The Saccharomyces cerevisiae v-SNARE Vti1p Is Required for Multiple Membrane Transport Pathways to the Vacuole

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The interaction between v-SNAREs on transport vesicles and t-SNAREs on target membranes is required for membrane traffic in eukaryotic cells. Here we identify Vti1p as the first v-SNARE protein found to be required for biosynthetic traffic into the yeast vacuole, the equivalent of the mammalian lysosome. Certain vti1-ts yeast mutants are defective in alkaline phosphatase transport from the Golgi to the vacuole and in targeting of aminopeptidase I from the cytosol to the vacuole. VTI1 interacts genetically with the vacuolar t-SNARE VAM3, which is required for transport of both alkaline phosphatase and aminopeptidase I to the vacuole. The v-SNARE Nyv1p forms a SNARE complex with Vam3p in homotypic vacuolar fusion; however, we find that Nyv1p is not required for any of the three biosynthetic pathways to the vacuole. v-SNAREs were thought to ensure specificity in membrane traffic. However, Vti1p also functions in two additional membrane traffic pathways: Vti1p interacts with the t-SNAREs Pep12p in traffic from the TGN to the prevacuolar compartment and with Sed5p in retrograde traffic to the cis-Golgi. The ability of Vti1p to mediate multiple fusion steps requires additional proteins to ensure specificity in membrane traffic.

INTRODUCTION

In eukaryotic cells protein traffic between different organelles is mediated by transport vesicles budding from the donor compartment and fusing with the acceptor compartment (Rothman, 1994). The yeast Saccharomyces cerevisiae has emerged as a powerful model system to study membrane traffic. Genetic screens have identified numerous genes involved in specific trafficking steps. In addition, the complete sequencing of the yeast genome gives an inventory of all yeast proteins and allows for the identification of new members of protein families and proteins with homologues in higher eukaryotes.

Proteins enter the secretory pathway via translocation into the lumen of the endoplasmic reticulum (ER). After transport from the ER to the cis-Golgi, proteins passage through the Golgi stacks and arrive in the trans-Golgi network (TGN). In the TGN proteins targeted for the plasma membrane or for secretion are sorted into secretory vesicles away from those destined for the vacuole. Two different pathways that lead from the TGN to the vacuole, the equivalent of the mammalian lysosome, have been identified to date (Bryant and Stevens, 1998; Odorizzi et al., 1998). Genetic screens have identified >50 genes as being required for transport of the soluble vacuolar hydrolase carboxypeptidase Y (CPY) from the Golgi to the vacuole, including the VPS, PEP, and VAM genes (Jones, 1977; Bankaitis et al., 1986; Rothman and Stevens, 1986; Bryant and Stevens, 1998). CPY and most other vacuolar enzymes are packaged into transport vesicles, which fuse with the prevacuolar/endosomal compartment (PVC; Vida et al., 1993). The PVC also receives proteins that are endocytosed from the plasma membrane (Vida et al., 1993; Piper et al., 1995). A second
fusion step is required for transport from the PVC to the vacuole. It is unclear whether this occurs via a maturation process or is mediated by transport vesicles budding from the PVC.

The vacuolar membrane protein alkaline phosphatase (ALP) does not follow the same pathway as CPY from the Golgi to the vacuole but instead is transported in vesicles separate from those carrying CPY (Cowles et al., 1997; Piper et al., 1997). ALP does not pass through the PVC, whereas CPY does transit this organelle. It is not known yet whether ALP reaches the vacuole directly or through an as yet unidentified intermediate compartment. The adaptor complex AP3 is required for ALP but not for CPY transport to the vacuole (Cowles et al., 1997; Stepp et al., 1997). Several proteins have been identified as being involved in both ALP and CPY transport to the vacuole, for example, Vam3p, Vam7p and a vacuolar protein complex consisting of Vps18p, Vps11p, Vps16p, and Vps33p (Darsow et al., 1997; Piper et al., 1997; Wada et al., 1997; Rieder and Emr, 1997; Sato et al., 1998; Srivastava and Jones, 1998).

Aminopeptidase I (API) is transported from the cytosol to the vacuole without passing through either the ER or the Golgi apparatus (Harding et al., 1995; Klionsky, 1997). API oligomerizes in the cytosol and is enclosed in a double membrane to form cytosol to vacuole transport (CVT) vesicles. A number of genes (CVT) have been identified that are required for API transport, and some CVT genes are identical to genes involved in autophagy or to genes required for vacuolar fusion in the CPY and ALP pathways (Klionsky, 1998). It is unclear whether traffic of API, CPY, and ALP converge at a common compartment before transport to the vacuole or whether membranes from these pathways fuse directly with vacuolar membranes. Recent EM studies suggest that the outer membranes of the double membranes that surround autophagosomes and CVT vesicles fuse with the vacuole (Baba et al., 1997).

According to the soluble NSF attachment protein receptor (SNARE) hypothesis the specific recognition between a transport vesicle and its target membrane is achieved through complexes of specific SNARE proteins (Rothman, 1994; Götte and Fischer von Mollard, 1998). Members of the target membrane-associated SNARE (t-SNARE) family are localized on the target membrane and mark this compartment for different types of incoming transport vesicles. Transport vesicles contain vesicle-associated SNAREs (v-SNAREs), which bind a specific t-SNARE and are responsible for specificity in membrane traffic. The interaction between v- and t-SNAREs leads to changes in their structure, which probably drives membrane fusion (Hanson et al., 1997).

Eight syntaxin-related t-SNAREs have been identified in the yeast genome (Götte and Fischer von Mollard, 1998; Holthuis et al., 1998a). Ufe1p is needed for retrograde traffic to the ER and for ER homotypic fusion (Lewis and Pelham, 1996; Patel et al., 1998). Sed5p is involved in anterograde and retrograde traffic to the cis-Golgi compartment (Hardwick and Pelham, 1992). Sso1p and Sso2p are required for secretion (Aalto et al., 1993). Tgl1p and Tgl2p are required for endocytosis, maintenance of wild-type levels of TGN proteins, and the correct localization of chitin synthase III (Holthuis et al., 1998a,b). Tgl2p has been implicated either in the internalization (Abeliovich et al., 1998) or degradation (Séron et al., 1998) of endocytic markers. Some CPY is secreted in tgl1Δ and tgl2Δ cells (Abeliovich et al., 1998; Nichols et al., 1998). Tgl2 has been localized to the TGN (Holthuis et al., 1998a), to the TGN and endosomes (Abeliovich et al., 1998), and to endosomes (Séron et al., 1998), and Tgl1p has been localized to the putative early endosome and to the chitosome (Holthuis et al., 1998a,b). Pep12p is needed for traffic from the Golgi to the PVC (Becherer et al., 1996). The vacuolar Vam3p is involved in homotypic vacuolar fusion and in the trafficking of CPY, ALP, and API along three different biosynthetic routes to the vacuole (Darsow et al., 1997; Nichols et al., 1997; Piper et al., 1997; Wada et al., 1997; Rieder and Emr, 1997; Sato et al., 1998; Srivastava and Jones, 1998).

Several v-SNAREs with functions in traffic from the ER to the Golgi apparatus or in retrograde traffic within the Golgi apparatus have been identified in yeast: Sec22p/Sly2p, Bet1p/Sly12p, Bos1p, Sft1p, Ykt6p, and Gos1p (Newman et al., 1990; Ossig et al., 1991; McNew et al., 1997; Holthuis et al., 1998a). The v-SNAREs Snclp and Sncl2p interact with Sso1p and Sso2p in secretion (Protopopov et al., 1993). Nyv1p forms a complex with Vam3p in homotypic vacuolar fusion (Nichols et al., 1997). Vti1p is the only other v-SNARE known to be involved in endosomal or vacuolar trafficking steps. Vti1p functionally interacts with Pep12p in traffic from the Golgi to the PVC and with Sed5p in retrograde traffic to the cis-Golgi (Fischer von Mollard et al., 1997; Lupashin et al., 1997). Allele-specific differences among several vti1-ts mutants allowed us to distinguish between these traffic steps (Fischer von Mollard et al., 1997). vti1-1 and vti1-2 cells exhibit defects in TGN to PVC transport at the nonpermissive temperature. vti1-11 cells display a block in traffic to the PVC and an additional defect in retrograde traffic to the cis-Golgi. Genetic interactions of VTI1 with YKT6 and SFT1 confirm a role for Vti1p in retrograde traffic to the cis-Golgi (Lupashin et al., 1997). A complex of Vti1p, Ykt6p, and Sft1p may bind to Sed5p to ensure specificity in this trafficking step.
Vti1p can also bind to Vam3p, Tlg1p, and Tlg2p, although the functional relevance of these complexes identified by coimmunoprecipitations has not yet been determined (Holthuis et al., 1998a).

Here we report that Vti1p interacts functionally with Vam3p in two different trafficking steps, in transport of ALP from the Golgi to the vacuole and in traffic of API from the cytoplasm to the vacuole. Nyv1p was found not to be required for biosynthetic pathways of ALP or API. These trafficking steps are unexpected and poses the question of how specificity in membrane traffic is controlled.

MATERIALS AND METHODS

Materials

Reagents were used from the following sources: enzymes for DNA manipulation from New England Biolabs (Beverly, MA); and Boehringer Mannheim (Indianapolis, IN); secondary antibodies from Promega (Madison, WI), Amersham (Arlington Heights, IL), and Jackson ImmunoResearch (West Grove, PA); 35S-Express label and ECL solution from New England Nuclear (Boston, MA); fixed Staphylococcus aureus cells (lgGorb) from The Enzyme Center (Malden, MA); Oxalyticase from Enzogenetics (Corvallis, OR); Glusulase from DuPont (Boston, MA); and Zymolyase from Seikagaku (Tokyo, Japan). All other reagents were purchased from Sigma (St. Louis, MO).

Plasmid manipulations were performed in the Escherichia coli strains MC1061 or XL1Blue using standard media.

Yeast strains (Table 1) were grown in rich media (1% yeast extract, 1% peptone, 2% dextrose; YEPD) or standard minimal medium (SD) with appropriate supplements. To induce expression from the GAL1 promoter, dextrose was replaced by 2% raffinose and 2% galactose.

Table 1. Yeast strains used in this study

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<th>Strain</th>
<th>Genotype</th>
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<td>MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel</td>
<td>(Robinson et al., 1988)</td>
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<tr>
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</tr>
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<td>FvMY22</td>
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<td>This study</td>
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<td>FvMY36</td>
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<td>nyv1Δ::HIS5p</td>
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Immunoprecipitation of 35S-labeled Proteins

CYP, ALP, and API were immunoprecipitated as described earlier (Klionsky et al., 1992; Vater et al., 1997; Nothwehr et al., 1993). For CYP immunoprecipitations, log-phase growing yeast cells were labeled with 35S-Express label for 10 min with 10 μCi/μl OD unit of cells followed by a 30-min chase with cysteine and methionine. The medium was separated and the cell pellet, spheroplasted, and lysed. CPY was isolated from the medium and cellular extracts. For ALP immunoprecipitations yeast cells were labeled for 7 min and chased for the indicated periods. To investigate API traffic at 36°C, 0.25 units OD of yeast cells in 500 μl of medium were labeled with 35S-Express label for each time point. After a 10-min pulse, cells were chased for the indicated periods and spheroplasted. Yeast cells (0.5 OD unit per time point) were labeled with 20 μl of 35S-Express label for 15 min to follow API traffic at 24°C. Extracts were prepared by boiling in 50 μl of 50 mM NaPO4, pH 7.0, 1% SDS, and 3 M urea and dilution with 950 μl of 50 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, and 0.1 mM EDTA. The API antisera was kindly provided by D. Klionsky (University of California, Davis, CA). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. A phosphorimager was used for quantification.
**Immunofluorescence Microscopy**

Indirect immunofluorescence microscopy was performed as previously described (Raymond et al., 1992; Piper et al., 1997). FvMY36 cells (GAL1-PHO8 vti1-2) were grown at 24°C in YEP and 2% raffinose. Synthesis of ALP was induced for 1 h by addition of 2% galactose at 24°C or simultaneously with a shift to 35°C. Cells were fixed, spheroplasted, and permeabilized with 1% SDS in 1.2 M sorbitol for 2 min. Cells were transferred to coverslips, blocked with 2% goat serum in PBS, and incubated overnight at 4°C with ALP antiserum (Raymond et al., 1992), which had been preabsorbed against pho8α cells; 1:100 diluted biotin-conjugated goat anti-rabbit IgG (heavy and light chains) and 1:100 diluted FITC-conjugated streptavidin were used for detection.

**RESULTS**

**Vti1p Interacts with Vam3p in ALP Transport to the Vacuole**

Newly synthesized vacuolar proteins are transported from the Golgi apparatus to the vacuole through two different pathways. CPY and most other vacuolar proteins reach the vacuole via the PVC. The vacuolar membrane protein ALP is transported to the vacuole without passage through the PVC (Bryant and Stevens, 1998).

Traffic of ALP to the vacuole requires the vacuolar t-SNARE Vam3p (Darsow et al., 1997; Piper et al., 1997). The v-SNARE involved in this step has not yet been identified, but it has been shown that Vam3p binds to Vti1p (Holthuis et al., 1998a). Allele-specific differences between different vti1-ts mutants revealed that Vti1p serves as a v-SNARE in two different membrane-trafficking pathways (Fischer von Mollard et al., 1997), vti1-1 and vti1-2 mutant cells are completely blocked in transport of CPY from the TGN to the PVC, but in contrast to vti1-11 cells, do not exhibit a defect in retrograde traffic to the cis-Golgi (Fischer von Mollard et al., 1997, see Figure 3).

To determine whether Vti1p functions in the alternative (ALP) pathway to the vacuole, we tested whether the various temperature-sensitive vti1 mutants display ALP processing defects. Wild-type, vti1-1, and vti1-2 cells were pulse–chase labeled with 35S-Met/-Cys at 24 or at 36°C after a 15-min preincubation at 36°C. ALP was immunoprecipitated from these cells, and the precursor form of ALP (pALP) and the mature vacuolar ALP (mALP) separated by SDS-PAGE. ALP was delivered to the vacuole and proteolytically processed with wild-type kinetics in vti1-1 cells at both 24 and 36°C, as indicated by the appearance of mALP (Figure 1, compare upper and lower panels). By contrast, vti1-2 cells exhibited a strong block in ALP processing at the nonpermissive temperature but transported ALP normally at the permissive temperature (Figure 1, middle panel). ALP processing was partially blocked in yeast cells carrying either of two other vti1 alleles that exhibit a complete block in CPY processing at the nonpermissive temperature (our unpublished results).

These data indicate that Vti1p is required for an additional trafficking step, transport of ALP to the vacuole. The different vti1 mutants exhibited allele-specific differences in their effect on ALP traffic, even though all mutants showed a tight block in CPY traffic at the high temperature.

Next we tested whether the ALP processing defect in vti1-2 cells could be suppressed by overexpression of different yeast t-SNAREs (Figure 2). Overproduction of the vacuolar t-SNARE Vam3p in vti1-2 cells resulted in the appearance of a significant amount of mALP. The suppression of the ALP transport defect indicates that VTTI and VAM3 interact functionally and suggests that Vti1p and Vam3p form a SNARE complex in transport of ALP to the vacuole. Both Sed5p and Pep12p interact with Vti1p in retrograde traffic to the Golgi and in traffic from the Golgi to the PVC, respectively (Fischer von Mollard et al., 1997). Overexpression of the cis-Golgi t-SNARE Sed5p or the prevacuolar t-SNARE Pep12p did not have an effect on ALP processing in vti1-2 cells. This indicates that the suppression is specific for Vam3p, the t-SNARE that acts in vacuolar transport of ALP.

Vam3p is also required for delivery of CPY from the PVC to the vacuole (Darsow et al., 1997). We next tested whether overexpression of VAM3 in vti1 mutant cells had an effect on CPY sorting. CPY traffic was followed by pulse–chase labeling with 35S and immunoprecipitation. In vti1-1 cells overproduction of Vam3p did not suppress the CPY sorting defect (Figure 3A). As described before, overexpression of Pep12p resulted in a suppression of the CPY sorting defect, whereas overexpression of Sed5p had no effect.
The CPY sorting defect in \textit{vti1-2} cells was not suppressed by overproduction of either Pep12p or Vam3p at 36°C (Figure 3B). By contrast, at 31°C overproduction of Pep12p suppressed the partial CPY sorting defect quite efficiently. These results suggest that the \textit{vti1-2} protein retains partial folding and activity at 31°C, which enables Vti1p to function in the presence of high levels of Pep12p, but that this partial function is lost at 36°C. These data also indicate that \textit{vti1-2} cells are defective in Golgi-to-PVC traffic, which hinders analysis of CPY traffic from the PVC to the vacuole.

Deletion of \textit{vps} genes necessary for fusion of transport vesicles with the PVC or required for fusion of membranes derived from the PVC and from the ALP pathway with the vacuole results in a growth defect at 37°C (Piper et al., 1994; Becherer et al., 1996; Rieder and Emr, 1997). \textit{vti1-1} cells do not have a growth defect at the nonpermissive temperature. By contrast, \textit{vti1-2} cells displayed a growth defect at 37°C (Figure 4). This defect was more pronounced in strains with an integrated copy of \textit{vti1-2} than in the strains with the \textit{vti1-2} allele on a centromeric plasmid used in the earlier study (Fischer von Mollard et al., 1997). The temperature-sensitive growth defect in \textit{vti1-2} cells was partially suppressed by overexpression of either Vam3p or Pep12p. Overproduction of Sed5p in \textit{vti1-2} cells did not suppress the growth defect at 37°C, even though overproduction of Sed5p allows for growth at 30°C in the complete absence of Vti1p (\textit{vti1D}), because the Golgi traffic block is overcome (Fischer von Mollard et al., 1997; our unpublished data). The partial suppression of the growth defect in \textit{vti1-2} cells by both Vam3p and Pep12p indicates that some traffic either through the ALP pathway or through the PVC is restored and that this is sufficient for slow growth of \textit{vti1-2} cells at 37°C.

Indirect immunofluorescence was used as an independent method to study traffic of ALP in \textit{vti1-2} cells. Expression of \textit{PHO8} (encoding ALP) was placed un-

(Fischer von Mollard et al., 1997; Figure 3A). This experiment confirms that suppression is specific and that elevated levels of Vam3p cannot restore traffic through the PVC in \textit{vti1-1} cells.
der control of the GAL1 promoter. vti1-2 cells were grown at 24°C in the absence of galactose. Synthesis of ALP was induced at 24°C or concomitant with a shift to 35°C for 1 h. The localization of this newly synthesized ALP was determined by indirect immunofluorescence. At the permissive temperature ALP reached the vacuoles, as indicated by the ring-like staining that colocalized with the vacuolar indentation seen in the differential interference contrast picture (Figure 5, middle panel). This staining was similar to ALP staining in wild-type cells (Figure 5, top panel). In approximately half of the cells examined the vacuolar membranes were not stained homogeneously. Intensely stained dots outlined the vacuolar membrane, indicating that labeling was restricted or concentrated in parts of the vacuolar membrane. Greater than 90% of the cells exhibited vacuolar staining. By contrast, the ALP staining at the nonpermissive temperature appeared very diffuse (Figure 5, bottom panel). ALP clearly had not traveled to the vacuole, and the staining pattern is consistent with an accumulation of ALP in nonvacuolar vesicular or membrane intermediates in 60% of the cells. In addition, some vacuolar staining was observed in ~40% of the cells, especially after induction of ALP synthesis for 2 h at 35°C, indicating that the vti1-2 allele is leaky. This is consistent with the presence of some mALP observed in immunoprecipitations of newly synthesized ALP after long times (see Figure 1). The immunofluorescence results confirm that vti1-2 cells have a temperature-sensitive defect in ALP transport to the vacuole and indicate that ALP accumulates in nonvacuolar vesicular or membrane intermediates.

Vti1p Interacts with Vam3p in Traffic of API to the Vacuole
API reaches the vacuole from the cytoplasm independently of the secretory pathway (Klionsky, 1997, 1998). API is synthesized in the cytoplasm and packaged into vesicles with a double membrane, which fuse with the vacuole in a Vam3p-dependent reaction (Darsow et al., 1997). Therefore, we tested whether Vti1p is required for API transport to the vacuole. vti1-11 cells were used because they exhibit the strongest trafficking defects. vti1-11 cells are blocked in retrograde traffic to...
the cis-Golgi and in traffic of CPY from the TGN to the vacuole at the nonpermissive temperature (Fischer von Mollard et al., 1997). Cells were pulse labeled with 35S-Met/-Cys at the indicated temperatures and chased for 0, 30, 60, or 120 min with unlabeled cysteine and methionine. API was immunoprecipitated, and pAPI was separated from the vacuolar mAPI by SDS-PAGE. In wild-type cells 67% of the immunoprecipitated API was processed to mAPI after a 120-min chase period at 24°C (Figure 6A). In vti1-11 cells mAPI represented 48% of the total API after a 120-min chase at 24°C (Figure 6B), indicating that API matured with a slight kinetic delay in vti1-11 cells at the permissive temperature. At 36°C API matured with a half-time of <30 min in wild-type cells. By contrast, in vti1-11 cells processing of API was almost completely blocked at the nonpermissive temperature (Figure 6B). This block was almost as tight as the block in vam3Δ cells (Darsow et al., 1997; Figure 6A).

API processing was partially but reproducibly blocked in vti1-2 and vti1-1 cells at 36°C (Figure 7A). Whereas API was almost fully processed after a 60- or 120-min chase period in wild-type cells (>80 or >90% mAPI, respectively; see Figure 6A), only 24% of the total API was processed after 60 min, and 41% was processed after 120 min in vti1-1 cells at the restrictive temperature.

**Figure 5.** In vti1-2 cells newly synthesized ALP localizes to the vacuole at 24°C but remains in vesicles at 35°C. Expression of ALP was placed under the control of the GAL1 promoter in vti1-2 cells. ALP expression was induced for 1 h at 24°C or simultaneously with a shift to 35°C, and cells were prepared for indirect immunofluorescence. At 24°C ALP antibodies stained ring-like structures, which were identified as vacuoles by the indentations in the differential interference contrast picture (DIC, arrowheads). At 35°C ALP staining was not vacuolar but very diffuse, which is a pattern typical of vesicles. For comparison, ALP staining in wild-type cells (WT) is shown.
temperature. An average of 26% mAPI was found after a 60-min chase period, and 34% was found after a 120-min chase period in \( vti1-2 \) cells (four independent experiments). Considerably higher levels of mAPI were found in \( vti1-2 \) cells overproducing Vam3p (Figure 7B). In four independent experiments an average of 46% mAPI was observed after a 60-min chase period, and 62% mAPI was observed after a 120-min chase period. By contrast, overproduction of Pep12p did not improve API processing in \( vti1-2 \) cells.

This partial but t-SNARE–specific suppression of the API processing defect in \( vti1-2 \) cells by overproduction of Vam3p indicates that Vti1p is directly involved in an additional traffic step and that Vam3p and Vti1p form a SNARE complex in API traffic to the vacuole. VAM3 overexpression had no effect on API processing in \( vti1-11 \) cells (our unpublished results), suggesting that the suppression was allele specific and not a result of bypass suppression. Even though both ALP and API transport to the vacuole require Vam3p and Vti1p, the severity of the block induced by different \( vti1 \) alleles varied in the different membrane transport pathways. \( vti1-1 \) cells processed ALP with wild-type kinetics, whereas \( vti1-2 \) cells were completely blocked in ALP traffic. By contrast, both \( vti1-1 \) and \( vti1-2 \) cells displayed partial blocks in API transport. The trafficking defects in different \( vti1-ts \) mutants are summarized in Table 3.

**Role of Nyv1p in Biosynthetic Pathways to the Vacuole**

The only known v-SNAREs in vacuolar and endosomal traffic are Vti1p and Nyv1p. Nyv1p forms a SNARE complex with Vam3p in homotypic vacuolar fusion (Nichols *et al.*, 1997). It has been reported that \( nyv1\Delta \) cells contain mature CPY and mature ALP under steady-state conditions, but the kinetics of vacuolar transport were not studied (Nichols *et al.*, 1997). We therefore investigated the kinetics of CPY, ALP, and API transport to the vacuole in \( nyv1\Delta \) cells. We also explored possible genetic interactions between \( NYV1 \) and \( VTI1 \) by creating \( nyv1\Delta vti1-ts \) double mutants and by overproduction of Nyv1p in the various \( vti1-ts \) mutant cells.

CPY sorting was analyzed in wild-type and \( nyv1\Delta \) cells after a 10-min pulse followed by a 0-, 10-, or 20-min chase period (Figure 8A). Even directly after the pulse period (0-min chase) most of the immuno precipitated CPY was vacuolar mCPY in \( nyv1\Delta \) cells. Processing of CPY to the vacuole in \( nyv1\Delta \) cells was only slightly slower than in wild-type cells at 24°C. Processing of API was severely inhibited in \( vti1-ts \) cells at the restrictive temperature.
of CPY were secreted into the medium (20-min chase; Figure 8E) in 

nyvΔ cells, and this secretion level was comparable with that of wild-type cells. To investigate genetic interactions in CPY traffic, 

vti1-2 and 

nyvΔ vti1-2 cells were grown at 24°C, preincubated for 15 min at 31°C, and radiolabeled at 31°C as before. A semipermissive temperature was used, because this is the most sensitive condition in which to identify weak genetic interactions. The absence of Nyv1p slightly but reproducibly worsened the partial CPY trafficking defect in 

vti1-2 cells at 31°C (Figure 8A). Deletion of 

nyv1 

was also investigated. 

nyv1Δ as well as wild-type cells contained predominantly mAPI after a 60-min chase period, indicating that 

nyv1Δ cells processed API with normal kinetics (Figure 8C). Overexpression of 

NYV1 did not suppress the ALP sorting defect of 

vti1-2 cells at any temperature. Deletion of 

NYV1 in 

vti1-1 cells did not result in an ALP processing defect (our unpublished results). Therefore, Nyv1p is not required for ALP transport to the vacuole.

The effect of the 

nyv1Δ mutation on API transport was also investigated. 

nyv1Δ cells processed ALP with normal kinetics compared with wild-type cells (Figure 8B). The absence of Nyv1p did not aggravate the ALP transport defect in 

vti1-2 cells at the semipermissive temperature. By contrast, ALP processing was slightly more efficient in 

nyv1Δ vti1-2 cells compared with 

vti1-2 cells in four independent experiments. As seen at 31°C (Figure 8B), a slight improvement of ALP processing was also observed in 

vti1-2 cells upon deletion of 

nyv1 at 36°C. Overexpression of 

NYV1 did not suppress the ALP sorting defect of 

vti1-2 cells at any temperature. Deletion of 

NYV1 in 

vti1-1 cells did not result in an ALP processing defect (our unpublished results). Therefore, Nyv1p is not required for ALP transport to the vacuole.

Deletion of 

nyv1Δ cells at either 24 or 37°C (our unpublished results). These experiments demonstrate that Nyv1p does not play a demonstrable role in any of the pathways for biosynthetic delivery of proteins to the vacuole. In addition, there is no evidence for any genetic interaction between 

VTI1 and 

NYV1 in these biosynthetic trafficking pathways. By contrast, both 

NYV1 (Nichols et al., 1997; Ungermann et al., 1998) and 

VTI1 (Ungermann, Fischer von Mollard, Stevens, and Wickner, unpublished results) are required for homotypic vacuolar fusion and form a SNARE complex. The interaction of 

NYV1 and 

VTI1 in homotypic fusion may indirectly cause the slight worsening of CPY transport at semipermissive temperature in 

vti1-2 cells and the improvement of ALP transport in 

vti1-2 cells upon deletion of 

nyv1. Therefore, 

NYV1 seems to function exclusively in homotypic vacuolar fusion (Nichols et al., 1997; Ungermann et al., 1998).

Table 3. Transport defects in different 

vti1-ts mutants

<table>
<thead>
<tr>
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<th>vti1-1</th>
<th>vti1-2</th>
<th>vti1-11</th>
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<tbody>
<tr>
<td>Retrograde to cis-Golgi</td>
<td>+</td>
<td>+</td>
<td>ts</td>
</tr>
<tr>
<td>TGN to PVC (CPY)</td>
<td>ts</td>
<td>ts</td>
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<tr>
<td>TGN to vacuole (ALP)</td>
<td>+</td>
<td>ts</td>
<td>nd</td>
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<tr>
<td>Cytosol to vacuole (API)</td>
<td>ts</td>
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+ no defect; ts, transport defect at 36°C; nd, not determined, because pALP that accumulated could be due to a block in traffic either to the cis-Golgi or from the TGN to the PVC.

*a Very slight block at 37°C.
DISCUSSION

This study identifies Vti1p as a v-SNARE required for both the API and the ALP membrane traffic pathways to the yeast vacuole, indicating that Vti1p is required for all three biosynthetic pathways to the vacuole. Here we demonstrate that a single v-SNARE, yeast Vti1p, is required for at least four transport steps and is able to interact functionally with three different t-SNAREs. Given the assumption that v-SNAREs determine specificity in membrane traffic, these results raise serious questions about how transport vesicles recognize the correct target membrane.

Implication for Vacuolar Membrane Traffic

Here we show that Vti1p interacts functionally with the vacuolar t-SNARE Vam3p in at least two different biosynthetic pathways to the vacuole (Figure 9). Vti1p is required for transport of ALP from the Golgi apparatus to the vacuole. In addition, Vti1p is involved in traffic of API from the cytosol to the vacuole through the CVT pathway. We conclude that Vti1p is directly involved in these trafficking steps for two reasons. First, processing of ALP and API is rapidly blocked upon shift to nonpermissive temperature, thus greatly minimizing the possibility of the indirect effects seen in deletion mutants. API transport could still be affected indirectly if efficient traffic to the PVC, which is blocked in vti1-ts mutants, is required for API transport. For example, the PVC could serve as the unknown membrane source for CVT vesicles. Second, overproduction of the t-SNARE Vam3p partially but reproducibly suppressed the ALP and API processing defects in certain vti1-ts mutants. This suppression was specific because overproduction of NYV1 did not influence API processing in a vti1-2 background. These data indicate that NYV1 is not required for CPY, ALP, or API traffic, and that VTI1 and NYV1 do not interact genetically.

Figure 8. Nyv1p is not required for transport of CPY, ALP, or API into the vacuole. Transport was analyzed in wild-type cells, nyv1Δ cells, vti1-2 cells, vti1-2 nyv1Δ double mutant cells, and vti1-2 cells overexpressing NYV1 from a 2μ plasmid. (A) CPY traffic was followed by pulse-chase immunoprecipitations at 31°C. CPY was transported to the vacuole and processed in nyv1Δ cells (mCPY). CPY traffic at the semipermissive temperature was affected to a similar degree in vti1-2, vti1-2 nyv1Δ double mutant and vti1-2 cells overexpressing NYV1. (B) ALP traffic was analyzed at 31°C by pulse-chase immunoprecipitations. ALP was delivered to the vacuole with normal kinetics in nyv1Δ cells. Deletion or overexpression of NYV1 did not change ALP traffic in a vti1-2 background. (C) API traffic was studied by pulse-chase immunoprecipitations at 36°C. API transport was unaffected in nyv1Δ cells. Deletion or overexpression of NYV1 did not influence API processing in a vti1-2 background. These data indicate that NYV1 is not required for CPY, ALP, or API traffic, and that VTI1 and NYV1 do not interact genetically.
biosynthetic pathways into the vacuole. Therefore, the SNARE complex involved in trafficking of ALP and API to the vacuole contains Vam3p (Darsow et al., 1997; Piper et al., 1997), Vam7p (Sato et al., 1998) and Vt1p but probably not Nyv1p. This means that different SNARE complexes form in homotypic vacuolar fusion and in biosynthetic traffic to the vacuole.

Our data indicate that the SNARE complexes involved in ALP and API transport to the vacuole are different from each other. Yeast cells carrying the temperature-sensitive allele \textit{vti1-2} display a complete block in ALP processing at the nonpermissive temperature. By contrast, ALP is delivered to the vacuole with normal kinetics in \textit{vti1-1} cells at the nonpermissive temperature for CYP transport. Delivery of API to the vacuole is partially blocked and affected to a similar degree in both \textit{vti1-1} and \textit{vti1-2} cells at the non-permissive temperature. These allele-specific differences suggest that different sets of additional proteins are required for ALP versus API traffic in addition to Vti1p and Vam3p. These results also imply that the ALP and API pathways do not converge before fusion with the vacuole. Instead, membranes from either pathway form separate SNARE complexes with Vam3p in fusion with the vacuolar membrane.

In the CVT pathway a double membrane engulfs oligomerized cytosolic API to form CVT vesicles (Klionsky, 1997, 1998). Because Vti1p is required for their vacuolar delivery, we assume that Vti1p is localized on the CVT vesicles. However, it is still unclear from which compartment the membranes forming the CVT vesicles originate. Vti1p has to be targeted to this membrane source. It also remains to be established whether ALP travels to the vacuole directly in Golgi-derived transport vesicles or through an intermediate compartment. Because Vti1p is localized to the Golgi, it could be incorporated into ALP-containing vesicles at this point. The role of Vti1p in fusion of transport vesicles with the vacuolar membrane means that Vti1p itself is transported to the vacuole. Under normal conditions little Vti1p is localized in the vacuole, even though Vti1p is a very stable protein (Fischer von Mollard et al., 1997; Bryant et al., 1998). Only upon overexpression does a significant amount of Vti1p redistribute to the vacuole (our unpublished observations). This suggests that Vti1p may be able to recycle from vacuolar membranes to participate in new rounds of vesicle transport. We have recently discovered a retrograde traffic pathway out of the vacuole to the PVC (Bryant et al., 1998). Vti1p accumulates in the vacuole in \textit{vac7} mutant cells, indicating that Vti1p indeed is transported to the vacuole and that Vac7p is involved in retrograde traffic out of the vacuole in addition to its role in vacuolar inheritance (Bonangelino et al., 1997; Bryant et al., 1998).

\textbf{Implication for the Role of v-SNAREs in Specificity}

This study demonstrates that Vti1p interacts functionally with the vacuolar \textit{t-SNARE} Vam3p in two different biosynthetic pathways to the vacuole. Earlier we demonstrated that Vti1p interacts with Pep12p in traffic from the Golgi to the prevacuole and with Sed5p in retrograde traffic to the \textit{cis-Golgi} compartment (Fischer von Mollard et al., 1997). Different trafficking steps are affected in various \textit{vti1-ts} mutants. \textit{vti1-1} mutants are completely blocked in traffic from the TGN to the PVC and partially in API traffic to the vacuole. The \textit{vti1-1} protein contains the amino acid exchanges E145K and G148R (Fischer von Mollard and Stevens, 1998). \textit{vti1-2} mutants are blocked in traffic from the Golgi to the TGN, in traffic of ALP to the vacuole, and partially in API traffic to the vacuole. The amino acid alterations are S130P and I151T in the \textit{vti1-2} protein. The \textit{vti1-11} protein is defective in interactions with Sed5p, Pep12p, and Vam3p and contains the amino acid exchanges E145G and L155F. These amino acid exchanges are all clustered in a short, evolutionary conserved domain. This domain is predicted to form an \textit{a}-helical structure, and all mutations are localized on the hydrophobic face of the predicted helix (Fischer von Mollard and Stevens, 1998). This domain probably represents the interaction site between Vti1p and different t-SNAREs. Amino acid exchanges on this surface seem to affect interactions with different t-SNAREs in specific ways. This hypothesis is strengthened by the recent finding that the equivalent domains in the synaptic SNARE complex form a four-
helix bundle with leucine zipper-like layers and a central ionic interaction between an arginine and three glutamine residues (Sutton et al., 1998).

It was also shown that Vti1p is able to bind to five of the eight syntaxin-related t-SNAREs identified in the yeast genome (Fischer von Mollard et al., 1997; Lupashin et al., 1997; Holthuis et al., 1998). Vti1p does not interact with Sso1p/Sso2p in secretory traffic from the Golgi to the plasma membrane and does not interact with Ufe1p in retrograde traffic to the ER. Vti1p binds Tlg1p and Tlg2p in addition to Sed5p, Pep12p, and Vam3p (Holthuis et al., 1998a). Both Tlg1p and Tlg2p are t-SNAREs required for endocytosis and for maintenance of normal levels of TGN proteins (Abeliovich et al., 1998; Holthuis et al., 1998a; Sérón et al., 1998).

Tlg2p has been localised to the TGN and endosomes, whereas Tlg1p was found in a novel compartment that may represent the early endosome in yeast. These data indicate that Vti1p may function in additional steps in post-Golgi/endosomal/vacuolar traffic.

A single v-SNARE or combinations of different v-SNAREs may be required for interactions with different t-SNAREs. Aside from Vti1p, Sec22p/Sly2p is the only other v-SNARE implicated in more than one fusion step. Sec22p interacts with Sed5p in traffic from the ER to the Golgi. Efficient binding of Sec22p to Sed5p requires the presence of the v-SNAREs Bet1p/Sly12p and Bos1p (Sacher et al., 1997; Stone et al., 1997). Sec22p is also required for retrograde traffic to the ER and binds to the ER t-SNARE Ufe1p. Bos1p and Bet1p were not found in the Sec22p–Ufe1p complex (Lewis et al., 1997). Therefore a Sec22p–Bet1p–Bos1p complex could serve as a targeting signal for anterograde traffic to the cis-Golgi, whereas Sec22p alone could direct retrograde traffic to the ER.

Vti1p interacts genetically with Sty1p (Lupashin et al., 1997). Sty1p is a v-SNARE involved in retrograde traffic within the Golgi apparatus and binds to Sed5p (Banfield et al., 1995). In addition, Vti1p interacts both genetically and physically with Ykt6p (Lupashin et al., 1997). The v-SNARE Ykt6p is also found in a complex with Sed5p (Sogaard et al., 1994). Therefore, it is possible that Vti1p requires the presence of both Sty1p and Ykt6p for functional interaction with Sed5p in retrograde traffic to the cis-Golgi, despite the fact that recombinant Sed5p and Vti1p bind each other.

The presence of additional v-SNAREs could explain specificity in the binding interactions between Vti1p and the other t-SNAREs. Nyv1p is the only other known v-SNARE localized to endosomal or vacuolar membranes. Nyv1p is involved in homotypic vacuolar fusion (Nichols et al., 1997). In this study we demonstrate that Nyv1p is not required for any of the three biosynthetic pathways into the vacuole and that Nyv1p does not interact with Vti1p in these pathways. Therefore, Nyv1p seems to be involved specifically in a single fusion step, homotypic vacuolar fusion.

Binding has been detected between Tlg1p and Snc1p (Holthuis et al., 1998a) and between Tlg2p and Snc2p (Abeliovich et al., 1998). Snc1p and Snc2p are v-SNAREs that are localized on secretory vesicles and interact with the plasma membrane t-SNAREs Sso1p and Sso2p in secretion (Protopenkov et al., 1993). The functional relevance of the interactions between Tlg1p and Vti1p, between Tlg1p and Snc1p, and between Tlg2p and Snc2p are not yet known. Still, not enough other v-SNAREs have been identified that could contribute to specificity in interactions of Vti1p with several t-SNAREs. It is possible that v-SNAREs were missed by sequence comparisons, because v-SNAREs share less amino acid identity with each other, and they lack a clearly identifiable conserved sequence motif in contrast to t-SNAREs. Therefore, it seems more likely that Vti1p has a role as a general fusion protein and is only one factor contributing to specificity in membrane traffic. Whether Vti1p is unique in this capacity cannot be determined from the data currently available.

Members of the Ypt/rab family of small GTPases are thought to contribute to specificity in membrane traffic (Lazar et al., 1997). A recent study demonstrates that Ypt1p is required for membrane binding of the protein Usolp (Cao et al., 1998). Usolp acts before the assembly of the SNARE complex and probably tethers ER-derived transport vesicles to Golgi membranes (Sapperstein et al., 1996). Recently, the TRAPP protein complex was identified on cis-Golgi membranes (Sacher et al., 1998). This protein complex may also be involved in tethering, because genetic interactions with SNAREs required for ER to cis-Golgi traffic and with USO1 were found. The tethering step is followed by SNARE-dependent docking and fusion. Therefore, it is possible that specificity in membrane traffic could be achieved through the sequential reactions of Ypt-regulated initial tethering and SNARE-dependent docking. Several Ypt proteins are involved in endosomal and vacuolar transport pathways. Vps21p/Ypt51p has been implicated in Golgi to prevacuolar and in endosomal traffic. Ypt7p is needed for traffic to the vacuole and for homotypic vacuolar fusion. Ypt6p is probably involved in retrograde traffic to the Golgi apparatus. Two additional Ypt proteins, Ypt10p and Ypt11p, were identified by sequence homology. Their localization and function has not yet been determined. Deletion of the YPT10 or YPT11 gene does not cause the severe phenotypes expected for proteins required in the secretory pathway, making it possible that Ypt10p and Ypt11p also play a role in endosomal or vacuolar trafficking (Lazar et al., 1997). Therefore, Ypt/rab proteins are good candidates to contribute to specificity in Vti1p dependent trafficking steps.

In conclusion, our work reveals that Vti1p regulates multiple vesicle transport steps to different organelles.
Therefore, trafficking of Vti1p itself must be very complex and highly regulated.

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