Thus, inactivation of clathrin would block Golgi-membrane proteins from reaching the endosome and thereby prevent their retrieval to the TGN. The sorting receptor Vps10p, which also contains a retrieval sorting signal, cycles between the TGN and the endosome in a manner similar to Kex2p and DPAP A to mediate the anterograde transport of CPY (Conibear and Stevens, 1998). Our study provides direct evidence that CCVs transport both Kex2p and Vps10p. Furthermore, Vps10p sorting into CCVs is independent of sorting signals in the cytoplasmic domain.

The observation that Vps10p is associated with clathrin by coimmunoprecipitation correlates with previous results showing that a rapid inactivation of temperature sensitive clathrin results in mislocalization of CPY (Seeger

and Payne, 1992a). Both Kex2p and Vps10p contain a tyrosine-sorting motif in their C-terminal domains, which allows them to be recycled back to the TGN. Substitution of this motif or deletion of the entire domain leads to rapid degradation of these proteins in the vacuole. Kex2p and Vps10p C-terminal mutant proteins have been shown to transit through the endosome to reach the vacuole for degradation (Brickner and Fuller, 1997; Bryant and Stevens, 1997). In this regard, *vps45Δ*, *pep12Δ*, and *vps34Δ* mutants that affect the anterograde transport of Vps10p to the endosome also block the transport of Vps10C_iΔp. This suggests that both Vps10p and Vps10C_iΔp are transported by the same population of vesicles. Because we have been able to coimmunoprecipitate Vps10C_iΔp-HA with clathrin, we propose that CCVs represent this population of vesicle and therefore, mediate the anterograde transport of proteins from the TGN to the endosome. Support for this view comes from the observation that clathrin genetically interacts with *vps1*, a dynamin that is believed to be required for the formation of vesicles at the TGN, and *pep12*, a t-SNARE involved in the fusion of post-Golgi vesicles with the endosome (Bensen *et al.*, 2000). Consistently, we failed to coimmunoprecipitate Vps10p with clathrin antibodies in these two mutants (Figure 5; our unpublished results). Although we cannot exclude the possibility that other types of vesicle mediate transport from the TGN to the endosome, the observation that Golgi membrane proteins such as Kex2p and DPAP A are rerouted to the cell surface in clathrin mutants affirms this view (Seeger and Payne, 1992b; Redding *et al.*, 1996). In this regard, we have observed that both Vps10p and Vps10C_iΔp are rerouted to the cell surface in a *chc1* mutant (unpublished data).

The cytoplasmic domain of membrane cargo proteins may serve directly to facilitate the recruitment of coat proteins. Previous studies showed that a specific recognition of tyrosine or dileucine motifs by APs is essential for the packaging of cargo proteins into CCVs in mammalian cells (Kirchhausen *et al.*, 1997; Marks *et al.*, 1997; for review, see Bonifacino and Dell'Angelica, 1999; Heilker *et al.*, 1999). Cargo recruitment into yeast CCVs does not depend solely on any of the three identified AP complexes (Huang *et al.*, 1999; Yeung *et al.*, 1999). Mutants containing multiple deletions of APs did not display any phenotypes associated with clathrin deficiency, including slowed growth and defects in

clathrin-dependent protein sorting in the endocytic or biosynthetic pathways. These results are extended by our data showing that coimmunoprecipitation of Vps10p and Kex2p (our unpublished results) with clathrin is not decreased in the absence of Apl2p. Thus, an alternative mechanism in which distinct clathrin-binding proteins or clathrin itself can substitute for AP function is possible. Indeed, accessory proteins provide alternative strategies for cargo selection (for review, see Jarousse and Kelly, 2000).

The efficient incorporation of Vps10C₁Δp-HA in CCVs rules out the possibility that the cytoplasmic tail of Vps10p has a prominent role in clathrin coat formation and for its recruitment in CCVs. Other domains of Vps10p may interact indirectly with clathrin-associated proteins. However the principle sorting step determining the fate of TGN proteins may be in their selective retrieval from the endosome. Kex2p, which is transported efficiently from the TGN in CCVs, must be actively recycled from the endosome to maintain its steady-state location in the Golgi (Redding *et al.* 1996; Brickner and Fuller, 1997). The anterograde limb of this cycling pathway may be less cargo-selective than the retrieval event. For example, overexpression of Golgi membrane proteins does not saturate transport from the TGN to the endosome, whereas the retrieval pathway that is signal-directed is saturated (Roberts *et al.*, 1992; Wilcox *et al.*, 1992). In addition, Vam3p and ALP, two proteins that require a specific sorting signal to transit through the AP-3-dependent pathway to the vacuole, are rerouted through the endosome in AP-3-deficient strains (Cowles *et al.* 1997a; Stepp *et al.*, 1997; Darsow *et al.*, 1998). Thus, clathrin may provide a default pathway for the transport of certain mutant or heterologous proteins to the vacuole in *S. cerevisiae*.

How membrane proteins are recruited into vesicles without reference to a cytoplasmic signal remains to be elucidated. Perhaps the membrane anchor domains and a special phospholipid environment are essential to form CCVs from the yeast *trans*-Golgi organelle. Recently, Chen *et al.* (1999) have reported that the integral membrane P-type ATPase (DRS2), a potential aminophospholipid translocase, is required for clathrin function at the late Golgi. Drsp2 may create a special bilayer environment into which protein substrates for clathrin vesicles may partition.

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