

# Retrograde Traffic Out of the Yeast Vacuole to the TGN Occurs via the Prevacuolar/Endosomal Compartment

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**Abstract.** A large number of trafficking steps occur between the last compartment of the Golgi apparatus (TGN) and the vacuole of the yeast *Saccharomyces cerevisiae*. To date, two intracellular routes from the TGN to the vacuole have been identified. Carboxypeptidase Y (CPY) travels through a prevacuolar/endosomal compartment (PVC), and subsequently on to the vacuole, while alkaline phosphatase (ALP) bypasses this compartment to reach the same organelle. Proteins resident to the TGN achieve their localization despite a continuous flux of traffic by continually being retrieved from the distal PVC by virtue of an aromatic amino acid-containing sorting motif. In this study we report that a hybrid protein based on ALP and containing this

retrieval motif reaches the PVC not by following the CPY sorting pathway, but instead by signal-dependent retrograde transport from the vacuole, an organelle previously thought of as a terminal compartment. In addition, we show that a mutation in *VAC7*, a gene previously identified as being required for vacuolar inheritance, blocks this trafficking step. Finally we show that Vti1p, a v-SNARE required for the delivery of both CPY and ALP to the vacuole, uses retrograde transport out of the vacuole as part of its normal cellular itinerary.

Key words: endosome • SNARE • TGN • vacuole • VPS

PROTEINS traveling to the lysosome of mammalian cells and the analogous vacuole in yeast cells reach their destination by following one of many pathways (Griffiths et al., 1988; Ludwig et al., 1991; Rabouille et al., 1993; Hunziker and Geuze, 1996; Liou et al., 1997; Bryant and Stevens, 1998). With the exception of resident lysosomal/vacuolar proteins, which tend to be degradative enzymes, most of these proteins are ultimately degraded and as such the lysosome and vacuole are viewed as the terminal compartments of the endosomal system (DeDuve, 1963; Kornfeld and Mellman, 1989). The endosomal system is clearly a very dynamic system with many proteins cycling between various compartments within it as part of their normal cellular itinerary (Kornfeld and Mellman, 1989; Nothwehr and Stevens, 1994). To date, recycling of proteins is thought to occur from compartments proximal to the lysosome/vacuole, and the possibility that proteins can recycle out of these hydrolytic compartments has been largely overlooked because of the difficulty of measuring such movement. Recent studies, however, indicate that lysosomes are far more dynamic than was previously appre-

ciated, and that they are in close communication with endocytic compartments perhaps making retrograde traffic from the lysosome more likely (Hunziker and Geuze, 1996; Storrie and Desjardins, 1996). These studies indicate that transport to the lysosome involves the fusion of late endosomes with pre-existing lysosomes (van Deurs et al., 1995; Futter et al., 1996; Storrie and Desjardins, 1996; Bright et al., 1997; Mullock et al., 1998), or possibly involves a distinct class of transport vesicles (Berg et al., 1995). Either scenario invokes the need to reacquire at least the specific transport proteins, such as putative SNAP receptor (SNARE)<sup>1</sup> proteins, that may mediate such a process.

Studies on the trafficking of lysosomal membrane proteins in the presence of cycloheximide, wortmanin, or the vacuolating toxin, VacA, indicate that proteins such as Lamp-1 continually cycle between lysosomes and late endosomes (Lippincott-Schwartz and Fambrough, 1987; Akasaki et al., 1993, 1995; Reaves et al., 1996). Using a different model system, Brachet et al. (1997) showed protein

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1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; CPY, carboxypeptidase Y; DIC, differential interference contrast; DPAP, dipeptidyl aminopeptidase; PVC, prevacuolar/endosomal compartment; SNARE, SNAP receptor; V-ATPase, vacuolar ATPase; VAC, vacuolar inheritance; VPS, vacuolar protein sorting.

traffic out of the lysosome-like MHC class II compartment is possible for MHC class II a/b chain complexes when degradation of the invariant chain is blocked. Other experiments indicate that soluble lysosomal constituents may also participate in retrograde transport (Jahrous et al., 1994). A mechanism for how proteins could be retrieved from the lysosome is provided by the observation that AP-2/clathrin coats can assemble specifically on the surface of lysosomes thus providing a specific mechanism for the budding of transport intermediates (i.e., vesicles) from the lysosome (Traub et al., 1996). While these above studies provide support for the occurrence of retrograde transport from the lysosome, they are complicated by the use of pharmacological experimental manipulations that may compromise the distinction and function of late endosomes and lysosomes. Furthermore, it has not been possible to find a particular protein that clearly follows such a pathway under normal conditions.

Vacuolar biogenesis in *Saccharomyces cerevisiae* has very strong parallels with lysosomal biogenesis in mammalian cells (Stack et al., 1995; Bryant and Stevens, 1998). The delivery of vacuolar hydrolases such as carboxypeptidase Y (CPY) relies on the cycling of the CPY receptor, Vps10p, between the TGN and the prevacuolar/endosomal compartment (PVC) in a manner analogous to the delivery of modified hydrolases by the mannose-6-phosphate receptor (Stack et al., 1995). In this pathway the fusion of Golgi-derived vesicles that contain Vps10p with the PVC is mediated by the Sec1p-like protein, Vps45p, the target (t)-SNARE protein Pep12p, the Rab protein, Vps21p/Ypt51, and the vesicle (v)-SNARE protein Vti1p (Cowles et al., 1994; Horazdovsky et al., 1994; Piper et al., 1994; Singer-Kruger et al., 1994; Becherer et al., 1996; Fischer von Mollard et al., 1997). Efflux of traffic from the PVC is controlled by class E Vps proteins such as Vps27p and Vps4p and these proteins may function by creating the necessary transport intermediates that ultimately fuse with the vacuole (Piper et al., 1995; Babst et al., 1997). Fusion to the vacuole itself is controlled in part by the vacuolar t-SNARE Vam3p, although it is unclear whether final delivery of CPY from the PVC requires a vesicular carrier or the fusion of a larger endosomal compartment with the vacuole as has been proposed for the delivery to lysosomes (Futter et al., 1996; Storie and Desjardins, 1996; Bright et al., 1997). A second intracellular route to the vacuole from the TGN is taken by the membrane proteins alkaline phosphatase (ALP) and Vam3p (Cowles et al., 1997; Piper et al., 1997). These proteins reach the vacuole by a transport pathway that is independent of Vps45p, Pep12p, and Vps27p function implying that they do not enter into Golgi-derived vesicles that fuse with the PVC (Piper et al., 1997). Transport of ALP to the vacuole does require Vam3p and the adaptor complex AP-3 as well as the dynamin homologue Vps1p indicating that ALP may be specifically sorted into a separate class of transport vesicles that rely on the vacuolar t-SNARE Vam3p for fusion to the vacuole (Nothwehr et al., 1995; Cowles et al., 1997; Stepp et al., 1997). This pathway appears to parallel a similar pathway in mammalian cells where a subset of lysosomal proteins such as LIMP-II and tyrosinase may be sorted by AP-3 in the TGN for ultimate delivery to the lysosome (Honing et al., 1998).

Previous studies have shown that the cytosolic tail of ALP contains sorting information sufficient for direction into the alternative pathway (Cowles et al., 1997; Piper et al., 1997). Our laboratory has previously reported that the motif FXXFD identified within the cytosolic tail of the resident TGN protein dipeptidyl aminopeptidase (DPAP) A mediates retrieval from the PVC back to the TGN (Nothwehr et al., 1993; Bryant and Stevens, 1997). Transplantation of this motif into the cytosolic tail of ALP results in a protein, RS-ALP (retention sequence-ALP), which achieves steady state localization to the TGN through continual retrieval from a post-Golgi compartment. In RS-ALP, the FXXFD retrieval motif is adjacent to a sorting domain for the alternative pathway. In this present study we report that like the wild-type ALP protein, RS-ALP follows the alternative route to the vacuole in a manner that was independent of Vps45p and Vps27p function. Furthermore, this protein was efficiently retrieved from the vacuole to the TGN via the PVC. We have identified *vac7* mutants as being defective in this retrograde trafficking pathway out of the vacuole and also found that the v-SNARE protein Vti1p uses this pathway to cycle out of the vacuole as part of its normal cellular itinerary.

## Materials and Methods

### Materials

Enzymes used in DNA manipulations were purchased from New England Biolabs Inc. (Beverly, MA), Boehringer Mannheim Corp. (Indianapolis, IN), Bethesda Research Laboratories (Gaithersburg, MD), or United States Biochemical Corp. (Cleveland, OH). Secondary antibodies used for indirect immunofluorescence (all cross-species absorbed) were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). mAbs specific for ALP (1D3-A10) are available from Molecular Probes, Inc. (Eugene, OR). Fixed *Staphylococcus aureus* cells (Ig sorb) were obtained from The Enzyme Center (Malden, MA). [<sup>35</sup>S]Express label was from New England Nuclear (Boston, MA). Oxalyticase was from Enzogenetics (Corvallis, OR). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### Strains, Media, and Microbiological Techniques

Yeast strains used in this study are listed in Table I. Strains were constructed by standard genetic techniques and grown in rich media (1% yeast extract, 1% peptone, 2% dextrose; YEPD) or standard minimal medium with appropriate supplements (Sherman et al., 1986). Strain NBY88 was derived from RPY2 (Piper et al., 1995) by transforming with pSN111 (*pho8Δ-X* construct) linearized with Sall (Nothwehr et al., 1995). Ura<sup>+</sup> transformants were plated onto media containing 5-FOA to select for Ura<sup>-</sup> loopouts and *pho8Δ-X* colonies were identified through immunoblot analysis. NBY72, NBY86, NBY73, and NBY89 were similarly derived from SF838-9D (Rothman et al., 1989), RHY6210 (Gomes de Mesquita et al., 1996), and RPY3 (Piper et al., 1995) and LWY2809, respectively. LWY2809 is a sister spore of LWY2806 (Bonangelino et al., 1997). (NBY85 was derived from NBY89 by transforming cells with EcoRI-linearized pLO2010 (Nothwehr et al., 1995). Ura<sup>+</sup> transformants were plated onto media containing 5-FOA and *pep4Δ-X* colonies were identified using the APNE plate assay (Wolf and Fink, 1975). NBY100 was made by using pSN111 to delete *PHO8* in RPY1017. RPY1017 was made by transforming SEY6210 to His<sup>+</sup> using a PCR product encompassing the *HIS3* gene flanked by 49–100 and 1,351–1,402 relative to the start codon. This *HIS3* disruption resulted in the deletion of the region corresponding to amino acids 33–450 of the *APM3* gene was confirmed by PCR analysis of the resulting *APM3* locus.

### Plasmid Construction

Plasmids used in this study are listed in Table II. DNA manipulations and DNA-mediated transformation of *E. coli* strains CJ236 and XL-1 blues

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
AACY28	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 pho8<math>\Delta</math>::LEU2</i>	Cooper and Stevens, 1996
NBY68	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps45<math>\Delta</math>-X pho8<math>\Delta</math>-X</i>	Piper et al., 1997
NBY88	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps27<math>\Delta</math>::LEU2 pho8<math>\Delta</math>-X</i>	This study
NBY72	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 pho8-<math>\Delta</math>X pep4-3</i>	This study
NBY83	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps45<math>\Delta</math>-X pho8<math>\Delta</math>-X pep4<math>\Delta</math>-X</i>	Bryant et al., 1998
NBY60	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps27<math>\Delta</math>::LEU2 pho8<math>\Delta</math>-X pep4-3</i>	Bryant et al., 1998
NBY84	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps27<math>\Delta</math>::LEU2 vps45<math>\Delta</math>-X pho8-<math>\Delta</math>X pep4-3</i>	Bryant et al., 1998
RPY103	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps27-ts pep4-3 PS10::LEU2::vps10-10*</i>	Piper et al., 1997
NBY73	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his4-519 ade6 vps27-ts pho8<math>\Delta</math>-X pep4-3</i>	This study
NBY86	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-901 lys2-801 suc2-<math>\Delta</math>9 pho8<math>\Delta</math>-X pep4-<math>\Delta</math>1137</i>	This study
SEY6210	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-901 lys2-801 suc2-<math>\Delta</math>9</i>	Robinson et al., 1988
LWY2809	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 lys2-801 ade28::HIS3 suc2-<math>\Delta</math>9 vac7-1</i>	This study
NBY85	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 lys2-801 ade8::HIS3 suc2-<math>\Delta</math>9 vac7-1 pho8<math>\Delta</math>-X pep4<math>\Delta</math>-X</i>	This study
NBY89	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 lys2-801 ade8::HIS3 suc2-<math>\Delta</math>9 vac7-1 pho8<math>\Delta</math>-X</i>	This study
SEY6210 vpt13	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-901 lys2-801 suc2-<math>\Delta</math>9 vpt13</i>	Robinson et al., 1988
NBY100	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-901 lys2-801 suc2-<math>\Delta</math>9 pho8-<math>\Delta</math>X apm3::HIS3</i>	This study

were performed by routine procedures (Sambrook et al., 1989). pSN123, a *CEN-URA3* plasmid encoding (F/A)RS-ALP was constructed by oligonucleotide directed mutagenesis of pSN97 according to the method of Kunkel et al. (1987). The resulting plasmid encodes a version of RS-ALP in which the two phenylalanine residues contained within the Golgi localization motif (RRESFQFNDI) transplanted from Ste13p into the cytosolic tail of ALP (Nothwehr et al., 1993) have been mutated to alanine residues. pNB7, a *CEN-LEU2* plasmid encoding RS-ALP, was constructed by subcloning the 4-kb BamHI fragment from pSN97 into the BamHI site of pRS315 (Sikorski and Hieter, 1989). pNB8, a *CEN-LEU2* plasmid encoding (F/A)A-ALP, was constructed by subcloning the SacI-EcoRV fragment encompassing the *STE13-PHO8* gene fusion encoding (F/A)A-ALP from pSN100 into the SacI-SmaI sites of pRS315 (Sikorski and Hieter, 1989).

### Radiolabeling and Immunoprecipitation

[<sup>35</sup>S]Methionine labeling and immunoprecipitation of ALP fusion proteins, Vps10p-related proteins, and CPY were performed as previously described (Piper et al., 1994; Nothwehr et al., 1995; Cooper and Stevens, 1996). In brief, yeast cultures were grown overnight in selective synthetic media without methionine to OD<sub>600</sub> = 1. Cells were harvested and resuspended in fresh media to the same OD<sub>600</sub>. Cells were pulse labeled for 10 min with 100  $\mu$ Ci <sup>35</sup>S-Express label per 0.5 OD<sub>600</sub>, followed by the addition of unlabeled methionine and cysteine both to 50  $\mu$ g/ml. At specified times samples were removed and treated by the addition of sodium azide to 10 mM at 4°C. Vps10p, CPY, ALP, and related proteins were quantitatively immunoprecipitated from protein extracts of these cells using the relevant specific polyclonal antibodies as previously described (Piper et al., 1994; Nothwehr et al., 1995; Cooper and Stevens, 1996). Half-times of processing of ALP- and Vps10p-related proteins were determined as previously described (Nothwehr et al., 1993; Cooper and Stevens, 1996) using an AMBIS Radioanalytic Imaging System (Ambis, San Diego, CA) and lin-

ear regression analysis plotting percentage total protein processed as a function of time.

### Immunofluorescence Microscopy

The preparation of fixed yeast cell spheroplasts, their attachment to microscope slides, and co-staining of ALP fusion proteins using the mouse anti-ALP mAb 1D3-A10 (Molecular Probes Inc.) and Vph1p using affinity-purified polyclonal antibodies was carried out as previously described (Cooper and Stevens, 1996; Piper et al., 1997). Before use, the mouse anti-ALP mAb 1D3-A10 (Molecular Probes Inc.) was adsorbed against *pho8 $\Delta$*  yeast cells to increase the ALP-specific signal. *pho8 $\Delta$*  yeast cells were fixed, converted to spheroplasts, and then permeabilized using 1% SDS for 1 min. These were resuspended in 1D3-A10, diluted in PBS containing 5 mg/ml BSA, and then incubated for 1 h at 25°C. Spheroplasts were removed by centrifugation and the resultant supernatant was used to stain fixed cells attached to microscope slides. Essentially, fixed spheroplasts attached to slides were incubated with the following solutions, followed by extensive washing with PBS containing 5 mg/ml BSA after each step (all antibody incubations were performed at 25°C for 1 h with the exception of those involving the mouse anti-ALP mAb 1D3-A10 cultured supernatant; these were performed at 4°C for 12–16 h): (a) PBS-BSA containing a 1:3 dilution of adsorbed 1D3-A10 cultured supernatant; (b) 1:500 dilution of a biotin-conjugated donkey anti-mouse IgG (H + L) and a 1:20 dilution of affinity-purified rabbit anti-Vph1p polyclonal antibody; and (c) 1:500 dilution of FITC-conjugated streptavidin and 1:2,000 dilution of Texas red-conjugated goat anti-rabbit IgG (H + L). Co-staining of ALP with Vti1p was performed using 1D3-A10 cultured supernatant at a dilution of 1:3 in conjunction with affinity-purified antibodies specific for Vti1p as previously described (Fischer von Mollard et al., 1997). In brief, the incubations were as follows: (a) PBS-BSA containing a 1:50 dilution of the affinity-purified polyclonal antibody, (b) followed by a 1:500 dilution of a bi-

Table II. Plasmids Used in This Study

Plasmid	Description	Source
pSN92	<i>CEN-URA3</i> plasmid encoding ALP	Nothwehr et al., 1993
pSN97	<i>CEN-URA3</i> plasmid encoding RS-ALP	Nothwehr et al., 1993
pSN123	<i>CEN-URA3</i> plasmid encoding (F/A)RS-ALP	This study
pSN55	<i>CEN-URA3</i> plasmid encoding A-ALP	Nothwehr et al., 1993
pSN100	<i>CEN-URA3</i> plasmid encoding (F/A)A-ALP	Nothwehr et al., 1993
pNB7	<i>CEN-LEU2</i> plasmid encoding RS-ALP	This study
pNB8	<i>CEN-LEU2</i> plasmid encoding (F/A)A-ALP	This study
pSN111	integrating plasmid for <i>pho8-<math>\Delta</math>X</i> allele (loop in/loop out)	Nothwehr et al., 1995
pLO2010	integrating plasmid for <i>pep4-<math>\Delta</math>H3</i> allele (loop in/loop out)	Nothwehr et al., 1995
pTS63	<i>CEN-LEU2</i> plasmid encoding Vps10p- $\Delta$ 10*	Cooper and Stevens, 1996
pRCP39	<i>GAL-PEP4</i> in <i>CEN-URA</i> plasmid	Piper et al., 1997

otin-conjugated donkey anti-rabbit IgG (H + L) and a 1:3 dilution of 1D3-A10, and (c) 1:500 dilution of FITC-conjugated streptavidin and 1:2,000 dilution of Texas red-conjugated goat anti-mouse IgG (H + L). Similarly, co-staining of RS-ALP and Vps10p was performed as previously described (Cooper and Stevens, 1996) using a 1:200 dilution of affinity-purified polyclonal antibodies that recognize Vps10p in conjunction with a 1:3 dilution of 1D3-A10. Staining for Pep12p was performed using affinity-purified rabbit anti-Pep12p antibodies (Fischer von Mollard et al., 1997) in conjunction with biotin/streptavidin amplification as described above. Images were captured on 35 mm T<sub>max</sub>-400 film with a 100× oil immersion lens on an Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Film negatives were digitized using a Polaroid SprintScan 35. Images were adjusted with standard settings using Photoshop™ (Adobe Systems Inc., Mountain View, CA).

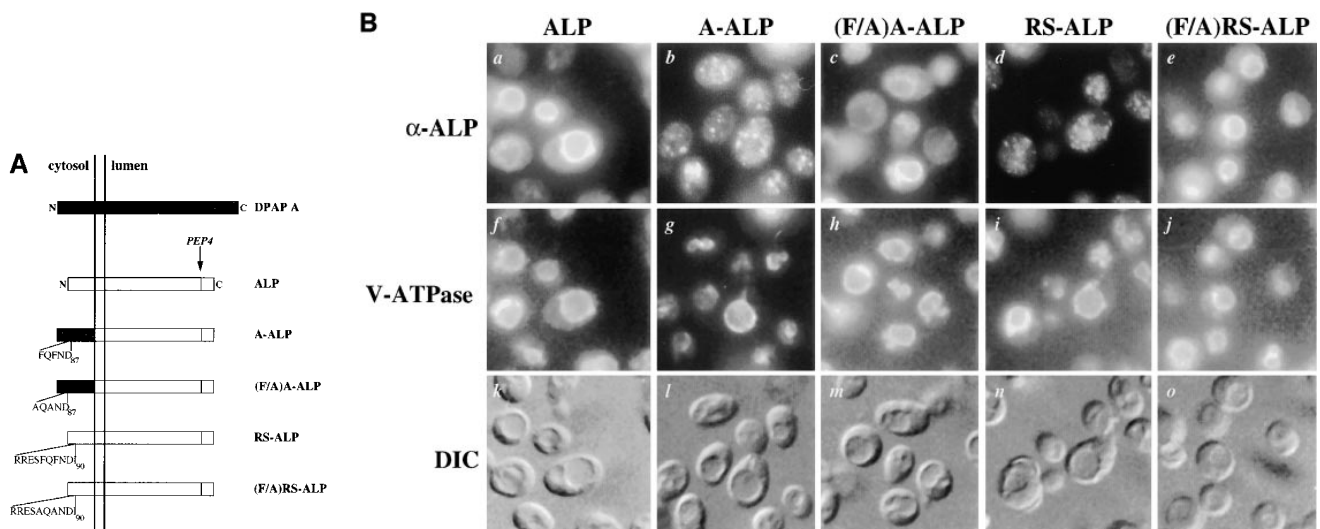
## Results

### Ablation of the Retrieval Motif of the Model Recycling Golgi Membrane Protein, RS-ALP, Causes Mislocalization to the Vacuole

Our laboratory has previously described the protein A-ALP, which was constructed by fusing the cytosolic domain of the TGN protein DPAP A to the transmembrane and luminal domains of the vacuolar protein ALP (Nothwehr et al., 1993). A-ALP is found in the TGN of wild-type cells, and achieves this localization through a combination of static retention mechanisms and retrieval from the post-Golgi PVC (Nothwehr et al., 1993; Bryant and Stevens, 1997). Fig. 1 shows a schematic representation of A-ALP and the other hybrid proteins used in this study, as well as their cellular localization in wild-type cells. In (F/A)A-ALP, the two phenylalanine residues contained within the FXFXD motif responsible for the retrieval of DPAP A and A-ALP from the PVC back to the Golgi have been mutated to alanine residues (Nothwehr et al., 1993). Because of this mutation, (F/A)A-ALP cannot be retrieved

from the PVC to the TGN, and consequently travels on to the vacuolar membrane (Nothwehr et al., 1993; Bryant and Stevens, 1997). RS-ALP was constructed by transplanting a 10-residue stretch from DPAP A, encompassing the FXFXD motif, into the cytosolic domain of ALP (Nothwehr et al., 1993). This transplantation inserted the retrieval motif (RRESFQFNDI) in place of residues 11–17 (TRLVPGS) from the 33-residue tail of ALP, and the resulting protein localizes to the TGN of wild-type cells (Nothwehr et al., 1993). Using indirect immunofluorescence and subcellular fractionation, previous studies have shown that RS-ALP, like A-ALP, colocalizes with the TGN marker protein Kex2p (Nothwehr et al., 1993; Bryant and Stevens, 1997). Like A-ALP, RS-ALP achieves its localization by continuous retrieval from a post-Golgi compartment but does not use the static retention mechanism(s) used by DPAP A and A-ALP (Bryant and Stevens, 1997).

Fig. 1 B shows double-labeling immunofluorescence for the 100-kD subunit of the vacuolar ATPase (V-ATPase), Vph1p, and the various ALP-based proteins depicted in Fig. 1 A. Whereas ALP was found on the vacuolar membrane of wild-type cells (Fig. 1 B, a), both A-ALP and RS-ALP localized to punctate structures (Fig. 1 B, b and d) characteristic of markers of the TGN (Franzoso et al., 1991; Redding et al., 1991; Nothwehr et al., 1993). This localization requires the presence of the two phenylalanine residues within the FXFXD motif, as demonstrated by the co-localization of both (F/A)A-ALP and (F/A)RS-ALP with Vph1p on the vacuolar membrane (Fig. 1 B, c, h, e, and j). Like (F/A)A-ALP (Nothwehr et al., 1993), (F/A)RS-ALP was constructed by mutation of the two phenylalanine residues within the FXFXD motif to alanine residues. The differential localization of RS-ALP and (F/A)RS-ALP (Fig. 1 B, d and e) demonstrates that it is the



**Figure 1.** (A) Schematic representation of proteins used in this study. Sequences derived from DPAP A are shown shaded and those from ALP are unshaded. (B) Localization of ALP, A-ALP, (F/A)A-ALP, RS-ALP, and (F/A)RS-ALP in wild-type cells. NBY72 (*pho8Δ-X pep4-3*) cells harboring pSN92 (ALP; a, f, and k), pSN55 (A-ALP; b, g, and l), pSN100 ((F/A)A-ALP; c, h, and m), pSN97 (RS-ALP; d, i, and n), or pSN123 ((F/A)RS-ALP; e, j, and o) were prepared for double labeling indirect immunofluorescence using the α-ALP mAb, 1D3-A10 (a–e) and affinity-purified antibodies against the 100-kD subunit of the V-ATPase, Vph1p, to show the localization pattern of the V-ATPase (f–j) as described in Materials and Methods. Cells were also visualized using DIC microscopy (k–o).

FXFXD motif that allows RS-ALP to achieve its TGN localization and in its absence, the protein behaves like ALP and is delivered to the vacuole.

### ***Neither RS-ALP nor (F/A)RS-ALP Depend on VPS45 for Their Localization***

At the TGN, recycling Golgi membrane proteins and vacuolar proteins that follow the *VPS*-dependent (or CPY) pathway to the vacuole enter transport vesicles whose fusion with the PVC is controlled by the Sec1p-like protein Vps45p (Cowles et al., 1994; Piper et al., 1994, 1997; Bryant et al., 1998). In *vps45* mutant cells vacuolar proteins such as the 100-kD subunit of the V-ATPase, Vph1p, become trapped in these Golgi-derived transport vesicles, which are unable to fuse with the PVC (Bryant et al., 1998). This mislocalization can be observed using indirect immunofluorescence microscopy where proteins caught in these vesicles display a diffuse staining pattern (Piper et al., 1994, 1997; Bryant et al., 1998). Golgi membrane proteins, such as the CPY receptor Vps10p, that continually cycle between the TGN and the PVC, also enter into Vps45p-controlled vesicles and consequently Vps10p localizes to diffuse vesicular structures in *vps45* mutant cells (Bryant et al., 1998). In contrast to this, proteins that follow the alternative, or ALP, pathway from the TGN to the vacuole do not depend on Vps45p to reach their final destination and are localized to the vacuolar membrane in *vps45* mutant cells (Piper et al., 1997). This is demonstrated in Fig. 2 A, which shows that ALP was delivered to the vacuolar membrane in *vps45Δ* cells (Fig. 2 A, a; Piper et al., 1997). In these same cells, in contrast to the vacuolar staining pattern observed in wild-type cells, Vph1p displayed a disperse, clearly non-vacuolar staining pattern consistent with its entrapment inside the vesicles that accumulated in *vps45Δ* cells (compare Fig. 1 B, f with Fig. 2 A, d). To determine whether the vacuolar proteins (F/A)A-ALP or (F/A)RS-ALP entered into Vps45p-controlled vesicles, we immunolocalized these proteins in *vps45Δ* cells. Like Vph1p, (F/A)A-ALP (a version of A-ALP that cannot recycle from the PVC back to the Golgi and consequently travels to the vacuole via the CPY pathway) accumulated in transport vesicles in *vps45Δ* cells. This is in contrast to its localization in wild-type cells where it was found on the vacuolar membrane (compare Fig. 1 B, c with Fig. 2 A, b). Fig. 2 A also shows that like ALP, whose trafficking to the vacuole is not dependent on *VPS45*, (F/A)RS-ALP was localized on the vacuolar membrane while Vph1p accumulated in transport vesicles in *vps45Δ* cells (compare Fig. 2 A, c and f). These data demonstrate that while both (F/A)A-ALP and (F/A)RS-ALP transit to the vacuole in wild-type cells, they do so by different routes. (F/A)A-ALP enters the Golgi-derived transport vesicles controlled by Vps45p, whereas (F/A)RS-ALP bypasses these trafficking intermediates as it travels to the vacuole.

The observation that (F/A)RS-ALP, like ALP, does not enter Vps45p-controlled transport vesicles demonstrated that sorting information necessary to enter the alternative pathway to the vacuole was fully functional within this protein. We reasoned that the unmutated RS-ALP protein would have both sorting information to enter the alternative pathway to the vacuole, and a functional retrieval sig-

nal. In both wild-type and *vps45* mutant cells, RS-ALP displayed a punctate distribution (Fig. 1 B, d; data not shown) consistent with its previous colocalization with the TGN protein Kex2p (Nothwehr et al., 1993). However, it was difficult to quantitatively assess the degree to which RS-ALP had a vesicular pattern in *vps45* mutant cells compared with its punctate distribution in wild-type cells (Fig. 1 B, d). Thus, to determine quantitatively whether RS-ALP did bypass Vps45p-controlled vesicles, we performed a series of pulse-chase immunoprecipitation experiments.

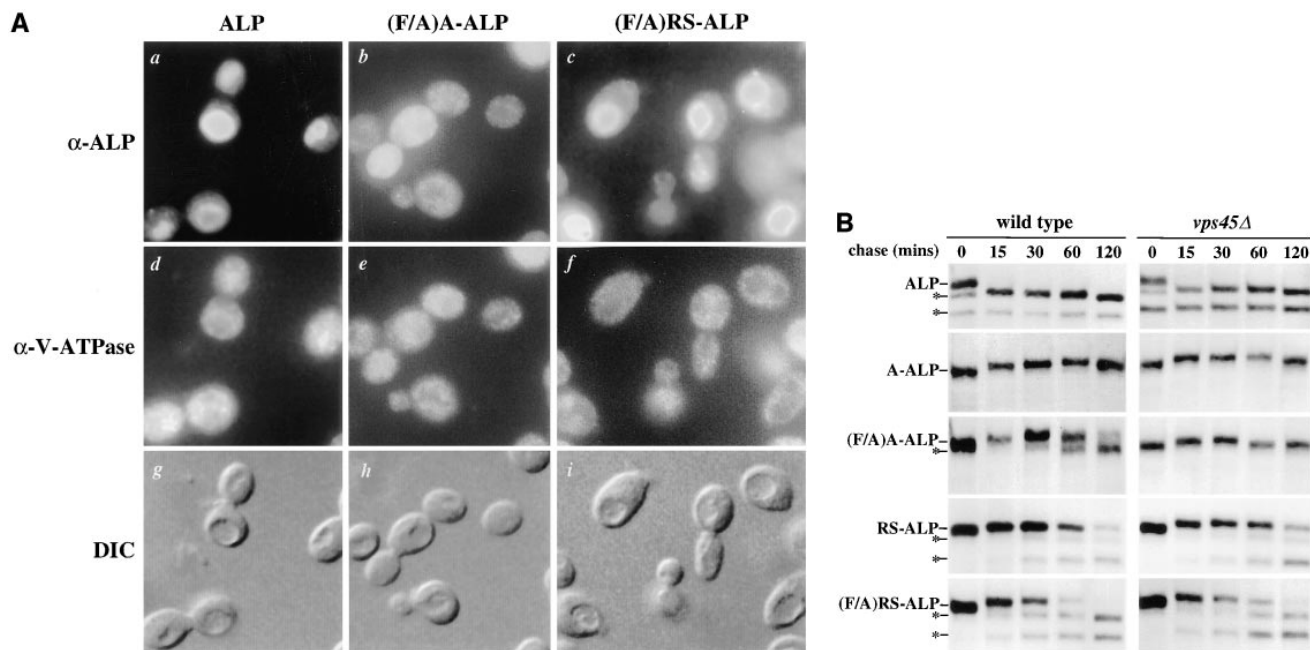
Upon delivery to the vacuole, proteins containing the luminal domain of ALP are processed in a *PEP4*-dependent manner to lower molecular mass forms (Ammerer et al., 1986; Klionsky and Emr, 1989; Nothwehr et al., 1993). For (F/A)A-ALP, this processing occurred with a half-time of ~60 min in wild-type cells (Fig. 2 B; Nothwehr et al., 1993). In *vps45Δ* cells, this processing was blocked since (F/A)A-ALP was trapped inside transport vesicles and unable to gain access to a proteolytically active compartment (Fig. 2 B). As expected from the observation that ALP does not require *VPS45* to reach the vacuolar membrane, the kinetics of processing of ALP were similar in wild-type and *vps45Δ* cells (Fig. 2 B; Piper et al., 1997). Consistent with the vacuolar localization of (F/A)RS-ALP in *vps45Δ* cells (Fig. 2 A, c), this protein was proteolytically processed with similar kinetics in wild-type and *vps45Δ* cells (Fig. 2 B). RS-ALP was also processed with similar kinetics in wild-type and *vps45Δ* cells (Fig. 2 B). These data indicate that RS-ALP does not use *VPS45*-controlled vesicles as part of its normal cellular itinerary, and like (F/A)RS-ALP, follows the *VPS45*-independent route out of the TGN.

The *VPS45*-independent route taken by ALP to the vacuole requires the adaptor protein complex AP-3 (Cowles et al., 1997; Stepp et al., 1997). Therefore we tested whether transport of (F/A)RS-ALP and RS-ALP to the vacuole was AP-3 dependent. Pulse-chase immunoprecipitation experiments revealed that the processing of ALP was significantly impaired in cells defective for the AP-3 complex (*apm3Δ*), as previously reported (Cowles et al., 1997; Stepp et al., 1997). The same set of experiments (Fig. 3) also revealed that the vacuolar delivery of (F/A)A-ALP, which transits to the vacuole through the CPY pathway, is unaffected by the *apm3Δ* mutation. As observed for ALP, the proteolytic processing of (F/A)RS-ALP was significantly slowed in *apm3Δ* cells (Fig. 3). The vacuolar delivery of RS-ALP was similarly impaired in *apm3Δ* cells (data not shown). These data indicate that (F/A)RS-ALP and RS-ALP transport to the vacuole is AP-3 dependent.

The data presented thus far fit with a model evoking the existence of an as yet undescribed retrograde trafficking pathway out of the vacuole (Fig. 2 C, *broken arrow*). RS-ALP and (F/A)RS-ALP would travel from the TGN to the vacuole along the alternative pathway taken by ALP, and after recognition of its FXFXD motif, RS-ALP (but not (F/A)RS-ALP) would be retrieved to the TGN via the PVC by the retrograde pathway.

### ***RS-ALP Reaches the Prevacuolar/Endosomal Compartment via the Vacuole***

Proteins such as ALP and Vam3p that follow the alternative pathway to the vacuole (Cowles et al., 1997; Darsow



**Figure 2.** (A) Localization of ALP, (F/A)A-ALP, and (F/A)RS-ALP in *vps45* $\Delta$  cells. NBY83 (*vps45* $\Delta$ -X *pho8* $\Delta$ -X *pep4* $\Delta$ -X) harboring pSN92 (ALP; *a*, *d*, and *g*), pSN100 ((F/A)A-ALP; *b*, *e*, and *h*) or pSN123 ((F/A)RS-ALP; *c*, *f*, and *i*) were prepared for double labeling indirect immunofluorescence using the  $\alpha$ -ALP mAb, 1D3-A10 (*a-c*) and affinity-purified antibodies against the 100-kD subunit of the V-ATPase, Vph1p (*d-f*), as described in Materials and Methods. Cells were also visualized using DIC microscopy (*g-i*). (B) Kinetics of processing of ALP, A-ALP, (F/A)A-ALP, RS-ALP, and (F/A)RS-ALP in wild-type and *vps45* $\Delta$  cells. AACY28 (wild type) and NBY68 (*vps45* $\Delta$ -X) *pho8* $\Delta$ -X *PEP4* cells harboring pSN92 (ALP), pSN55 (A-ALP), pSN100 ((F/A)A-ALP), pSN97 (RS-ALP), or pSN123 ((F/A)RS-ALP) were labeled with [<sup>35</sup>S]Met for 10 min and chased by adding unlabeled methionine and cysteine, each to a final concentration of 50  $\mu$ g/ml. At the indicated times, proteins were immunoprecipitated from cell extracts using polyclonal antibodies against ALP. The resulting immunoprecipitates were subjected to SDS-PAGE and fluorography. The products of *PEP4*-dependent proteolysis are indicated using asterisks. (C) Model depicting the pathways taken by CPY and ALP to the vacuole. CPY reaches the vacuole by firstly transiting through a prevacuolar/endosomal compartment (PVC). Entry of proteins into this compartment requires the product of *VPS45*. Exit of proteins from the PVC, both back to the TGN and on to the vacuole requires the product of *VPS27*. ALP follows an alternative pathway to the vacuole bypassing the trafficking intermediates defined by mutations in *VPS45* and *VPS27*. The large shaded arrow depicts the retrograde membrane trafficking pathway proposed to be taken by RS-ALP out of the vacuole to reach the PVC.

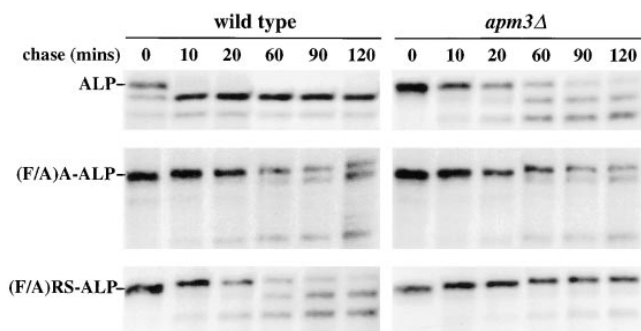
ml. At the indicated times, proteins were immunoprecipitated from cell extracts using polyclonal antibodies against ALP. The resulting immunoprecipitates were subjected to SDS-PAGE and fluorography. The products of *PEP4*-dependent proteolysis are indicated using asterisks. (C) Model depicting the pathways taken by CPY and ALP to the vacuole. CPY reaches the vacuole by firstly transiting through a prevacuolar/endosomal compartment (PVC). Entry of proteins into this compartment requires the product of *VPS45*. Exit of proteins from the PVC, both back to the TGN and on to the vacuole requires the product of *VPS27*. ALP follows an alternative pathway to the vacuole bypassing the trafficking intermediates defined by mutations in *VPS45* and *VPS27*. The large shaded arrow depicts the retrograde membrane trafficking pathway proposed to be taken by RS-ALP out of the vacuole to reach the PVC.

et al., 1997; Piper et al., 1997) do not transit through the PVC defined by mutations in the class E *VPS* gene, *VPS27* (Fig. 2 C). *vps27* mutants accumulate an exaggerated form of the PVC because of a block of traffic out of this compartment both on to the vacuole and back to the TGN (Piper et al., 1995). This class E compartment contains both endocytosed proteins and recycling late-Golgi membrane proteins as well as vacuolar proteins that follow the *VPS*-dependent, or CPY, pathway to the vacuole (Piper et al., 1995). Vacuolar proteins such as Vph1p and recycling Golgi membrane proteins such as Vps10p, enter the class E compartment in Golgi-derived, Vps45p-controlled transport vesicles (Bryant et al., 1998) while proteins that follow the alternative pathway to the vacuole bypass this compartment (Piper et al., 1997).

We examined the behavior of RS-ALP and (F/A)RS-ALP in *vps27* mutant cells to gain further insight into the trafficking routes of these proteins. Fig. 4 A shows that while Vph1p accumulated in the perivacuolar class E com-

partment (Fig. 4 A, *f-j*), ALP was localized to the vacuolar membrane of *vps27* $\Delta$  cells (Fig. 4 A, *a*; Piper et al., 1997). Entry into the class E compartment from the TGN is controlled by Vps45p (Bryant et al., 1998), and as expected, proteins that get packaged into Vps45p vesicles become trapped in the class E compartment of *vps27* $\Delta$  cells. This is demonstrated in Fig. 4 A where *b* and *c* show that A-ALP and (F/A)A-ALP colocalized with Vph1p (*g* and *h*) in the class E compartment. By contrast, (F/A)RS-ALP did not require *VPS45* to reach the vacuole (Fig. 2 A, *c*) and further evidence that this protein followed the alternative pathway to the vacuole is shown in Fig. 4 A (*e*). (F/A)RS-ALP did not accumulate in the class E compartment of *vps27* $\Delta$  cells but instead localized to the vacuole. Interestingly, RS-ALP does localize to the class E compartment of *vps27* mutant cells (Fig. 4 A, *d*; Bryant and Stevens, 1997).

The observation that RS-ALP localizes to the class E compartment is not inconsistent with the observation that it does not travel via the CPY pathway in Vps45p-con-

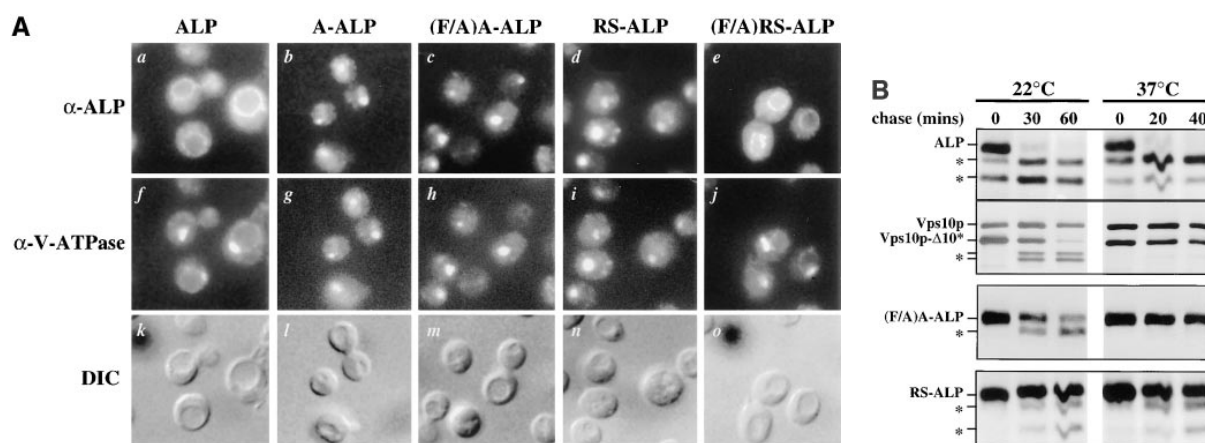


**Figure 3.** Kinetics of processing of ALP, (F/A)A-ALP, and (F/A)RS-ALP in wild-type and *apm3Δ* cells. SNY17 (*wild-type*) and NBY100 (*apm3::HIS3 pho8Δ-X PEP4*) cells harboring pSN92 (ALP), pSN55 (A-ALP), or pSN123 ((F/A)RS-ALP) were labeled with [<sup>35</sup>S]Met for 10 min and chased by adding unlabeled methionine and cysteine each to a final concentration of 50 μg/ml. At the indicated times, proteins were immunoprecipitated from cell extracts using polyclonal antibodies against ALP. The resulting immunoprecipitates were subjected to SDS-PAGE and fluorography.

trolled vesicles. To test the model that RS-ALP visits the vacuole before its arrival at the PVC, we used a regimen in which the PVC of *vps27* mutant cells can be made proteolytically inactive while the vacuole remains proteolytically active. This manipulation uses a temperature-sensitive allele of *VPS27* and the phenomenon of phenotypic lag (Zubenko et al., 1982). *vps27-ts* cells harboring a plasmid in which the *PEP4* open reading frame was placed under the control of the inducible *GALI* promoter were grown in media containing galactose as their sole carbon source at the permissive temperature of 22°C. We have re-

ported previously that these cells are Pep<sup>+</sup> and that when cells are exposed to glucose to shut off production of Pep4p, active vacuolar proteases are flushed from prevacuolar/endosomal compartments (Piper et al., 1997). Shifting these cells to the restrictive temperature of 37°C induces the formation of a proteolytically inactive PVC (Piper et al., 1997). Under this strategy, the vacuole itself remains proteolytically active because of the phenomenon of phenotypic lag, where the autocatalytic activation cycle of protease B results in this and other proteases remaining active for many cell divisions after elimination of Pep4p synthesis (Zubenko et al., 1982; Jones, 1991).

Cells were grown at 22°C in media containing 2% galactose for 24 h, to induce Pep4p production. Expression of *PEP4* in these cells was turned off when the cultures were diluted in media containing 2% glucose and incubated at 22°C for an additional 24 h to ensure that Pep4p was flushed out of the biosynthetic pathway to the vacuole. Cells were then either shifted to 37°C for 10 min, or left at 22°C, and subjected to radiolabel pulse/chase analysis to follow the fate of both newly synthesized ALP and newly synthesized Vps10p-Δ10\*. Vps10p-Δ10\* is a mutant form of the CPY receptor, Vps10p, which lacks the information allowing the protein to cycle between the PVC and the TGN and is instead mislocalized to the vacuole (Cooper and Stevens, 1996). Consequently, Vps10p-Δ10\* follows the *VPS*-dependent pathway to the vacuole and has been shown previously to depend on both *VPS27* and *VPS45* for its trafficking to the vacuole (Cooper and Stevens, 1996; Piper et al., 1997). Fig. 4 B shows that at 22°C both ALP and Vps10p-Δ10\* were proteolytically processed indicating that both had reached the vacuole. These data indicate that the vacuole in these cells is fully capable of processing both ALP and Vps10p-Δ10\* despite the fact that there had been no Pep4p biosynthesis for several generations.



**Figure 4.** (A) Localization of ALP, A-ALP, (F/A)A-ALP, RS-ALP, and (F/A)RS-ALP in *vps27Δ* cells. NBY60 (*vps27Δ pho8Δ-X pep4-3*) cells harboring pSN92 (ALP; a, f, and k), pSN55 (A-ALP; b, g, and l), pSN100 ((F/A)A-ALP; c, h, and m), pSN97 (RS-ALP; d, i, and n), or pSN123 ((F/A)RS-ALP; e, j, and o) were prepared for double-labeling indirect immunofluorescence using the α-ALP mAb, 1D3-A10 (a-e) and affinity-purified antibodies against the 100-kD subunit of the V-ATPase, Vph1p (f-j) as described in Materials and Methods. Cells were also visualized using DIC microscopy (k-o). (B) Rate of *PEP4*-dependent cleavage of ALP, (F/A)A-ALP, RS-ALP, and Vps10p-Δ10\* in *vps27-ts* cells. RPY103 (*VPS10::LEU2::vps10-10\* pep4-3 vps27-ts*) cells and NBY73 (*pho8Δ-X pep4-3 vps27-ts*) carrying the *GALI-PEP4* plasmid, pRCP39 and either pNB8 ((F/A)A-ALP) or pNB7 (RS-ALP). Cells were grown in galactose-containing media for 24 h at 22°C, and then shifted to glucose-containing media for 24 h. Before labeling with [<sup>35</sup>S]Met for 10 min, cells were either maintained at 22°C or shifted to 37°C for 10 min. Chase times used are indicated, after which aliquots were removed from which proteins were immunoprecipitated using α-ALP and α-Vps10p antibodies.

At 37°C, the *VPS*-dependent pathway was blocked as demonstrated by the severe inhibition of Vps10p- $\Delta$ 10\* processing. Since Vps10p- $\Delta$ 10\* localizes to the PVC of *vps27* mutant cells (Piper et al., 1997), the observation that no processing occurred indicates that the PVC within these *vps27*-ts cells at 37°C is proteolytically inactive. In contrast, ALP within the same cells was processed with the same kinetics as observed in wild-type cells, demonstrating that the vacuole was proteolytically active. Fig. 4 B also shows that the processing of (F/A)A-ALP, which is delivered to the vacuole in *vps27*-ts cells at 22°C (Piper et al., 1997), with 55% of the protein processed after 60 min. The processing of (F/A)A-ALP was blocked at 37°C, with no detectable processing after 40 min, consistent with the protein being trapped within the proteolytically inactive PVC that accumulates within these cells. Recall that like Vps10p- $\Delta$ 10\* and (F/A)A-ALP, RS-ALP becomes trapped in the PVC of *vps27 $\Delta$  cells (Fig. 4 A, d) and *vps27*-ts cells at 37°C (Bryant and Stevens, 1997). However, unlike Vps10p- $\Delta$ 10\* and (F/A)A-ALP, the processing of RS-ALP was not blocked in *vps27*-ts cells at 37°C whose PVC had been made proteolytically inactive (40% of RS-ALP has been processed after 40 min at 37°C compared with 35% of the protein after 60 min at 22°C). These data strongly suggest that RS-ALP visits the proteolytically active vacuole before becoming trapped in the PVC of *vps27* mutant cells.*

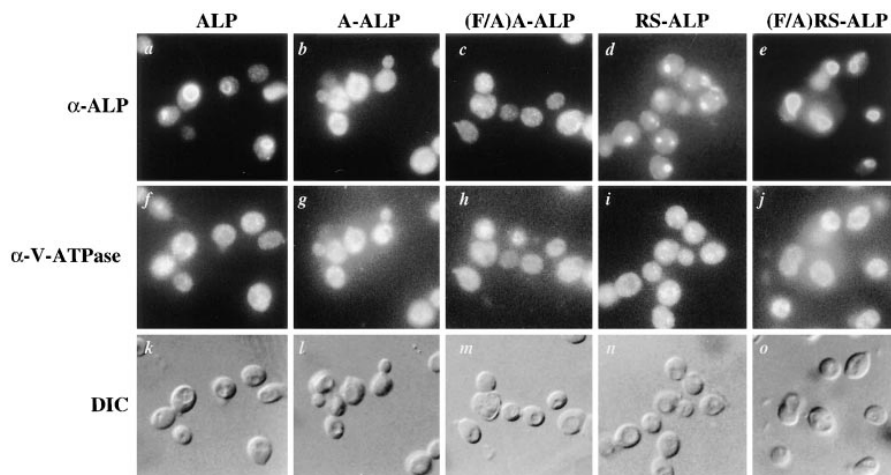
#### RS-ALP and A-ALP Follow Different Intracellular Routes to the PVC

We have previously reported that the entry of proteins such as Vph1p, that follow the CPY pathway to the vacuole, into the class E compartment of *vps27* cells requires the function of *VPS45* (Bryant et al., 1998). We found that for Vph1p trafficking, *VPS45* is epistatic to *VPS27* since mutations in *VPS45* prevent the accumulation of Vph1p in the PVC of *vps27* mutant cells. Similarly, *VPS45* is epistatic to *VPS27* for Vps10p trafficking, since the recycling receptor becomes trapped inside Golgi-derived transport vesicles and cannot gain entry into the PVC of cells carrying mutations in both *VPS45* and *VPS27* (Bryant et al., 1998). It is clear that these *vps45 vps27* double mutant cells

do accumulate a PVC since the endocytosed protein Ste3p, whose trafficking does not depend upon *VPS45*, accumulates there (Bryant et al., 1998).

While both A-ALP and RS-ALP use continuous retrieval from the PVC to achieve steady-state localization to the TGN (Bryant and Stevens, 1997), it appears that the two proteins are delivered to the PVC via different trafficking pathways. If RS-ALP reaches the PVC via retrograde transport from the vacuole (Fig. 2 C), we predict that while *VPS45* function is required for the entry of A-ALP and Vph1p into the PVC (Bryant et al., 1998), RS-ALP will not require Vps45p to enter the same compartment. Fig. 5 shows that both A-ALP and (F/A)A-ALP were prevented from reaching the PVC in *vps27 $\Delta vps45$  $\Delta$  double mutants. Neither A-ALP nor (F/A)A-ALP displayed characteristic staining of localization to the class E compartment in these cells, but instead displayed a disperse staining pattern, as seen for Vph1p within the same cells, consistent with their entrapment inside transport vesicles (Fig. 5, b, c, g, and h). These data are consistent with the finding that A-ALP and (F/A)A-ALP use the CPY pathway to transit between the TGN and the PVC (Figs. 2 and 3). Like A-ALP and (F/A)A-ALP, RS-ALP accumulated in the PVC of *vps27 $\Delta$  cells (Fig. 4 A, b-d) but its trafficking to this compartment did not require *VPS45*, RS-ALP localized to the PVC of *vps27 $\Delta vps45$  $\Delta$  double mutants while Vph1p accumulated within *VPS45* controlled vesicles within the same cells (compare Fig. 5, d and i).***

Like ALP, which does not require the function of *VPS45* or *VPS27* to reach the vacuole (Piper et al., 1997), (F/A)RS-ALP was found on the vacuolar membrane of *vps27 $\Delta vps45$  $\Delta$  double mutants (Fig. 5, e). This is consistent with the data presented in Figs. 2 and 3, which show that (F/A)RS-ALP followed the alternative pathway to the vacuole. This, taken with the observation that RS-ALP localized to the PVC of *vps27 $\Delta vps45$  $\Delta$  double mutants supports a model in which RS-ALP traffics to the vacuole along the alternative (ALP) pathway before traveling to the PVC and finally back to the TGN as part of its normal cellular itinerary. The unique properties of RS-ALP have thus provided the basis for an assay for the retrograde trafficking step from the vacuole to the prevacuolar/endosomal compartment in yeast.**



**Figure 5.** Localization of ALP, A-ALP, (F/A)A-ALP, RS-ALP, and (F/A)RS-ALP in *vps27 $\Delta vps45$  $\Delta$  double mutant cells. NBY84 (*vps27 $\Delta vps45$  $\Delta pho8\Delta-X pep4-3$ ) cells harboring pSN92 (ALP; a, f, and k), pSN55 (A-ALP; b, g, and l), pSN100 ((F/A)A-ALP; c, h, and m), pSN97 (RS-ALP; d, i, and n), or pSN123 ((F/A)RS-ALP; e, j, and o) were prepared for double labeling indirect immunofluorescence using the  $\alpha$ -ALP mAb 1D3-A10 (a-e) and affinity-purified antibodies against the 100-kD subunit of the V-ATPase, Vph1p (f-j), as described in Materials and Methods. Cells were also visualized using DIC microscopy (k-o).**

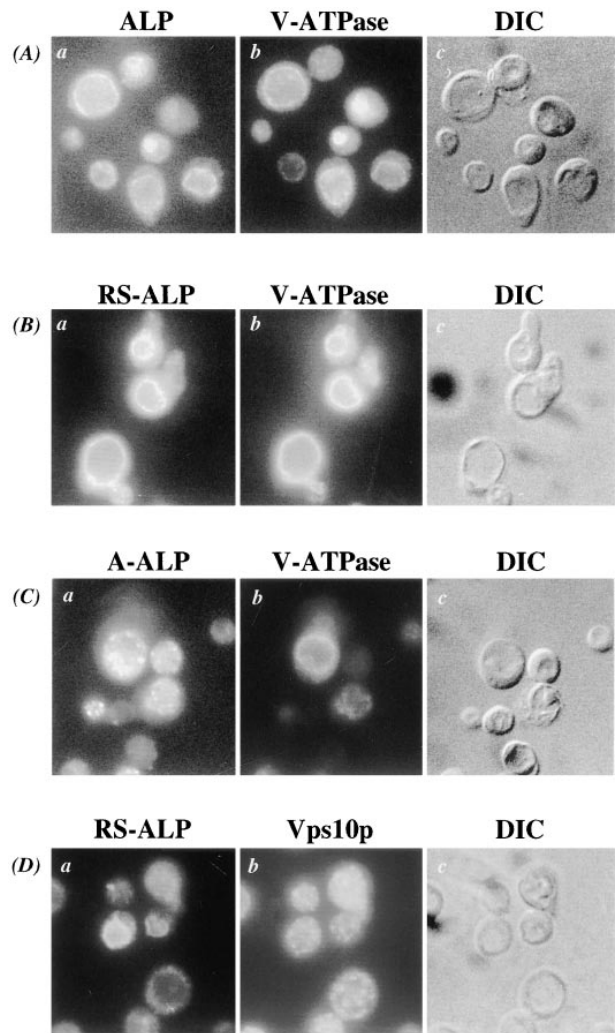


### RS-ALP Cannot Be Retrieved from the Vacuole of *vac7* Mutant Cells

The Class III *vac* mutant *vac7* is defective in vacuolar inheritance likely resulting from an inability to perform a scission step necessary to segregate the budding vacuole from the mother into the daughter cell (Bonangelino et al., 1997). We reasoned that the same machinery on the vacuolar membrane might be involved in a trafficking step out of the vacuole, and investigated whether *vac7* mutant cells were defective in the transport of RS-ALP out of the vacuole. RS-ALP and A-ALP colocalize with Kex2p to the TGN in wild-type cells (Nothwehr et al., 1993; Bryant and Stevens, 1997) showing the punctate staining pattern in indirect immunofluorescence that is characteristic of localization to this compartment (Fig. 1 B, *b* and *d*; Nothwehr et al., 1993). In *vac7-1* cells, RS-ALP was found on the vacuolar membrane, colocalizing with the V-ATPase (Fig. 6 B, *a* and *b*) while A-ALP still displayed a punctate staining pattern (Fig. 6 C, *a* and *b*). Fig. 6 D also shows double labeling of RS-ALP and the TGN protein Vps10p. These two proteins both localize to the TGN in wild-type cells (Nothwehr et al., 1993; Cooper and Stevens, 1996), but in *vac7-1* cells, RS-ALP was mislocalized to the vacuolar membrane while Vps10p maintained its punctate, non-vacuolar staining pattern (Fig. 6 D, *a* and *b*). Fig. 6 demonstrates that even though *vac7-1* cells possessed a morphologically recognizable TGN (as defined by A-ALP and Vps10p), RS-ALP was not found in this organelle as it is in wild-type cells but instead accumulated on the vacuolar membrane. These data suggest that *VAC7* is required for a membrane transport pathway out of the vacuole, which RS-ALP uses to achieve its localization to the TGN.

### The v-SNARE *Vti1p* Is Mislocalized in Cells That Block Retrograde Traffic Out of the Vacuole

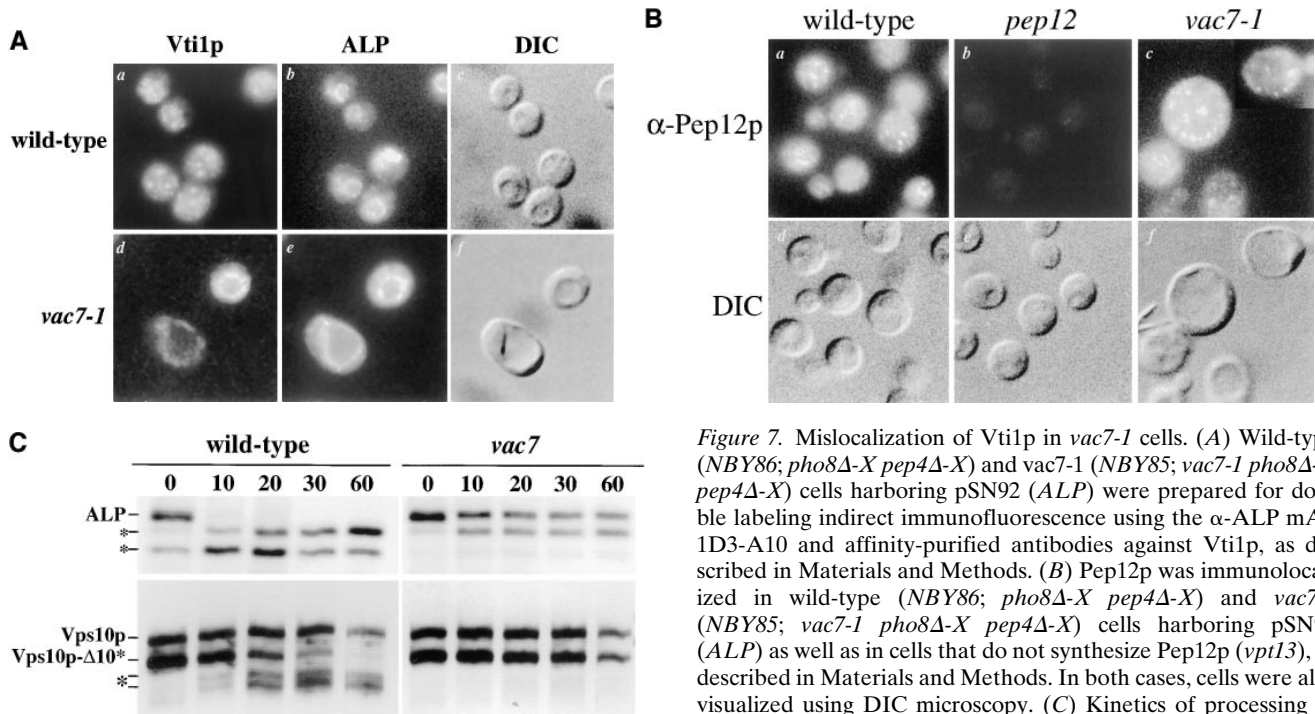
The SNARE hypothesis proposes that interactions between specific v-SNARE molecules on transport vesicles and cognate t-SNAREs on target membranes are involved in controlling the fidelity of membrane fusion (Sollner et al., 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994). In this model, vesicle fusion results in delivery of v-SNAREs to the target membrane and it is likely that these proteins are recycled back to the compartment from where their vesicles bud so that they can be involved in subsequent rounds of targeting. *Vti1p* is a v-SNARE that has been shown to interact with the t-SNARE *Pep12p* to control entry of proteins into the PVC (Fischer von Mollard et al., 1997). *Vti1p* has also been shown to interact with the t-SNARE *Sed5p* to control protein transport between the ER and the *cis*-Golgi (Fischer von Mollard et al., 1997; Lupashin et al., 1997). More recently, *Vti1p* has been shown to interact with *Vam3p* (Holthius et al., 1998) and to be involved in the alternative pathway taken by ALP and *Vam3p* to the vacuole (Fischer von Mollard, G., and T.H. Stevens, in preparation). Since *Vti1p* presumably travels to the vacuole in its role of directing ALP containing trafficking intermediates there, it is likely to be recycled back to the TGN so that it can be involved in further rounds of protein transport. Thus, we identified *Vti1p* as a candidate for an endogenous cargo protein of the retrograde pathway out of the vacuole. In wild-type cells, *Vti1p* displayed



**Figure 6.** RS-ALP is mislocalized to the vacuolar membrane of *vac7* mutant cells. (A) NBY85 (*vac7-1 pho8Δ-X pep4Δ-X*) cells harboring pSN92 (ALP), (B) pSN97 (RS-ALP), and (C) pSN55 (A-ALP) were prepared for double labeling indirect immunofluorescence using the  $\alpha$ -ALP mAb 1D3-A10, and affinity-purified antibodies against the 100-kD subunit of the V-ATPase, Vph1p, as described in Materials and Methods. Cells were also visualized using DIC microscopy (*k-o*). (D) NBY85 (*vac7-1 pho8Δ-X pep4Δ-X*) cells harboring pSN97 (RS-ALP) were prepared for double labeling indirect immunofluorescence using the  $\alpha$ -ALP mAb 1D3-A10 and affinity-purified antibodies against Vps10p, as described in Materials and Methods. Cells were also visualized using DIC microscopy.

a punctate immunofluorescence staining pattern (Fig. 7 A, *a*), but in *vac7-1* cells the protein was found on the vacuolar membrane colocalizing with ALP (Fig. 7 A, *d* and *e*).

The endosomal t-SNARE protein *Pep12p* (Becherer et al., 1996) was also immunolocalized in *vac7-1* cells to determine whether this protein would maintain a non-vacuolar localization. Consistent with subcellular fractionation localization of *Pep12p* (Becherer et al., 1996), *Pep12p* displayed a punctate, non-vacuolar staining pattern in wild-type cells (Fig. 7 B, *a*) (similar to that seen for *Vti1p* in wild-type cells [Fischer von Mollard et al., 1997; Fischer von Mollard, G., unpublished data]). Wild-type cells con-



**Figure 7.** Mislocalization of Vti1p in *vac7-1* cells. (A) Wild-type (*NCY86; pho8Δ-X pep4Δ-X*) and *vac7-1* (*NCY85; vac7-1 pho8Δ-X pep4Δ-X*) cells harboring pSN92 (*ALP*) were prepared for double labeling indirect immunofluorescence using the  $\alpha$ -ALP mAb 1D3-A10 and affinity-purified antibodies against Vti1p, as described in Materials and Methods. (B) Pep12p was immunolocalized in wild-type (*NCY86; pho8Δ-X pep4Δ-X*) and *vac7-1* (*NCY85; vac7-1 pho8Δ-X pep4Δ-X*) cells harboring pSN92 (*ALP*) as well as in cells that do not synthesize Pep12p (*vpt13*), as described in Materials and Methods. In both cases, cells were also visualized using DIC microscopy. (C) Kinetics of processing of ALP and Vps10p- $\Delta$ 10\* in *vac7-1* cells. SEY6210 (*wild-type*) and

LWY2809 (*vac7-1*) *PEP4* cells were labeled with [<sup>35</sup>S]Met for 10 min and chased by adding unlabeled methionine and cysteine each to a final concentration of 50  $\mu$ g/ml. At the indicated times proteins were immunoprecipitated from cell extracts using polyclonal antibodies against ALP and Vps10p. The resulting immunoprecipitates were subjected to SDS-PAGE and fluorography. The products of *PEP4*-dependent proteolysis of both ALP and Vps10p- $\Delta$ 10\* are indicated using asterisks.

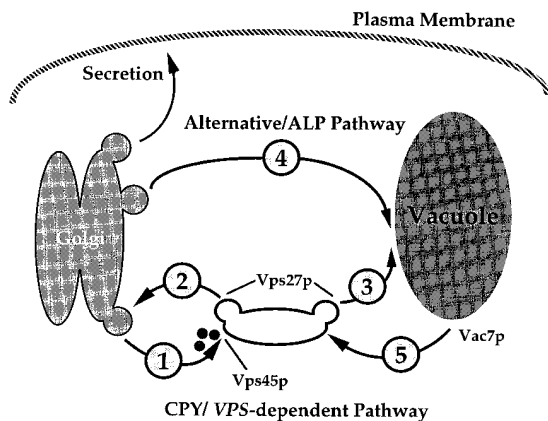
tained 30–50 of these punctate structures per cell, not seen in cells that do not produce Pep12p (Fig. 7 B, b). Pep12p was observed to maintain a punctate, non-vacuolar distribution in *vac7-1* cells (Fig. 7 B, c) and was not mislocalized to the vacuole, arguing that Pep12p does not use the retrograde pathway controlled by *VAC7* to maintain its localization.

Vti1p has been shown to be involved in both the *VPS*-dependent, or CPY pathway, and the alternative, or ALP pathway to the vacuole (Fischer von Mollard et al., 1997; Fischer von Mollard, G., and T.H. Stevens, manuscript in preparation). Since *vac7* mutant cells accumulate Vti1p on the vacuolar membrane, *vac7* mutant cells could be defective for transport along both the ALP and CPY pathways. In fact, it has been reported that *vac7* mutant cells accumulate Golgi-modified forms of CPY intracellularly (Gomes de Mesquita et al., 1996). Fig. 7 C shows pulse-chase immunoprecipitation experiments, which revealed that *vac7* mutant cells were also defective in processing of Vps10p- $\Delta$ 10\*, a membrane protein that follows the *VPS*-dependent pathway to the vacuole (Cooper and Stevens, 1996; Piper et al., 1997; Fig. 4 B). In wild-type cells, Vps10p- $\Delta$ 10\* underwent *PEP4*-dependent processing with a half-time of  $\sim$ 15 min (Fig. 7 C; Cooper and Stevens, 1996), but in *vac7-1* cells, little processing was observed even after chase times of 60 min. Fig. 7 C also shows that the processing of ALP was significantly slower in *vac7* mutant cells than in wild-type cells, with unprocessed forms of ALP still present after 60 min of chase. These data suggest that the vacuolar delivery of ALP is slower in *vac7* mutant cells

than in wild-type cells, but it is worth noting that processed forms of ALP are obvious after longer chase times, consistent with the immunolocalization of ALP to the vacuole in *vac7* mutant cells (Fig. 6 A, a; Fig. 7 A, d; and B, d). This defect in both the CPY and the alternative pathway to the vacuole in *vac7-1* cells may reflect mislocalization of proteins such as Vti1p in these cells, since Vti1p uses retrograde transport out of the vacuole as part of its normal cellular itinerary (Fig. 7 A).

### Discussion

In this study we report that a hybrid protein (RS-ALP) resident to the TGN of the yeast *Saccharomyces cerevisiae* achieves its localization by transiting through the vacuole (Fig. 8). Directed by sorting information contained within its cytosolic tail, RS-ALP follows the alternative pathway, as taken by ALP, to the vacuole thus bypassing Golgi-derived transport vesicles that carry proteins from the TGN to the PVC. By virtue of the retrieval motif (FXFXD) that it also carries in its cytosolic tail, RS-ALP then uses a retrograde membrane trafficking step out of the vacuole to reach the PVC from where it is delivered back to the TGN. This work has also revealed that *VAC7* function is required for membrane traffic out of the vacuole, and that the v-SNARE Vti1p uses this retrograde trafficking pathway as part of its normal cellular itinerary. This study represents the first report of retrograde membrane traffic out of the vacuole in the yeast *S. cerevisiae*.



### Pathways taken by proteins in this study

- ① → ② A-ALP
- ① → ③ (F/A)A-ALP, Vph1p
- ④ → ⑤ → ② RS-ALP
- ④ ALP, (F/A)RS-ALP

**Figure 8.** Model for membrane trafficking pathways between the TGN and the vacuole of *S. cerevisiae*. There are at least two routes taken by proteins from the TGN to the PVC. Proteins that follow the CPY pathway to the vacuole transit through the PVC in a *VPS45*, *VPS27*-dependent manner. From the PVC, these proteins are then delivered to the vacuole. Recycling TGN membrane proteins such as A-ALP are retrieved from the PVC to the TGN. The second route from the TGN to the vacuole does not transit through the PVC and is taken by ALP, RS-ALP, and (F/A)RS-ALP. After delivery to the vacuolar membrane, RS-ALP follows the newly identified retrograde membrane trafficking step in a *VAC7*-dependent manner by virtue of its FXXFD retrieval motif.

### Identification of a Retrograde Membrane Trafficking Step Out of the Vacuole

The yeast vacuole and its mammalian counterpart the lysosome have typically been thought of as terminal destinations for proteins with regard to membrane trafficking (Kornfeld and Mellman, 1989; Bryant and Stevens, 1998). Recently however, evidence that this might not be the case has been building from work carried out on mammalian cells (see Introduction for details), suggesting that the lysosome acts as a donor membrane in protein trafficking and is therefore a more dynamic organelle than has been widely appreciated. Although the possible existence of a retrograde pathway out of the yeast vacuole has been suggested (Wilcox et al., 1992), there has been no experimental test of this idea. In this study, we have taken advantage of the unique properties of a hybrid protein, RS-ALP (Nothwehr et al., 1993) to demonstrate the existence of a retrograde membrane trafficking step out of the vacuole in the yeast *S. cerevisiae*. RS-ALP carries a motif (FXXFD) in its cytosolic tail that is sufficient to specify retrieval of the protein from the PVC to the TGN, affording steady

state localization to the TGN (Bryant and Stevens, 1997). RS-ALP was constructed in such a way that information sufficient to direct ALP away from the CPY pathway and into the alternative pathway taken by ALP to the vacuole remains present (Nothwehr et al., 1993; Piper et al., 1997).

The transport of ALP, RS-ALP, and (F/A)RS-ALP to the vacuole has been found to be Vps45p independent but requires the adaptor protein complex AP-3. Although ALP, RS-ALP, and (F/A)RS-ALP all travel to the vacuole along the alternative pathway, they are not all proteolytically processed with the same kinetics. Vowels and Payne (1998) recently identified the amino acid residues LV as being the most important signal within the ALP cytosolic domain for targeting into the alternative pathway. The residues LV are missing in (F/A)RS-ALP, and although it is clear that this protein uses the alternative pathway (as does a version of ALP lacking residues 2–21 and therefore the LV signal [Piper et al., 1997]), its slower transport rate to the vacuole likely results from its compromised targeting information. Like (F/A)RS-ALP, RS-ALP also lacks the LV motif, and therefore enters the alternative pathway at a rate slower than ALP, but likely at the same rate as (F/A)RS-ALP. However, RS-ALP is processed in wild-type cells with even slower kinetics than (F/A)RS-ALP. This difference can be explained if the signal-dependent retrieval from the vacuole occurs rapidly enough to prevent RS-ALP from becoming fully processed during one round of transit through the vacuole.

The AP-3-dependent, alternative pathway to the vacuole is unaffected by mutations in either *VPS45* or *VPS27*, which control transit through the PVC (Cowles et al., 1994, 1997; Piper et al., 1994, 1995, 1997; Bryant et al., 1998). We have demonstrated that the trafficking of RS-ALP occurs independently of Vps45p and that it reaches the PVC by first traveling through the proteolytically active vacuole. The observation that RS-ALP localizes to the PVC of *vps27* mutant cells (Bryant and Stevens, 1997), whereas a mutant version of the same protein (F/A)RS-ALP is found on the vacuolar membrane, was crucial to the formation of our model in which RS-ALP reaches the PVC from the vacuole via a retrograde membrane trafficking step (Fig. 8).

Although both RS-ALP and the hybrid protein A-ALP achieve TGN localization through continual retrieval from the PVC (Bryant and Stevens, 1997), the route taken by the two proteins to reach the PVC differs. A-ALP consists of the cytosolic domain of the TGN protein DPAP A fused to the transmembrane and luminal domains of ALP (Nothwehr et al., 1993). A-ALP and its derivative (F/A)A-ALP leave the TGN by entering the CPY pathway out of the Golgi (Fig. 8, pathway 1). Fusion of the vesicles that these proteins enter with the PVC is controlled by the Sec1p-like protein Vps45p. After recognition of its FXXFD motif, A-ALP is recycled from the PVC back to the TGN (Fig. 8, pathway 2) and thus achieves steady state localization to the TGN (Nothwehr et al., 1993; Bryant and Stevens, 1997). Since it does not carry an FXXFD motif, (F/A)A-ALP cannot be retrieved from the PVC to the TGN, but instead travels on from the PVC to the vacuole (Bryant and Stevens, 1997; Fig. 8, pathway 3). In contrast to this, RS-ALP and (F/A)RS-ALP do not enter Vps45p controlled vesicles and instead follow the alternative path-

way to the vacuole (Fig. 8, pathway 4). After delivery to the vacuolar membrane, the FXFXD motif of RS-ALP directs its retrograde transport from the vacuole to the PVC (Fig. 8, pathway 5) from where it follows the same pathway as A-ALP to return back to the TGN (Fig. 8, pathway 2). As a result of ablation of the FXFXD motif, (F/A)RS-ALP is not retrieved from the vacuole to the PVC and is therefore localized to the vacuolar membrane of both wild-type and *vps27* mutant cells.

This model can be used to explain why RS-ALP undergoes processing by vacuolar proteases in wild-type cells, whereas the CPY receptor, Vps10p, which leaves the TGN and enters the PVC with similar kinetics to RS-ALP, is not exposed to vacuolar proteases (Bryant and Stevens, 1997). RS-ALP becomes processed as it transits through the vacuole en route to the PVC, whereas Vps10p enters the PVC in Vps45p controlled vesicles (Bryant et al., 1998). These data also suggest that the levels of active vacuolar proteases contained within the PVC are not as high as those found in the vacuole.

### ***Machinery and Physiological Relevance of Retrograde Vacuolar Transport***

We have demonstrated that mutations in the class III VAC gene *VAC7* (Gomes de Mesquita et al., 1996; Bonangelino et al., 1997) cause mislocalization of RS-ALP to the vacuolar membrane. This implies that *Vac7p*, a vacuolar protein required for vacuolar inheritance and normal vacuolar morphology (Bonangelino et al., 1997), is required for the transport of RS-ALP from the vacuole to the PVC.

The identification of the v-SNARE Vti1p (Fischer von Mollard et al., 1997) as a cargo molecule of the retrograde membrane trafficking pathway out of the vacuole is important since it provides some insight into the physiological relevance of this pathway. Vti1p is required for the CPY pathway, where it interacts with the t-SNARE Pep12p to control the entry of proteins into the PVC (Fischer von Mollard et al., 1997). In addition, Vti1p is required for the trafficking of ALP to the vacuole as demonstrated by the observation that cells carrying a conditional allele of *VTII* accumulate unprocessed ALP under restrictive conditions (Fischer von Mollard, G., and T.H. Stevens, manuscript in preparation). Like RS-ALP, Vti1p accumulates on the vacuolar membrane of *vac7* mutant cells. In its role of directing trafficking intermediates to the vacuole along the ALP pathway at least a portion of Vti1p must be transported to the vacuolar membrane. Our observation that Vti1p follows the retrograde trafficking step defined by mutations in *vac7* allows us to propose a mechanism for recycling of this v-SNARE to allow it to be involved in further rounds of targeting. Although *vac7* mutant cells do not secrete CPY as might be expected from their mislocalization of Vti1p, they do accumulate Golgi-modified, or p2, precursor forms of CPY intracellularly consistent with a defect in TGN to PVC trafficking (Gomes de Mesquita et al., 1996; our unpublished data). In addition, *vac7* mutant cells are defective in the processing of ALP as well as in that of Vps10p- $\Delta 10^*$ , a membrane protein marker of the CPY pathway (Cooper and Stevens, 1996; Piper et al., 1997).

While *vac7* mutant cells mislocalize Vti1p, overexpression of *VTII* is not sufficient to suppress these processing

phenotypes (Bryant, N.J., and T.H. Stevens, unpublished data). This suggests that mislocalization of Vti1p alone is not responsible for the trafficking defects displayed by *vac7* mutants. Such an observation is not surprising since there are likely to be additional proteins that travel to the vacuole along with Vti1p by the alternative pathway and it is easy to imagine that these molecules will also be recycled using retrograde transport out of the vacuole. *VTII* is unusual among genes involved in vacuolar protein sorting in that it is an essential gene (Fischer von Mollard et al., 1997). The essential nature of *VTII* arises from the involvement of Vti1p in ER to *cis*-Golgi trafficking through interactions with the t-SNARE Sed5p (Fischer von Mollard et al., 1997; Lupashin et al., 1997). Although *vac7* mutant cells mislocalize Vti1p to the vacuole, these cells are still viable (although it is worth noting that *vac7* $\Delta$  cells do display a slow growth phenotype; Bonangelino et al., 1997). It may be that newly synthesized Vti1p in these cells is sufficient to allow enough membrane traffic to occur through the *cis*-Golgi to sustain life.

Our studies with RS-ALP and its derivative (F/A)RS-ALP indicate that the delivery of RS-ALP from the vacuole to the PVC depends on the FXFXD motif. There is no such motif within the cytosolic sequence of Vti1p (Fischer von Mollard et al., 1997), which suggests that there are at least two ways of entering this pathway, or perhaps overlapping signals. A similar phenomenon is seen with the signal-dependent alternative pathway taken by ALP to the vacuole (Bryant and Stevens, 1998). The t-SNARE Vam3p also follows this pathway and yet there are no obvious similarities between the sequences of ALP and Vam3p (Wada et al., 1997). To date, very little is known regarding the trafficking of SNARE proteins and it is likely that as more information regarding the intracellular pathways followed by these proteins is uncovered, signals that control the trafficking of Vam3p, Vti1p, and SNARE proteins in general will be identified.

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