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Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE–Sec1p complex composed of Tlg2p and Vps45p

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Aminopeptidase I (API) is imported into the yeast vacuole/lysosome by a constitutive non-classical vesicular transport mechanism, the cytoplasm to vacuole targeting (Cvt) pathway. Newly synthesized precursor API is sequestered in double-membrane cytoplasmic Cvt vesicles. The Cvt vesicles fuse with the vacuole, releasing single-membrane Cvt bodies containing pro-API into the vacuolar lumen, and maturation of API occurs when the Cvt body is degraded, releasing mature API. Under starvation conditions, API is transported to the vacuole by macroautophagy, an inducible, non-selective mechanism that shares many similarities with the Cvt pathway. Here we show that Tlg2p, a member of the syntaxin family of t-SNARE proteins, and Vps45p, a Sec1p homologue, are required in the constitutive Cvt pathway, but not in inducible macroautophagy. Fractionation and protease protection experiments indicate that Tlg2p is required prior to or at the step of API segregation into the Cvt vesicle. Thus, the early Vps45–Tlg2p-dependent step of the Cvt pathway appears to be mechanistically distinct from the comparable stage in macroautophagy. Vps45p associates with both the Tlg2p and Pep12p t-SNAREs, but API maturation is not blocked in a *pep12*^{ts} mutant, indicating that Vps45p independently regulates the function of multiple t-SNARES at distinct trafficking steps.

Keywords: autophagy/Cvt/rapamycin/Sec1p/Tlg2 t-SNARE

Introduction

The yeast vacuole is analogous to the lysosome of mammalian cells, and the transport pathways that mediate delivery of hydrolases to these organelles are similar in both yeast and mammals. In yeast, the vacuole receives membrane traffic through at least five different transport pathways: the carboxypeptidase Y (CPY) pathway, the alkaline phosphatase (ALP) pathway, the endocytic pathway, autophagy and the cytoplasm to vacuole targeting (Cvt) pathway. Each of these pathways has different cargo, transport intermediates and genetic requirements. This multiplicity allows the analysis of the specificity and degree of functional overlap between transport components that control these distinct, convergent pathways. Newly synthesized proteins that enter the early part of the secretory pathway reach the vacuole by one of two biosynthetic routes. The CPY pathway delivers proteins such as CPY to the vacuole via the late endosome, a point of intersection between biosynthetic and endocytic traffic, and requires the function of the endosomal syntaxin, Pep12p (Becherer *et al*., 1996; Burd *et al*., 1997). The ALP pathway does not require Pep12p function and is presumed to bypass endosomal intermediates, but requires the function of the AP-3 adaptor protein complex (Cowles *et al*., 1997a,b). Down-regulation and degradation of cell surface receptors, as well as turnover of other integral plasma membrane proteins (e.g. nutrient transporters), occurs via endocytosis (Vida *et al*., 1993; Babst *et al*., 1997; Hicke *et al*., 1997). Autophagy, an inducible membrane trafficking mechanism, is activated in response to nitrogen starvation or administration of the macrolide antibiotic rapamycin (Takeshige *et al*., 1992; Egner *et al*., 1993; Noda and Ohsumi, 1998). During autophagy, large double-membraned vesicles (500 nm) non-selectively surround cytoplasmic proteins and organelles to form autophagosomes. The outer autophagosomal membrane fuses with the vacuole to release a single membrane-bounded autophagic body into the vacuolar lumen. The autophagic bodies are then degraded in a step that depends on active vacuolar hydrolases (Baba *et al*., 1994, 1997). Finally, the Cvt pathway is a constitutive biosynthetic pathway which shares many common transport components with the autophagy pathway (Harding *et al*., 1996; Baba *et al*., 1997). The vacuolar enzyme aminopeptidase I (API) is synthesized on soluble ribosomes and transported to the vacuole via the Cvt pathway. Newly synthesized API undergoes homo-oligomerization and then becomes specifically associated with membranes. API is surrounded by these membranes to form the double-membrane Cvt vesicle (130–150 nm). The Cvt vesicle then fuses with the vacuole, releasing a single bilayer vesicle, the Cvt body, into the vacuolar lumen. Soluble API is released into the vacuolar lumen upon lysis of Cvt bodies, and the propeptide is processed to yield the mature protein. This view of the Cvt pathway involves at least two membrane fusion reactions, and indeed the process appears to depend on *SEC18*, which encodes yeast NSF as well as the vacuolar t-SNARE Vam3p. Aside from NSF, none of the known components of the protein targeting and membrane fusion machinery in yeast has been specifically implicated in the formation of Cvt bodies to date.

Membrane transport reactions in eukaryotic cells often involve vesicular carriers, which bud from a donor compartment and fuse with a target membrane (Rothman and Wieland, 1996). Small GTP-binding proteins of the rab family mediate reversible tethering events which are a prerequisite for the formation of a protein complex between integral proteins of the vesicle and target membrane, v-SNAREs and t-SNAREs, in what is called the

SNARE complex (Sollner *et al*., 1993; Pfeffer, 1996; Ungermann *et al*., 1998). Although the SNARE hypothesis was formulated with regard to vesicular trafficking, it has since been shown that SNARE proteins mediate homotypic fusion events of vacuolar membranes (Nichols *et al*., 1997), endoplasmic reticulum (ER) membranes (Patel *et al*., 1998) and Golgi membranes (Rabouille *et al*., 1998). There are two families of t-SNARE proteins: SNAP-25 homologues and syntaxin homologues (Weimbs *et al*., 1997). In yeast, the vacuolar/endocytic system contains at least three syntaxin homologues: Vam3p, Pep12p and Tlg2p. These three proteins constitute a subfamily of yeast syntaxins, related by sequence similarity. Vam3p is located at the vacuolar membrane and is required for membrane docking/fusion events at the vacuole (Darsow *et al*., 1997). In contrast, Pep12p is enriched on endosomal membranes and is required, together with the Sec1p homologue Vps45p, for biosynthetic traffic from the Golgi to the vacuole along the CPY pathway which traverses the pre-vacuolar endosome (Becherer *et al*., 1996).

The primary function of Tlg2p remains somewhat controversial. Initial studies of the phenotypes of *tlg2Δ* cells and of the general properties of the protein have been published (Abeliovich *et al*., 1998; Holthuis *et al*., 1998; Seron *et al*., 1998). Tlg2p is known to form SNARE complexes with the v-SNAREs Snc1p, Snc2p and Vti1p, and has been shown to localize to late Golgi and endosomes. Yeast lacking Tlg2p show reduced rates of endocytosis as well as very mild sorting defects in the trafficking of CPY to the vacuole. Apart from these kinetic effects on protein transport, no true block in any protein trafficking pathway has been described for *tlg2Δ* cells.

We have uncovered an essential role for Tlg2p in biosynthetic API trafficking to the vacuole, and show that it is involved at an early membrane trafficking event that precedes the sequestration of API into a protease-inaccessible Cvt vesicle. Surprisingly, although *tlg2Δ* cells are defective in the Cvt pathway, they are not defective in regulated autophagy. This implies that autophagy and the Cvt pathway diverge with respect to some aspects of their membrane trafficking requirements. Finally, we demonstrate that Vps45p, a Sec1p homologue, is required for API trafficking and that this reflects a physical interaction between Vps45p and Tlg2p. Because Vps45p also interacts with the endosomal t-SNARE Pep12p, our data demonstrate that Vps45p interacts with two different syntaxin homologues in regulating two separate membrane trafficking events.

Results

API maturation is the major biosynthetic trafficking defect in tlg2Δ cells

In wild-type cells, the core-glycosylated 67 kDa ER form of CPY (p1CPY) is modified further in the Golgi complex, resulting in the 69 kDa, p2 form of CPY, which is transported to the vacuole where it is processed to yield mature, 61 kDa CPY (mCPY). Likewise, ALP is a glycoprotein that reaches the vacuole in a 74 kDa proenzyme form (proALP). In the vacuole, proALP is processed to yield the mature, 72 kDa form (mALP). API is synthesized on cytoplasmic ribosomes as a 61 kDa precursor and its transport to the vacuole via the Cvt pathway can be monitored through its proteolytic conversion from the precursor (proAPI) to the 50 kDa mature form (mAPI).

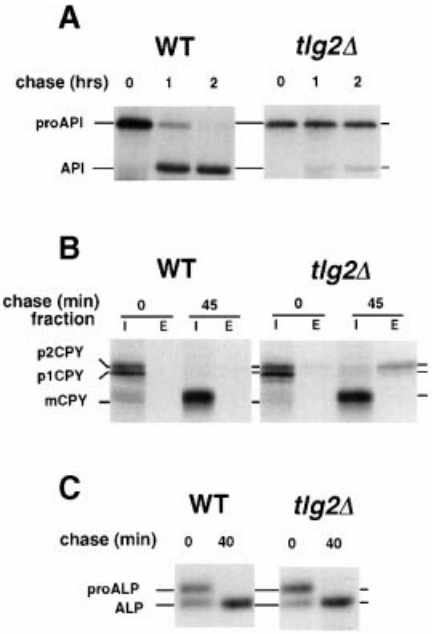


Fig. 1. The predominant biosynthetic trafficking defect of *tlg2Δ* cells involves API maturation. (**A**) Wild-type and *tlg2Δ* cells were pulsed with ^{35}S-labelled cysteine and methionine and chased as described in Materials and methods. Samples (2 A_{600} equivalents) were drawn at 0, 1 and 2 h of chase, and the lysates were immunoprecipitated with antiserum to API. (**B**) Wild-type and *tlg2Δ* spheroplasts were pulse–chased with ^{35}S-labelled cysteine and methionine. Aliquots were removed at 0 and 45 min of chase. Spheroplasts were separated from the medium, lysed, and both cell lysates and medium were subjected to immunoprecipitation with antibody to CPY. I, intracellular fraction; E, extracellular fraction. (**C**) Wild-type and *tlg2Δ* cells were pulse–chased as described in (A). Samples were drawn at 0 and 40 min of chase, and lysates were immunoprecipitated with antibody to ALP.

To examine the trafficking of these marker proteins in *tlg2Δ* cells, wild-type and *tlg2Δ* cells were metabolically labelled with Trans^{35}S-label and chased as described in the legend to Figure 1. Protein extracts from these cells were immunoprecipitated with antibodies to CPY, ALP and API. We find that in cells lacking Tlg2p, ALP maturation appears entirely normal (Figure 1C). CPY is matured normally, with a small fraction of p2 CPY (~10%) being missorted and secreted (Figure 1B). However, API maturation is blocked in *tlg2Δ* cells (Figure 1A). In wild-type cells, the half-time for API maturation has been shown to be 30–45 min in such experiments (Klionsky *et al*., 1992). We found that in *tlg2Δ* cells <3% of the newly synthesized API is found in the mature form after a 2 h chase at 30°C (Figure 1). We conclude that although loss of Tlg2p does not affect biosynthetic trafficking through the secretory pathway to the vacuole, it strongly affects maturation of API, suggesting that Tlg2p functions in the Cvt pathway.

Rapamycin-induced autophagy bypasses the API transport block in tlg2Δ cells

API transport to the vacuole is known to occur by two related mechanisms. Constitutive transport is carried out by the Cvt pathway, while inducible and selective uptake

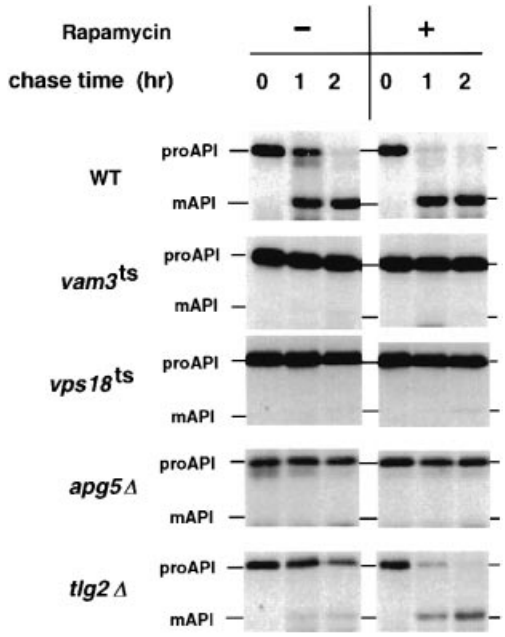


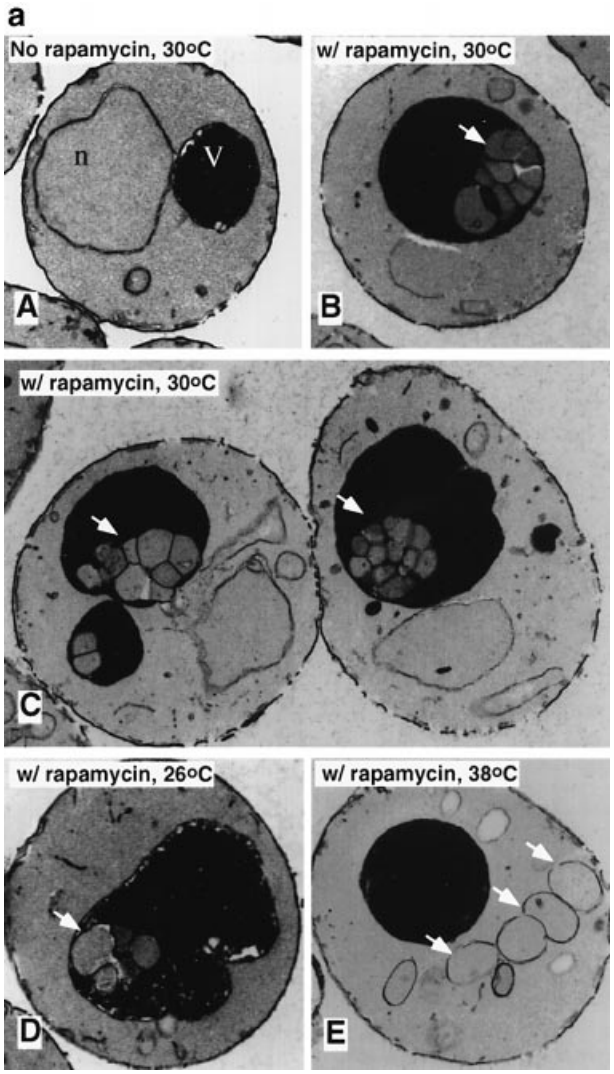
Fig. 2. The API maturation defect of *tlg2Δ* yeast is suppressed by the addition of rapamycin. *vam3*^{ts} and *vps18*^{ts} were grown overnight at 26°C and shifted to non-permissive temperature (37°C) for 20 min prior to labelling. The cells were then labelled for 15 min with [^{35}S]cysteine/methionine, and chased with unlabelled cysteine and methionine in the presence or absence of 0.2 μg/ml rapamycin (diluted from a 1 mg/ml stock dissolved in 10% Tween-20/90% ethanol). Wild-type, *apg5Δ* and *tlg2Δ* yeast were grown overnight at 30°C, and pulsed–chased as above, in the presence or absence or rapamycin. Samples were taken at the indicated time points and immunoprecipitated with antibody to API.

of API can also occur by the induction of autophagy through nitrogen starvation or addition of the macrolide antibiotic rapamycin to the medium. A significant degree of overlap has been uncovered between mutants isolated as defective in the Cvt pathway and mutants that were isolated as defective in autophagy (Harding *et al*., 1996; Scott *et al*., 1996). The two processes are, however, distinct in terms of both the morphology of the transport intermediates and the existence of *cvt* mutants that are not defective in autophagy, and vice versa (Harding *et al*., 1996). We were interested in defining whether Tlg2p is required only for constitutive API transport or also for autophagy-mediated transport of API to the vacuole. It has been shown previously that addition of rapamycin to yeast cells mimics the induction of autophagy by nitrogen starvation (Noda and Ohsumi, 1998). To assay the effects of macroautophagy on API maturation in *tlg2Δ* cells, we performed pulse–chase experiments as described above, except that after the radioactive labelling period, cells were diluted into medium (see Materials and methods) that either contained or did not contain 0.2 μg/ml rapamycin. When rapamycin is added to *tlg2Δ* cells during the chase period, rapid and near-complete maturation of proAPI is observed (Figure 2). Thus rapamycin-induced autophagy appears to bypass the requirement for Tlg2p.

If the rapamycin-induced bypass of the block to API maturation in *tlg2Δ* cells is the result of autophagy-dependent trafficking, then mutants known to block autophagy should be insensitive to rapamycin. Specific mutants such as *apg5Δ* cells, which are blocked in the formation of autophagosomes (Tsukada and Ohsumi, 1993; Noda and Ohsumi, 1998), or *vam3*^{ts} and *vps18*^{ts}, mutants that are blocked in the consumption of autophagosomes by the vacuole (Darsow *et al*., 1997; Scott *et al*., 1997), would not be sensitive to the addition of rapamycin. To test this, *apg5Δ*, *vam3*^{ts} and *vps18*^{ts} cells were pulse-labelled, chased in the presence or absence of rapamycin, and API was immunoprecipitated as above. In contrast to the ability of rapamycin to bypass the *tlg2Δ* defect, we find that rapamycin does not cause maturation of proAPI in *vam3*^{ts}, *vps18*^{ts} or *apg5Δ* cells, implying that autophagic transport into the vacuole is required for rapamycin-dependent maturation in *tlg2Δ* cells and that the bypass observed in *tlg2Δ* cells that are challenged with rapamycin reflects vacuolar processing of proAPI.

tlg2Δpep4Δ double mutants accumulate autophagic bodies in the vacuole in response to rapamycin and nitrogen starvation

The rapamycin suppression experiments suggest that *tlg2Δ* cells are not defective in macroautophagy. Autophagic bodies are normally degraded in the vacuole in a proteinase B (PrB)-dependent fashion (Takeshige *et al*., 1992). Their accumulation in the vacuole upon induction of autophagy can be observed by light and electron microscopy either in vacuolar protease mutants such as *pep4*, in which autophagic bodies are stable, or by inactivating PrB with protease inhibitors in wild-type cells. Thus, upon treatment with rapamycin, *pep4Δ* cells accumulate autophagic bodies in the vacuole (Noda and Ohsumi, 1998). To examine further the process of macroautophagy in *tlg2Δ* cells, we assayed the morphology of *tlg2Δpep4Δ* double mutants following administration of rapamycin, and compared them with *pep4Δ* cells and *vam3*^{ts} cells. Untreated *pep4Δ* cells appear wild-type in morphology, with a large, prominent vacuole and nucleus. However, when treated with 0.2 μg/ml rapamycin for 2 h, *pep4Δ* cells show a dramatic accumulation of autophagic bodies (~300 nm in diameter) in the vacuole as previously described. When *vam3*^{tsf}*pep4Δ* cells are treated with rapamycin at the permissive temperature (26°C), they appear identical to *pep4Δ* cells. However, at elevated temperatures (38°C), these cells accumulate autophagosomes in the cytoplasm, apparently unable to dock and/or fuse with the vacuole. These results are identical to those observed when autophagy is induced by nitrogen starvation (Darsow *et al*., 1997), providing further evidence that rapamycin treatment faithfully mimics induction of autophagy by nutrient starvation. When *tlg2Δpep4Δ* cells were tested in the same manner, we found that autophagic bodies accumulate in the vacuole. Thus, unlike its role in constitutive API trafficking, Tlg2p is not required for the induction and formation of autophagosomes or their targeting and fusion with the vacuole. Finally, to rule out the possibility that rapamycin-induced macroautophagy is different from starvation-induced macroautophagy, we tested whether *tlg2Δpep4Δ* cells can accumulate autophagic bodies under nitrogen starvation conditions, a classical assay for autophagy (Takeshige *et al*., 1992; Thumm *et al*., 1994). We find that, while the control *apg5*Δ*pep4Δ* cells do not accumulate autophagic bodies after a 3 h incubation in SD(–N) medium or acetate medium, both *pep4Δ* and



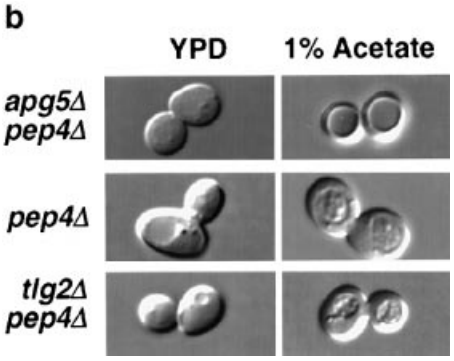


Fig. 3. *tlg2Δ pep4Δ* double mutants accumulate autophagic bodies in response to rapamycin and nitrogen starvation. (**a**) Yeast cells were grown in YPD (A, B and C) or YNB (D and E) to mid-log phase, harvested and transferred to fresh medium with or without 0.2 μg/ml rapamycin, as indicated. The cells were incubated for a further 2 h at the indicated temperature for induction of autophagy, and fixed for electron microscopy as described in Materials and methods. (A and B) Examples of *pep4Δ* cells incubated without (A) or with (B) rapamycin. The white arrow denotes the accumulation of autophagic bodies in the vacuole. (C) Examples of *tlg2Δ pep4Δ* cells following rapamycin treatment. The white arrow denotes the accumulation of autophagic bodies in the vacuole. (D and E) *vam3*^{tsf}*pep4Δ* cells were treated with rapamycin at the permissive (26°C; C and D) or non-permissive (38°C; E) temperature. Arrows denote the accumulation of autophagic bodies in the vacuole (D) or autophagosomes in the cytoplasm (E). n, nucleus; V, vacuole. (**b**) Yeast were grown in YPD as above, harvested, washed in water and resuspended in 1% acetate or YPD. The cells were incubated at 30°C for 3 h prior to viewing.

tlg2Δpep4Δ cells accumulated autophagic bodies in >90% of the cells examined (Figure 3b).

tlg2Δ cells accumulate proAPI in a membrane-associated, but protease-accessible state

Previous studies have defined stages in API synthesis and vacuolar transport, based on a combination of fractionation and protease protection assays (Harding *et al*., 1995; Scott *et al*., 1997). API is synthesized initially as a soluble monomeric form that rapidly oligomerizes to form dodecamers (Kim *et al*., 1997). The dodecameric form of the protein then associates with membranes, and the membrane-bound form is engulfed, generating the double-membrane Cvt vesicle, which in turn fuses with the vacuole. We utilized these protease protection and fractionation assays in determining the step at which API transport is blocked in *tlg2Δ* cells. Spheroplasted yeast cells were pulsed with Trans^{35}S-label, and chased for 90 min. The spheroplasts were then subjected to differential osmotic lysis (Scott and Klionsky, 1995), and fractionation by differential centrifugation (Scott *et al*., 1997) (Figure 4A). Under our conditions, >90% of API was matured in wild-type cells at this time point, while no mature API was observed in *tlg2Δ* spheroplasts, recapitulating the observations made with whole cells.

We find that the proAPI, which accumulates in *tlg2Δ* mutants, fractionates primarily in the 5000 *g* pellet, while >90% of the cytosolic marker glucose-6-phosphate dehydrogenase (G6PDH) was found in the supernatant. When fractionation was conducted in the presence of 0.5% Triton X-100, proAPI was found in the supernatant fraction, implying that the sedimentation of the protein into the 5000 *g* pellet reflects a membrane association. To understand whether proAPI was sequestered in a membrane-bound compartment in these cells, we performed protease protection assays. As controls, we compared *tlg2Δ* cells with *vam3*^{ts}, *pep4Δ* and *apg7Δ* mutants. In *vam3*^{ts} cells, the fusion of Cvt vesicles with the vacuole is inhibited at the non-permissive temperature, predicting the accumulation of proAPI in protease resistant Cvt vesicles. In protease-deficient *pep4Δ* cells, proAPI accumulates within the vacuole in stable Cvt bodies, again predicted to be protease resistant. In *apg7Δ* cells, the Cvt pathway has been shown to be blocked at a stage prior to vesicle completion, and proAPI accumulates in a protease-sensitive state (Kim *et al*., 1999).

Lysates prepared from these mutants by differential osmotic lysis were incubated in the presence or absence of proteinase K. We found that >90% of the proAPI that accumulated in *vam3*^{ts} and *pep4Δ* cells was resistant to

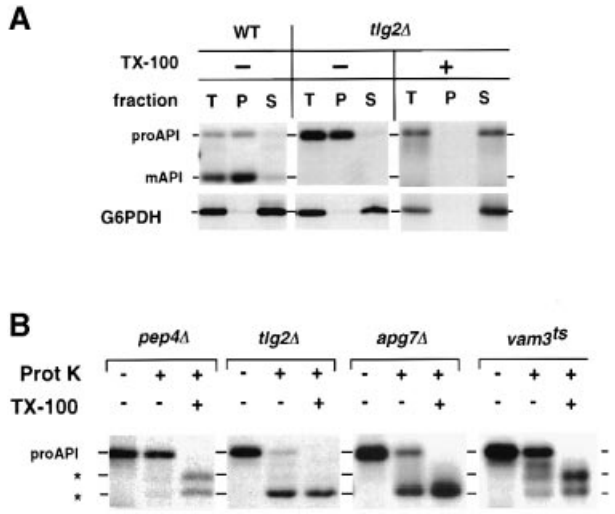


Fig. 4. *tlg2Δ* cells accumulate proAPI in a membrane-bound, non-sequestered form. (**A**) proAPI accumulates in *tlg2Δ* cells in a membrane-associated form. Wild-type and *tlg2Δ* spheroplasts (6 OD_{600} units) were pulse-labelled and chased for 90 min. Cells were lysed by treatment with PS100 buffer in the presence or absence of 0.5% Triton X-100, as indicated. The lysates were fractionated into 5000 *g* supernatant (S) and pellet (P). Equivalent amounts (1.5 OD_{600} units) of total (T), supernatant and pellet fractions were immunoprecipitated with antibodies to API, CPY and G6PDH. (**B**) proAPI that accumulates in *tlg2Δ* cells is accessible to exogenously added protease. The indicated yeast mutants were pulse–chased and lysed as above, and whole-cell extracts were treated with buffer control, 100 μg/ml proteinase K or 100 μg/ml proteinase K + 0.2% Triton X-100. *vam3*^{ts} mutants were pre-shifted to non-permissive temperature (37°C) for 20 min prior to labelling. *tlg2Δ* and *apg7Δ* cells were grown and labelled at 30°C.

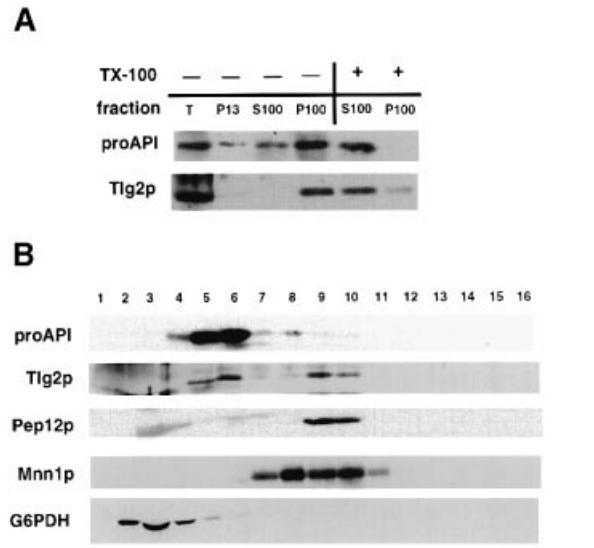


Fig. 5. A pool of Tlg2p co-fractionates with proAPI that accumulates in *vam3*^{ts} cells at non-permissive temperature. A culture of *vam3*ts cells was grown to exponential phase and shifted to 37°C for 1 h. The cells (50 A_{600} units) were harvested, lysed and the lysate was clarified by centrifugation at 300 *g* for 5 min. The clarified lysate (S3, total extract) was fractionated into a 13 000 *g* pellet (P13) and 13 000 *g* supernatant (S13), and the 13 000 *g* supernatant was fractionated further into 100 000 *g* supernatant (S100) and pellet (P100). The 13 000 *g* supernatant fraction was loaded at the top of a pre-formed Accudenz step gradient. Total proteins were precipitated from the fractions, separated by SDS–PAGE and transferred to nitrocellulose. Proteins were detected by immunoblotting and ECL fluorography: (**A**) differential centrifugation analysis of Tlg2p and API in total clarified extract (T), P13, S100 and P100, with and without 1% Triton X-100; (**B**) fractions from Nycodenz gradient fractionation of the S13 fraction were immunoblotted for G6PDH, API, Tlg2p, Pep12p and Mnn1p.

proteinase K, as is expected since these mutants should accumulate proAPI in a membrane-sequestered form. On the other hand, we found that >85% of the proAPI that accumulated in *tlg2Δ* cells is sensitive to proteolysis both in the presence and absence of detergent (Figure 4B). The protease sensitivity of proAPI in *tlg2Δ* cells is comparable with that seen in *apg7Δ* cells, which have been shown previously to accumulate unsequestered proAPI. We conclude that in *tlg2Δ* cells, the majority of proAPI is recruited to membranes, but is not sequestered in a protease-inaccessible compartment as observed in *vam3*^{ts} or *pep4Δ* yeast. This suggests a requirement for Tlg2p in the formation of the Cvt vesicle, perhaps at the engulfment step in which API is packaged into the double-membrane pre-vacuolar Cvt vesicle.

Tlg2p co-fractionates with accumulated pro-API in vam3^{ts} cells

If Tlg2p functions directly in the formation of Cvt vesicles, one expects that some Tlg2p will be found on these structures. *vam3*^{ts} cells accumulate numerous types of vacuole-bound vesicular transport intermediates, including Cvt vesicles, since Vam3p is required for all import into the vacuole (Darsow *et al*., 1997). In wild-type cells, API is found in the vacuole, which fractionates primarily in the 13 000 *g* pellet during differential centrifugation. In *vam3*^{ts} cells incubated for 1 h at non-permissive temperature, we find that proAPI accumulates in the 100 000 *g* pellet, presumably because the fusion of Cvt vesicles with the vacuole is blocked under these conditions. If Tlg2p is involved in the formation of Cvt vesicles, as suggested by our data, then fractionation of cell lysates from *vam3*^{ts} cells that were incubated at non-permissive temperature should reveal some degree of co-fractionation between Tlg2p and proAPI. Indeed, as shown in Figure 5, Tlg2p from such cells fractionated in Nycodenz gradients as two distinct pools, and the lighter of these fractions coincided with proAPI. This pool of accumulated proAPI is clearly separated from the cytosolic marker G6PDH as well as from Pep12p, which co-fractionated with the denser pool of Tlg2p. Thus, our data support the presence of a portion of the total cellular Tlg2p on Cvt vesicles, under conditions which promote the accumulation of proAPI.

vps45^{ts} yeast show an API defect similar to tlg2Δ cells

Syntaxin homologues have been shown to bind to Sec1p homologues, and this interaction is important in the function of SNARE complexes (reviewed in Pfeffer, 1996). Yeast contain seven syntaxin homologues, but only four known *SEC1*-like genes. Since all the vesicular transport steps elucidated to date require a Sec1p-like protein, it is reasonable to suggest that certain Sec1p homologues interact with more than one syntaxin homologue. Vps45p is the best candidate for a Sec1p homologue that controls Tlg2p function. Vps45p previously was shown to interact with the endosomal syntaxin Pep12p in mediating endosomal transport (Burd *et al*., 1997), and more recently

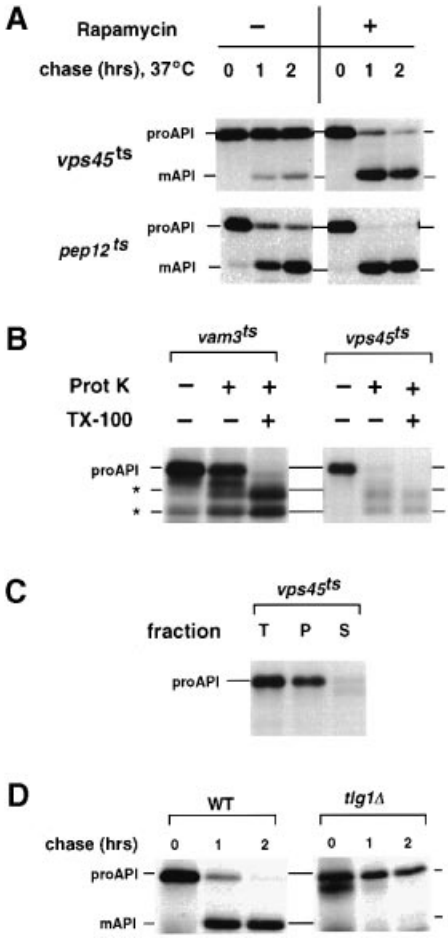


Fig. 6. *vps45*^{ts} yeast show an API trafficking defect that is suppressed by addition of rapamycin and accumulate proAPI in a membrane-bound, protease-sensitive form. (**A**) *vps45*^{ts} and *pep12*^{ts} cells were grown overnight at 26°C, shifted to 37°C for 20 min and pulse-labelled for 15 min with ^{35}S-labelled cysteine and methionine. The cells were chased by adding unlabelled cysteine and methionine in the presence or absence of rapamycin (0.2 μg/ml final concentration). Samples were drawn after 0, 1 and 2 h of chase, lysed, and immunoprecipitated with antiserum to API. (**B**) proAPI accumulates in *vps45*^{ts} cells in a protease-sensitive form. *vam3*^{ts} and *vps45*^{ts} spheroplasts (6 OD_{600} units) were grown at 26°C, pre-shifted to 37°C for 20 min and then pulse-labelled and chased for 90 min. Cells were lysed in PS100 buffer and incubated for 20 min on ice in the presence or absence of 0.2% Triton X-100 and 100 μg/ml proteinase K, as indicated. The reactions were quenched with 10% TCA and immunoprecipitated with anti-API antibody. (**C**) Cells were lysed as in (B), and the lysate was separated into 5000 *g* supernatant (S) and pellet (P) as above. Equivalent amounts (1.5 A_{600} units) of total (T), supernatant and pellet fractions were immunoprecipitated with antibodies to API. (**D**) *tlg1Δ* cells are defective in maturation of API. Wild-type and HAY322 (*tlg1Δ*) cells were pulse-labelled, chased and extracts were immunoprecipitated as described in Materials and methods.

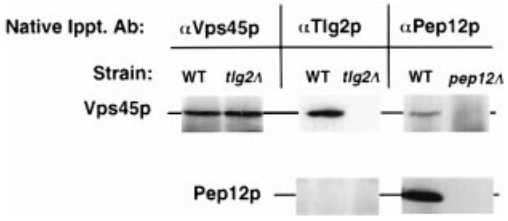


Fig. 7. Tlg2p and Pep12p form separate complexes with Vps45p. Wild-type, *pep12Δ* and *tlg2Δ* cells (10 A_{600} units each) were grown and spheroplasted as described in Materials and methods. Lysates prepared under native conditions and 1 mg of total protein were immunoprecipitated with either anti-Vps45p antiserum, anti-Pep12p antiserum or affinity-purified anti-Tlg2p antibody. The immunoprecipitate was eluted from the protein A–Sepharose, separated by SDS–PAGE and blotted for Vps45p and Pep12p.

it was also demonstrated to interact physically with Tlg2p, although no function was ascribed to this interaction (Nichols *et al*., 1998). To determine if Vps45p is functionally required for API maturation, we performed pulse–chase analysis as described above. We found that *vps45*^{ts} cells do not mature API at the non-permissive temperature (Figure 6A). To understand whether *vps45*^{ts} cells showed the same Cvt pathway-specific defect as described above for *tlg2Δ*, we treated these cells with rapamycin. As was shown for *tlg2Δ* cells, the API defect of *vps45*^{ts} cells was rapidly overcome by rapamycin, implying that Vps45p, like Tlg2p, functions at a membrane transport step specific to the Cvt pathway. In contrast, *pep12*^{ts} cells under the same conditions mature most of the precursor API in the presence or absence of rapamycin, data consistent with a specific role for Pep12p in the CPY pathway. This indicates that another syntaxin homologue other than Pep12p is interacting with Vps45p in the Cvt pathway. Thus it is likely that Vps45p functions as the Sec1p homologue that is required for the regulation of Tlg2p in membrane fusion reactions required for API trafficking, in addition to its role in the CPY pathway. To test this hypothesis, we assayed the fractionation properties and protease sensitivity of proAPI in *vps45*^{ts} cells. We find that, like *tlg2Δ* cells, *vps45*^{ts} cells accumulate proAPI in a membrane-associated yet protease-sensitive state at the non-permissive temperature (Figure 6B), again suggesting that Vps45p and Tlg2p are required at the same step in API maturation. In addition, *vps45Δ* mutants accumulate autophagic bodies in the vacuole in response to nitrogen starvation, and are therefore not defective in autophagy (not shown).

Tlg1p is a SNARE protein that participates in a SNARE complex that includes Vps45p and Tlg2p (Coe *et al*., 1999). Although Tlg1p was originally published as a non-essential gene that does not affect growth at 30°C, it was demonstrated recently that the protein is essential (Coe *et al*., 1999). Consistent with these studies, we found that in our genetic background, diploid *TLG1/tlg1Δ* cells (HAY166) give rise to a ratio of 2:2 viable:non-viable haploid spores. Sporadic survivors able to grow were recovered and were shown to contain extragenic suppressor mutations by backcrossing to wild-type haploid cells (H.Abeliovich, unpublished data). We determined whether these *tlg1Δ* survivors carrying the additional suppressor mutation were able to mature API. As shown in Figure 6D, these *tlg1Δ* mutants were strongly blocked in API maturation. Thus it is likely that Tlg1p participates with Tlg2p in mediating API transport via the Cvt pathway. However, further characterization of the suppression mechanism will be required to understand fully the role of Tlg1p in the Cvt pathway.

Vps45p function is required for transport through both the CPY and Cvt pathways. We wanted to determine the nature of the molecular interactions that underlie these activities of Vps45p. We immunoprecipitated Tlg2p from radiolabelled lysates under native conditions. These native immunoprecipitates were then re-immunoprecipitated

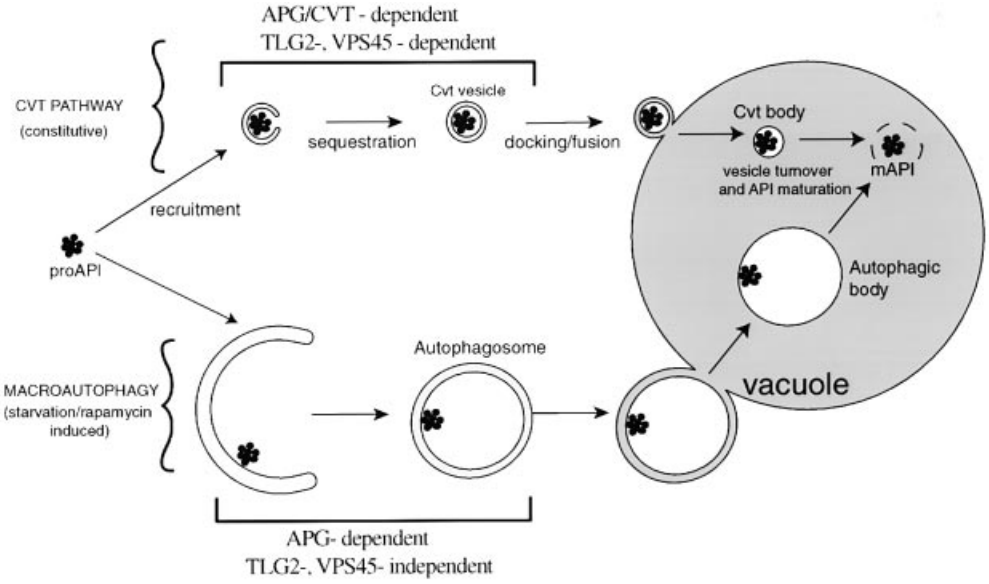


Fig. 8. Model for Tlg2p function in Cvt pathway trafficking. We propose that, under constitutive steady-state conditions, soluble API is recruited to membranes in a fashion dependent on *CVT* and *APG* gene products, as previously described by others. The engulfment of proAPI by the recruiting membranes requires a homotypic fusion event, which we suggest requires the functions of Tlg2p and Vps45p. Engulfment creates the previously described Cvt vesicle, which contains API within a double-bilayer structure. The Cvt vesicle then fuses with the vacuole, releasing proAPI into the vacuolar lumen in a Pep4p- and Cvt17p-dependent step. Under nutrient starvation conditions or treatment with rapamycin, API is packed into autophagosomes in a process that does not require Tlg2p or Vps45p function.

under denaturing conditions with antibodies to Vps45p or other proteins. In agreement with previous data (Nichols *et al*., 1998), we found that native Tlg2p immunoprecipitates contain a substantial fraction of the total precipitable Vps45p in the extract. As expected, anti-Pep12p antibodies were also able to co-precipitate Vps45p, but not nearly as well as the Tlg2p-specific antibodies (Figure 7). Significantly, we could not detect Pep12p in the α-Tlg2p immunoprecipitates. Thus, the two functions of Vps45p in separate trafficking events reflect its ability to form distinct complexes with t-SNAREs that are specific to these pathways.

Discussion

The yeast vacuole is a highly dynamic organelle that is involved in the maintenance of cellular pH and ion homeostasis, storage of amino acids and turnover of macromolecules (Klionsky *et al*., 1990). In addition, several distinct membrane trafficking pathways have been shown to converge on the vacuole, including the CPY pathway, the ALP pathway, the endocytic pathway, the Cvt pathway and macroautophagy.

API has been shown to reach the vacuole by a non-classical vesicular mechanism called the Cvt pathway (Kim *et al*., 1997; Scott *et al*., 1997; Klionsky, 1998), and the final step leading to delivery of API-containing membranes into the vacuole has been shown to depend on certain *VPS* genes products, Vam3p (Darsow *et al*., 1997) and Vps18p (Scott *et al*., 1997), which are thought to be required for fusion of membrane-bound transport intermediates with the vacuole. While the proposed model for the Cvt pathway involves complex membrane fusion steps, none of the yeast repertoire of t- and v-SNARE proteins has been implicated specifically in the early events of API maturation. We now demonstrate that the functions of Tlg2p, a yeast syntaxin-like protein, and Vps45p, a Sec1p homologue, are required in the Cvt pathway.

Tlg2p is required for an early event in API maturation

The delivery of API into the vacuole has been divided into steps that are defined by both immunoelectron microscopy and biochemical analysis. The association of API with membranes can be assayed by a simple fractionation procedure, and the topology of this association has been probed by protease protection experiments (Harding *et al*., 1995; Kim *et al*., 1997; Scott *et al*., 1997). We used this approach to define the stage at which Tlg2p functions in API trafficking. We find that *tlg2Δ* cells accumulate membrane-associated, yet protease-accessible proAPI, while proAPI that accumulates in *vam3*^{ts} cells and *pep4Δ* cells is resistant to digestion by exogenous protease. In the context of present models for API trafficking, the protease protection and fractionation analysis results can best be reconciled with an early role for Tlg2p. According to this, Tlg2p would be required for a step or steps that precede the engulfment of proAPI by the membrane of the Cvt vesicle. Since these engulfment events constitute, by definition, homotypic fusion, it is attractive to suggest that Tlg2p functions in this homotypic fusion event.

Autophagy is a non-biosynthetic membrane trafficking pathway which shares mechanistic and morphological similarities with the Cvt pathway (Harding *et al*., 1996; Scott *et al*., 1996). Unlike the Cvt pathway, autophagy is not a constitutive process, but rather is induced by nitrogen or carbon starvation or by treatment with drugs such as rapamycin. Rapamycin has been shown to mimic the starvation response that induces the G_0 programme in yeast. Rapamycin inhibits the action of Tor, a kinase that signals nutrient availability and is required for G_1

progression (Barbet *et al*., 1996; Zaragoza *et al*., 1998), and autophagy is induced as a part of this response (Noda and Ohsumi, 1998). Autophagy is generally a non-specific process that packages cytoplasm and organelles into autophagosomes which fuse with the vacuole. We find that Tlg2p is not required for autophagy, since administration of rapamycin effectively bypasses the API maturation defect seen in *tlg2Δ* cells. In addition, rapamycin causes an accumulation of autophagic bodies in the vacuoles of *tlg2Δ pep4Δ* double mutant cells, a defining feature of yeast macroautophagy. The fact that macroautophagy is normal in *tlg2Δ* cells implies a role for Tlg2p prior to the Vam3p-dependent fusion of Cvt vesicles with the vacuole. Our data indicate that although autophagy shares considerable mechanistic similarities with the Cvt pathway, the two pathways do not utilize identical membrane intermediates and/or membrane donors. This corroborates previous findings, in which the API maturation defects of *cvt3* and *cvt9* were found to be bypassed by the autophagy pathway, and two mutants defective in autophagy, *aut4* and *aut6*, were found to process API normally (Harding *et al*., 1996).

Vps45p controls Tlg2p-mediated membrane fusion

In all transport pathways analysed, both a t-SNARE and a Sec1p homologue have been shown to be required for efficient docking and fusion of donor and acceptor membranes. Yeast contain seven distinct syntaxin-like proteins, but only four *SEC1*-like genes. This suggests that Sec1p homologues may act on multiple t-SNAREs. In this study, we report that Vps45p, a previously characterized protein that interacts with the syntaxin homologue Pep12p in mediating Golgi to endosome trafficking (Burd *et al*., 1997), is also required in the Cvt pathway. Like Tlg2p, Vps45p is not required for rapamycin-induced maturation of API, and *vps45*^{ts} cells accumulate proAPI in a protease-sensitive, non-soluble form. Consistent with data published during the preparation of this manuscript (Nichols *et al*., 1998), we also find that Tlg2p forms a complex with Vps45p. The Vps45p–Tlg2p complex does not contain detectable amounts of Pep12p, suggesting that Vps45p independently controls Tlg2p function in the Cvt pathway and Pep12p function in the CPY pathway. Based on these findings, we suspect that other Sec1p family members will also be shown to function with more than one t-SNARE, in mediating separate trafficking events.

Previous studies have indicated that the Cvt and CPY pathways converge at the vacuole as both require a common set of transport components for their final docking and fusion with the vacuolar membrane (Darsow *et al*., 1997; Scott *et al*., 1997). This study has broadened the scope of the CPY pathway–Cvt pathway overlap by demonstrating a requirement for Vps45p in API trafficking. Although Vps45p is known to be required for Golgi to endosome trafficking, it has long been speculated that Vps45p has additional functions, since several of the phenotypes associated with *vps45* mutants are more severe than those of other class D *vps* mutants, such as *pep12*/*vps6* (Emr lab, unpublished observations). Indeed we find that *pep12*^{ts} mutants do not show an API transport block at 37°C. However, the residual maturation of API which we find in *tlg2Δ* mutants is abolished in a double *pep12tstlg2Δ* mutant (H.Abeliovich, unpublished data), implying that Pep12p may be able to compensate partially for the loss of Tlg2p.

During the preparation of this manuscript, it was demonstrated that Tlg2p and Vps45p participate in a SNARE complex containing Vti1p and Tlg1p (Coe *et al*., 1999). Tlg2p is known to interact with the v-SNAREs Snc2p and Vti1p. It was shown recently that Vti1p is required for API maturation (Fischer von Mollard and Stevens, 1999), but it is not known whether its function is required in the formation of the Cvt vesicles or in the docking and fusion of these vesicles with the vacuole, or in both processes. Thus, while it is possible that Vti1p functions together with Tlg2p in the sequestration of API into Cvt vesicles, further analysis of *vti1* alleles is necessary to demonstrate this.

Tlg1p was designated initially as a syntaxin homologue based on the presence of a juxtamembrane coiled-coil domain with weak similarity to the analogous regions in syntaxin homologues and its ability to participate in SNARE complexes (Holthuis *et al*., 1998). However, a thorough analysis of all coiled-coil domains from SNARE proteins shows that the juxtamembrane coiled-coil domain of Tlg1p (YDR468c) is most similar to coiled-coil domains from Sec9p and Spo20p (YMR017w), yeast SNAP25 homologues (Weimbs *et al*., 1997), rather than to a syntaxin. Present data also indicate that direct interaction with a Sec1p homologue is a hallmark of syntaxin function (Pevsner *et al*., 1994; Sogaard *et al*., 1994; reviewed in Pfeffer, 1996). The fact that Nichols *et al.* report that the Tlg1p–Vps45p interaction is indirect, and depends on the Tlg2p–Vps45p interaction, which is direct, suggests that Tlg1p does not function as a syntaxin, but rather may act as a SNAP-25-like protein within the Tlg2p–Tlg1p–Vti1p SNARE complex (Nichols *et al*., 1998). This places Tlg1p and Tlg2p not as equivalent syntaxin homologues with overlapping function but rather as two different components of the SNARE machinery. If Tlg1p is a SNAP25-like protein, it may associate with multiple syntaxins in different trafficking pathways (e.g. with both Tlg2p and Sed5p), and this may explain the fact that Tlg1p is an essential protein. Similarly, the essential v-SNARE Vti1p associates with the non-essential syntaxins Pep12p, Vam3p and Tlg2p, as well as the essential syntaxin Sed5p, to mediate different transport steps (Fischer von Mollard *et al*., 1997, 1999; Holthuis *et al.*, 1998). Consistent with the fact that Tlg1p and Tlg2p participate together in SNARE complexes, we have found that *tlg1Δ* cells carrying an additional unmapped suppressor mutation are blocked in API trafficking. Further study of the mechanism by which the suppressor mutation allows viability of *tlg1Δ* cells will be required for a definitive understanding of the role of tlg1p in API maturation.

Multiple intracellular roles for Tlg2p

Like Vps45p, which functions in more than one trafficking pathway, Tlg2p also appears to be involved in several cellular functions. Tlg2p has been implicated in endocytosis (Abeliovich *et al*., 1998; Seron *et al*., 1998). Tlg2p co-fractionates in a bimodal fashion with both late Golgi and endosomal membranes, and a functional green fluorescent protein (GFP)–Tlg2p fusion localizes to the class E compartment in *vps27* mutants. The rates of constitutive Ste3p endocytosis and uracil permease turnover are

delayed 2- to 3-fold, and lucifer yellow uptake is impaired in *tlg2Δ* yeast, implicating the protein, directly or indirectly, in an endocytosis-related transport step. It is possible, therefore, that Tlg2p functions independently in both API trafficking and endocytosis. Alternatively, it is possible that endosomal membranes function as the donor membranes in the formation of Cvt vesicles, and in *tlg2Δ* cells the defect in formation of Cvt vesicles causes an accumulation of aberrant endosomal membranes, resulting in a delay of endosomal trafficking.

Our data indicate that Tlg2p participates directly in the Cvt pathway, for several reasons. (i) Unlike the partial endocytic defects or CPY missorting previously described for *tlg2Δ* cells, the block in API trafficking is almost complete. Therefore, the Cvt phenotype is the most penetrant trafficking defect in these cells. (ii) *vps45*^{ts} mutants are blocked in API trafficking at the non-permissive temperature, suggesting a direct role for Vps45p in the pathway. Since Tlg2p co-precipitates with Vps45p, and since Pep12p is not required for API maturation, it leaves Tlg2p as the prime candidate for the Vps45p-associated t-SNARE that is functioning in the Cvt pathway. (iii) The fact that *tlg2Δ* cells can mature API efficiently upon treatment with rapamycin demonstrates that the Cvt phenotype of these cells does not arise from the lack of potential for API maturation, for example as a result of inefficient protease activity.

We propose that our findings can be explained by the following model for Tlg2p function in the Cvt pathway (Figure 8): API is recruited to Cvt membranes in a step that depends on *APG* and *CVT* gene products. Analysis of the *cvt*, *apg* and *aut* mutants that are defective in API maturation has revealed that all but one of these genes (*cvt17/aut5*) are required at or prior to Cvt vesicle formation (Harding *et al*., 1996; Scott *et al*., 1996). The membrane then sequesters API in a homotypic membrane fusion reaction that depends on Tlg2p and Vps45p, thus forming the Cvt vesicle. Once formed, the Cvt vesicle undergoes docking and fusion with the vacuolar membrane in a Vam3p- and Vps18p-mediated reaction. Finally, a Cvt17p- and Pep4p-dependent lytic step releases proAPI into the vacuolar lumen, where it is processed to yield mature API. In the presence of rapamycin, the requirement for Tlg2p and Vps45p is bypassed. We suggest that, under these conditions, API is recruited to different membranes, which sequester API into autophagosomes in a Tlg2p- and Vps45p-independent fashion, perhaps utilizing a different t-SNARE–*SEC1* homologue combination. Future work that defines the donor membrane required for formation of Cvt vesicles and the isolation of Cvt vesicles will allow a better understanding of the relative roles of Tlg2p, Tlg1p, Vti1p and Vps45p in this pathway.

Materials and methods

Materials

Protein A–Sepharose was from Amersham Pharmacia. Enzymes used in DNA manipulations were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN). Proteinase K was from Boehringer Mannheim. Other chemicals were from Sigma Chemical (St Louis, MO), standard sources or as indicated. Antisera to Pep12p (Becherer *et al*., 1996), Vps45p (Cowles *et al*., 1994) and Tlg2p (Abeliovich *et al*., 1998) were described previously. Antisera to the vacuolar proteins ALP (Klionsky and Emr, 1989) and CPY (Klionsky *et al*., 1988) were as described. Antibody to API was a gift of Dr Dan Klionsky.

Plasmids and DNA methods

DNA manipulations were conducted using standard methods (Maniatis *et al*., 1982). Deletion constructs for the *TLG2* gene were made either as previously described (Abeliovich *et al*., 1998), or by PCR amplification of the *Schizosaccharomyces pombe HIS5* gene from the ME3 plasmid (a gift of Dr Neta Dean) with primer overhangs that match sequences 3′ and 5′ of the *TLG2* reading frame. Transformation of yeast with plasmid DNA was as previously described (Guthrie and Fink, 1991). Plasmids pCB49, pCB34, pVPS45-37, pVAM3-6.414 and pVPS33-8.415 were described previously (Cowles *et al*., 1997b; Darsow *et al*., 1997).

Strains and media

Strain HAY70 was constructed by sequentially transforming SEY6210 **a**/α diploids with the *Sac*I–*Xba*I *tlg2Δ::URA3* fragment of pHAB1 and the *Cla*I–*Afl*III *pep12*Δ*::HIS3* fragment from pCB34. Strains HAY72 and HAY73 are haploid progeny from HAY70. Disruption of the *TLG2* locus was assayed by PCR and by immunoblotting for Tlg2p.

To make strain HAY214, strain TVY1 was transformed with the *Sac*I–*Xba*I *tlg2Δ::URA3* fragment of pHAB1, and disruption of the *TLG2* locus was determined as above. Strain HAY244 was made by transforming SEY6210 with a PCR product of the ME3 plasmid containing the *S.pombe HIS5* gene and flanked by sequences derived 3′ and 5′ of the *APG5* open reading frame. Disruption of the *APG5* locus was verified by PCR. Strain HAY322 is a slow-growing *tlg1Δ* haploid progeny of a diploid 6210 **a**/α strain heterozygous for a disruption at the *TLG1* locus (HAY166). Disruption at the *TLG1* locus was done with a PCR product of the ME3 plasmid as above. Targeted disruption in the diploid was confirmed by PCR and suppression of the *tlg1Δ* segregation pattern with a CEN plasmid carrying the wild-type *TLG1* gene. Other yeast used in this study are described in Table I.

Yeast were grown in YPD (1% yeast extract, 2% Bacto-peptone and 2% glucose) or in synthetic medium (YNB) containing 2% glucose and supplemented as necessary with essential amino acids (Sherman *et al*., 1979). Standard bacterial medium (Miller, 1972) supplemented with 100 μg/ml ampicillin for plasmid retention was used to propagate *Escherichia coli*.

In vivo labelling and immunoprecipitation

For whole-cell analyses, yeast cultures were grown to an A_{600} of 0.5 in SD supplemented with the required amino acids. For ALP precipitation, 5 A_{600} units per time point were harvested by centrifugation at 1800 *g* and resuspended in 1 ml per time point of SD medium with amino acids. A 5 min pulse with 100 μCi of [^{35}S]cysteine/methionine (ICN Biochemicals) per time point was followed by a 40 min chase with methionine, cysteine and yeast extract at concentrations of 5 mM, 1 mM and 0.2%, respectively. At the indicated time points, 1 ml samples were collected and precipitated with 10% trichloroacetic acid (TCA) followed by two washes with cold acetone. The pellet was dried and cell contents were solubilized by vortexing for 10 min in 100 μl of cracking buffer (6 M urea, 1 mM EDTA, 1% SDS, 40 mM Tris pH 6.8) in the presence of an equal volume of acid-washed glass beads. The lysate was diluted 10-fold in IP dilution buffer [150 mM NaCl, 0.5% Tween-20, 5 mM EDTA, 100 μg/ml bovine serum albumin (BSA), 50 mM Tris pH 7.5] for immunoprecipitation. For API immunoprecipitations, the following modifications were made to this protocol: 2 A_{600} units of cells were used per time point. The cells were labelled at a concentration of 6 A_{600} units/ml and were diluted in the chase to a concentration of 2 A_{600} units/ml with medium containing a final concentration of 5 mM methionine, 1 mM cysteine and 0.2% yeast extract.

For analysis of maturation and missorting of CPY, yeast cells were grown to an A_{600} of 0.5. The cells were then collected (2 A_{600} units per time point) and resuspended in softening buffer [10 mM dithiothreitol (DTT), 100 mM Tris pH 9.4] for 10 min, washed in spheroplast labelling medium (SD medium containing 1 M sorbitol), resuspended in spheroplast labelling medium plus 250 μg/ml zymolyase 100T (Seikagaku, Japan), and incubated for 30 min at 30°C. Spheroplasted cells were collected by gentle centrifugation at 1000 *g* and resuspended in spheroplast labelling medium at a concentration of 6 original A_{600} units/ml. The cells were labelled with a 5 min pulse of [^{35}S]cysteine/methionine and chased by the addition of methionine, cysteine and yeast extract at concentrations of 5 mM, 1 mM and 0.2%, respectively. At the indicated time points, 2 A_{600} units of original culture equivalents were collected by gentle centrifugation, the media supernatant was separated from the cell pellet, and the cells were lysed by incubation in urea

Table I. The *S.cerevisiae* strains used in this study

Strain	Genotype	Reference/source
SEY6210**a**/α	MAT**a**/α *ura3-52/ura3-52 his3-Δ200 trp1-Δ901 leu2-3, 112*/leu2-3, *112 lys 2-801/lys2-801 suc2-Δ9/suc2-Δ9*	Emr lab strain collection
SEY6210	MATα *leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al*. (1998)
HAY73	SEY6210; *tlg2Δ::URA3*	this study
HAY214	SEY6210; *pep4Δ::LEU2 tlg2Δ::URA3*	this study
HAY244	SEY6210; *apg5::S.p. HIS5*	this study
TDY	SEY6210; *vam3Δ::LEU2*	Darsow *et al*. (1997)
CBY31	SEY6210; *pep12Δ::HIS3*	Burd *et al*. (1997)
LBY317	SEY6210; *vps33Δ::HIS3*	Banta *et al*. (1990)
TDY10	SEY6210; *vam3Δ::HIS3 pepΔ::LEU2*	Darsow *et al*. (1997)
CCY120	SEY6210; *vps4Δ52::HIS3*	Cowles *et al*. (1994)
SRY18-T1	SEY6210; *vps18-1*	Rieder *et al*. (1997)
VDY1	SEY6210; *apgΔ7::LEU2*	D.Klionski
TVY1	SEY6210; *pep4Δ::LEU2*	T.Vida
TDY27	SEY6210; *vam3*^{ts}	T.Darsow
HAY322	SEY6210; *tgl1Δ::S.p. HIS5* carrying an unmapped suppressor	this study

cracking buffer at 95°C for 5 min. The cell extract and medium supernatant were then diluted 10-fold in IP dilution buffer as above and immunoprecipitated separately.

For native co-immunoprecipitation experiments, 10 A_{600} units of cells (at an A_{600} of 0.5) were collected and spheroplasted as above. Spheroplasts were then collected by gentle centrifugation and lysed by resuspension in 250 µl of native lysis buffer (500 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 1% Triton X-100, 20 mM HEPES pH 7.4) supplemented with protease inhibitors (5 µg/ml antipain, 1 µg/ml each of aprotinin, leupeptin and pepstatin, and 30 µg/ml α_2-macroglobulin). Lysates were diluted to 1 ml with native dilution buffer (100 mM KCl, 1 mM DTT, 5 mM EDTA, 0.5% Triton X-100, 20 mM HEPES pH 7.4). The cell lysate was cleared by centrifuging at 13 000 *g* for 10 min and the protein concentration was assayed with Bradford reagent (Bio-Rad). One milligram of total protein lysate was adjusted to 1 ml with the appropriate buffer and antiserum was added at a dilution of 1:200. Following a 3 h incubation with antibody at 4°C, protein A–Sepharose (Amersham Pharmacia) was added to a final concentration of 2% (v/v) and incubated a further 1.5 h. The immunoprecipitates were washed twice in native dilution buffer and three times in native wash buffer (500 mM KCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM HEPES pH 7.4). The immunoprecipitates were then separated by SDS–PAGE and immunoblotted for Vps45p and Pep12p.

Osmotic lysis, fractionation and protease protection analysis

To analyse the biochemical properties of proAPI that accumulated in mutant cells, 6 A_{600} units of cells were spheroplasted at 26°C for 40 min as above, except that oxolyticase (Zymogenetics) at 10 µg/ml was used instead of zymolyase. The cells were labelled in spheroplasting medium for 15 min as above and chased by addition of cysteine, methionine and yeast extract to 5 mM, 1 mM and 0.2%, respectively, and dilution in medium containing 1 M sorbitol to an A_{600} of 1. Following 90 min of chase, cells were harvested at 4°C by gentle centrifugation and resuspended for 10 min in 1 ml of PS100 buffer (100 mM sorbitol, 50 mM potassium acetate, 5 mM magnesium acetate, 100 mM KCl, 1 mM $CaCl_2$, 20 mM PIPES pH 6.8) on ice. For fractionation, a 400 µl sample was collected as total and the remaining suspension was centrifuged at 5000 *g* for 5 min. Supernatant (400 µl) was drawn and the pellet was resuspended in 600 µl of PS100 and 400 µl were collected. The total, pellet and supernatant fractions were precipitated with 10% TCA. The TCA precipitates were solubilized in 100 µl of urea cracking buffer, diluted to 1 ml with IP dilution buffer as above, and assayed for API, G6PDH and CPY.

For protease protection, 1 ml of spheroplasts was lysed in PS100 buffer as above and divided into three 300 µl aliquots. After 10 min in PS100, each aliquot was diluted 2-fold in PS100 buffer containing either 200 µg/ml proteinase K, 200 µg/ml proteinase K plus 0.4% Triton X-100, or control PS100. After a 20 min incubation on ice, the reactions were quenched with 100 µg/ml BSA and 1 mM AEBSF (Calbiochem) followed by 20% TCA. Immunoprecipitation of API was as above.

Subcellular fractionation

To analyse the fractionation properties of Tlg2p and proAPI in *vam3*^{ts} cells under non-permissive conditions, a culture of logarithmically growing TDY27 cells was transferred to 37°C for 1 h, then sodium azide and sodium fluoride were added to a final concentration of 10 and 1 mM, respectively. The cells (50 A_{600} units) were collected and incubated at room temperature for 10 min in 100 mM Tris pH 9.4, 50 mM β-mercaptoethanol, 10 mM NaN, washed and resuspended in spheroplasting buffer (1 M sorbitol, 10 mM NaN_3, 10 µg/ml oxolyticase, 40 mM HEPES–KOH pH 7.5) and incubated at 37°C until 90% of the cells were converted to spheroplasts (30–40 min). Spheroplasts were collected by centrifugation, resuspended in 2 ml of lysis buffer [0.2 M sorbitol, 50 mM potassium acetate, 5 mM EDTA, 40 mM HEPES–KOH pH 7, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml antipain, 1 µg/ml each of aprotinin, leupeptin and pepstatin, and 30 µg/ml α_2-macroglobulin] and lysed in a dounce homogenizer (tight pestle, 15 strokes).

The lysate was cleared by centrifugation at 300 *g* for 5 min, and the cleared lysate was centrifuged for 15 min at 13 000 *g*. The 13 000 *g* supernatant (1.5 ml) was layered over a pre-formed 10–45% Accudenz (Accurate Chemical & Scientific Co., NY) step gradient in lysis buffer (0.5 ml of 45% and 1 ml each of 40, 35, 31, 28, 25, 22, 19, 16, 13 and 10% w/v Accudenz) and centrifuged in a Beckman SW41 rotor at 170 000 *g* for 8 h at 4°C. Sixteen fractions were collected from the top and the Accudenz concentration was monitored by refractive index. After adding insulin (Sigma) to a final concentration of 200 µg/ml to each fraction, the proteins were precipitated by adding TCA to 10% (w/v). The precipitates were solubilized in 60 µl of SDS sample buffer, and distribution of proteins in the gradient was analysed by SDS–PAGE and immunoblotting.

Electron microscopy

To examine the formation and localization of autophagic intermediates by electron microscopy, cells were grown at mid-log phase for a period of 24 h at 26°C in YPD. Cells were harvested and resuspended in fresh medium to a concentration of 0.5 A_{600}/ml. To induce autophagy, rapamycin (Sigma) was added to the cultures at a final concentration of 0.2 µg/ml from a 1 mg/ml stock solution in 10% Tween-20/90% ethanol as above. An equivalent volume of Tween-20/ethanol carrier solution was added to control experiments in which autophagy was not induced. For experiments with temperature-sensitive strains, cultures were split into duplicates and then incubated at either 26 or 38°C during induction of autophagy. Following rapamycin treatment and/or temperature shift, the cells were incubated for 2 h to allow for the formation and accumulation of membrane intermediates and then processed as described previously for electron microscopy (Rieder *et al*., 1996).

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