The EMBO Journal Vol.17 No.1 pp.113–126, 1998

Two syntaxin homologues in the TGN/endosomal system of yeast

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Intracellular membrane traffic is thought to be regulated in part by SNAREs, integral membrane proteins on transport vesicles (v-SNAREs) and target organelles (t-SNAREs) that bind to each other and mediate bilayer fusion. All known SNARE-mediated fusion events involve a member of the syntaxin family of t-SNAREs. Sequence comparisons identify eight such proteins encoded in the yeast genome, of which six have been characterized. We describe here the remaining two, Tlg1p and Tlg2p. These have the expected biochemical properties of t-SNAREs, and are located in separable compartments which correspond to a putative early endosome and the yeast equivalent of the TGN, respectively. They co-precipitate with the v-SNARE Vti1p, which is implicated in Golgi–endosome traffic and, remarkably, binds to five different syntaxins. Tlg1p also binds the plasma membrane v-SNARE Snc1p. Both Tlg1p and Tlg2p are required for efficient endocytosis and to maintain normal levels of TGN proteins. However, neither is required for intra-Golgi traffic. Since no further syntaxins have been identified in yeast, this implies that the Golgi apparatus can function with a single syntaxin, Sed5p.

Keywords: endosome/Golgi apparatus/*Saccharomyces cerevisiae*/SNAREs/syntaxin

Introduction

The endocytic and secretory pathways of eucaryotic cells consist of multiple membrane-bound compartments which communicate by means of transport vesicles or by direct fusion. There has been much interest in determining how these compartments are formed and maintained, and how proteins pass between them. Analysis has been complicated by the morphological differences between cell types and species, and by the variety of traffic routes that have been detected or proposed. Central to the secretory pathway is the Golgi apparatus, which consists of a variable number of cisternae through which secreted proteins pass in an ordered fashion (Farquhar, 1985). At the exit point from this organelle is a structure known in animal cells as the trans-Golgi network (TGN), which is thought to be the first point at which the biosynthetic pathway is connected to the endocytic pathway.

Progress in unravelling the membrane fusion events in cells has been greatly stimulated by the identification of SNARE proteins. These are (mostly) integral membrane proteins found on vesicles and organelles which interact by the formation of helix bundles; these interactions are a necessary prelude to most known fusion events, and are thought to contribute significantly to the specificity of membrane recognition (Söllner *et al.*, 1993a,b; reviewed by Rothman, 1994; Bennett, 1995; Rothman and Wieland, 1996). The interactions are controlled in part by soluble proteins, NSF and SNAPs, which act at multiple steps in the secretory pathway. SNAREs have been divided conceptually into those associated primarily with vesicles (v-SNAREs) and those on target membranes (t-SNAREs). This is a convenient distinction but not an absolute one—the neuronal t-SNARE syntaxin 1 is found not only on the plasma membrane but also on synaptic vesicles (Walch-Solimena *et al.*, 1995), and in the case of homotypic vacuole fusion in yeast, t- and v-SNAREs operate in equivalent membranes (Nichols *et al.*, 1997). SNAREs also fall into families related in structure and sequence. Of these, the most widespread and well-defined is the syntaxin family of t-SNAREs (Weimbs *et al.*, 1997); all known SNARE-dependent fusion steps so far examined involve a member of this family.

A full understanding of the number, distribution and interactions of SNAREs should provide considerable insight into the organization of the intracellular membrane system, and the traffic routes between organelles. An issue of particular interest is the mode of transport through the Golgi apparatus. Two main types of model have been proposed (reviewed by Farquhar, 1985). In the vesicular transport model, cisternae with a stable existence communicate by means of vesicular traffic (Rothman and Wieland, 1996). This is likely to be bidirectional, to allow recycling of proteins such as v-SNAREs. In the cisternal maturation model, based largely on electron microscopic studies, cisternae form *de novo* by fusion of ER-derived vesicles, mature while passing through the Golgi stack, and disintegrate into vesicles again at the level of the TGN (see Farquhar, 1985). This model requires resident Golgi enzymes to be continually recycled to earlier compartments by retrograde vesicular traffic (Bannykh and Balch, 1997; Mironov *et al*., 1997; Pelham, 1998). Both models predict a requirement for SNAREs, and indeed it is well established that NSF and SNAP (Sec18p and Sec17p in yeast) are required for intra-Golgi transport as measured in mammalian cell-free systems and *in vivo* in yeast (Graham and Emr, 1991; Rothman, 1994; Rothman and Wieland, 1996). However, the two models predict somewhat different roles for the SNAREs. A full knowledge of Golgi SNAREs should give clues to the number of different

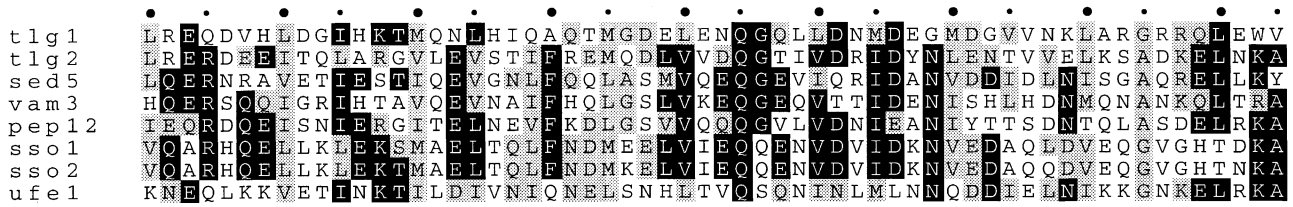

Fig. 1. Sequence similarities between yeast syntaxin family members. Only the C-terminal coiled-coil region is shown; the heptad repeats of predominantly hydrophobic residues are indicated by the black dots. Residues that are identical or similar in at least half the sequences are shown on a black or grey background respectively.

compartments and transport steps, provide constraints for the models, and perhaps distinguish between them (Schekman and Mellman, 1997).

The completion of the yeast genome sequence offers a chance to define all the SNAREs in a eucaryotic cell and to attempt to fit them into such models. We have focused on the syntaxin family of t-SNAREs, because these proteins are the easiest to identify by sequence homology. We and others, using a variety of algorithms, have been able to detect just eight putative syntaxins in yeast (Weimbs *et al.*, 1997). Six of these have already been described: Ufe1p in the ER, Sso1p and Sso2p on the plasma membrane, Vam3p on the vacuole, Pep12p in an endosomal compartment, and Sed5p in the earliest part of the Golgi apparatus (Hardwick and Pelham, 1992; Aalto *et al.*, 1993; Becherer *et al.*, 1996; Lewis and Pelham, 1996; Nichols *et al.*, 1997; Wada *et al.*, 1997).

In this paper we characterize the remaining two syntaxins, Tlg1p and Tlg2p (t-SNAREs affecting a late Golgi compartment). We show that both are required for efficient endocytosis and for the maintenance of resident proteins in the TGN. The TGN does not have a morphological definition in yeast, but is defined as the compartment where proteins destined for the cell surface are sorted from those destined for delivery to the vacuole, and contains processing proteases such as Kex2p and DPAP A (Ste13p). All known TGN proteins in yeast are integral membrane proteins which are thought to cycle continuously through endosomes (Stack *et al.*, 1995; Cooper and Stevens, 1996; Bryant and Stevens, 1997). Tlg2p co-localizes with TGN markers, while Tlg1p appears to be in an early endosomal compartment through which TGN proteins cycle. Both the Tlg proteins interact with v-SNAREs implicated in Golgi–endosome and Golgi–plasma membrane traffic. However, they do not bind the putative intra-Golgi v-SNAREs Sft1p and Gos1p, and neither protein is essential for transport through the Golgi apparatus. These results indicate that Tlg1p and Tlg2p mediate interactions between the exocytic and endocytic pathways, and also imply that traffic through the Golgi apparatus can be sustained with a single Golgi syntaxin, Sed5p, a result which seems more compatible with a cisternal maturation model than with the standard vesicular model for anterograde intra-Golgi transport.

Results

Identification of TLG1 and TLG2

The most conserved domain of the syntaxins is the ~60-residue coiled-coil motif adjacent to the C-terminal membrane anchor. BLAST searches with this portion of known yeast syntaxin family members identified two new yeast genes, corresponding to ORFs YDR468c and YOL018c, which we have named *TLG1* and *TLG2* (Figure 1). Tlg2p shows strongest homology to the endosomal and vacuolar syntaxins Pep12p and Vam3p, although it is unusual in having a substantial extracytoplasmic domain of 62 residues. Tlg1p is one of the most divergent members of the family, but retains the overall structure characteristic of the syntaxins. A more sophisticated profile search for syntaxin homologues found the same two proteins (Weimbs *et al.*, 1997). The only other related proteins found in the yeast genome were the putative SNAREs Bet1p, Sft1p, Vam7p, Sec9p, and a protein related to Sec9p. Though showing some sequence similarity to the syntaxins these are clearly more divergent, and none of them shares the domain structure of the syntaxins, which in addition to the conserved region includes two other coiled-coil motifs and a C-terminal transmembrane domain (Weimbs *et al.*, 1997). Thus, Tlg1p and Tlg2p are the only syntaxins in yeast that have not previously been characterized.

Location of Tlg1p and Tlg2p

As a preliminary guide to their intracellular location, we expressed epitope-tagged versions of Tlg1p and Tlg2p from centromere vectors and visualized them by immunofluorescence. Both proteins revealed a punctate staining pattern, which is characteristic of the yeast Golgi apparatus, and examples of stained cells are shown in Figure 2A and B. Since no syntaxin had previously been assigned to the yeast equivalent of the TGN, we co-stained cells for Kex2p, a TGN marker (Stack *et al.*, 1995). Endogenous levels of Kex2p are difficult to detect (Redding *et al.*, 1991), so we transformed the cells with a multi-copy vector containing the *KEX2* gene. Moderate overexpression has previously been shown not to alter the qualitative pattern of Kex2p immunofluorescence, nor to prevent its co-localization with the Golgi marker Sec7p (Franzusoff *et al.*, 1991; Redding *et al.*, 1991). As illustrated in Figure 2A, some Tlg1p-positive structures seemed to contain Kex2p, but in general the staining patterns were not very similar. There was a much more striking similarity between the Tlg2p and Kex2p patterns, although close examination showed that there were also some differences; an example is shown in Figure 2B. These results suggested that Tlg2p might be associated with the TGN, with Tlg1p having a somewhat different distribution.

We also compared Tlg2p with the early Golgi t-SNARE, Sed5p. In this case, we were able to detect the endogenous Sed5p, and used a strain in which sequences encoding a C-terminal influenza epitope tag had been introduced into

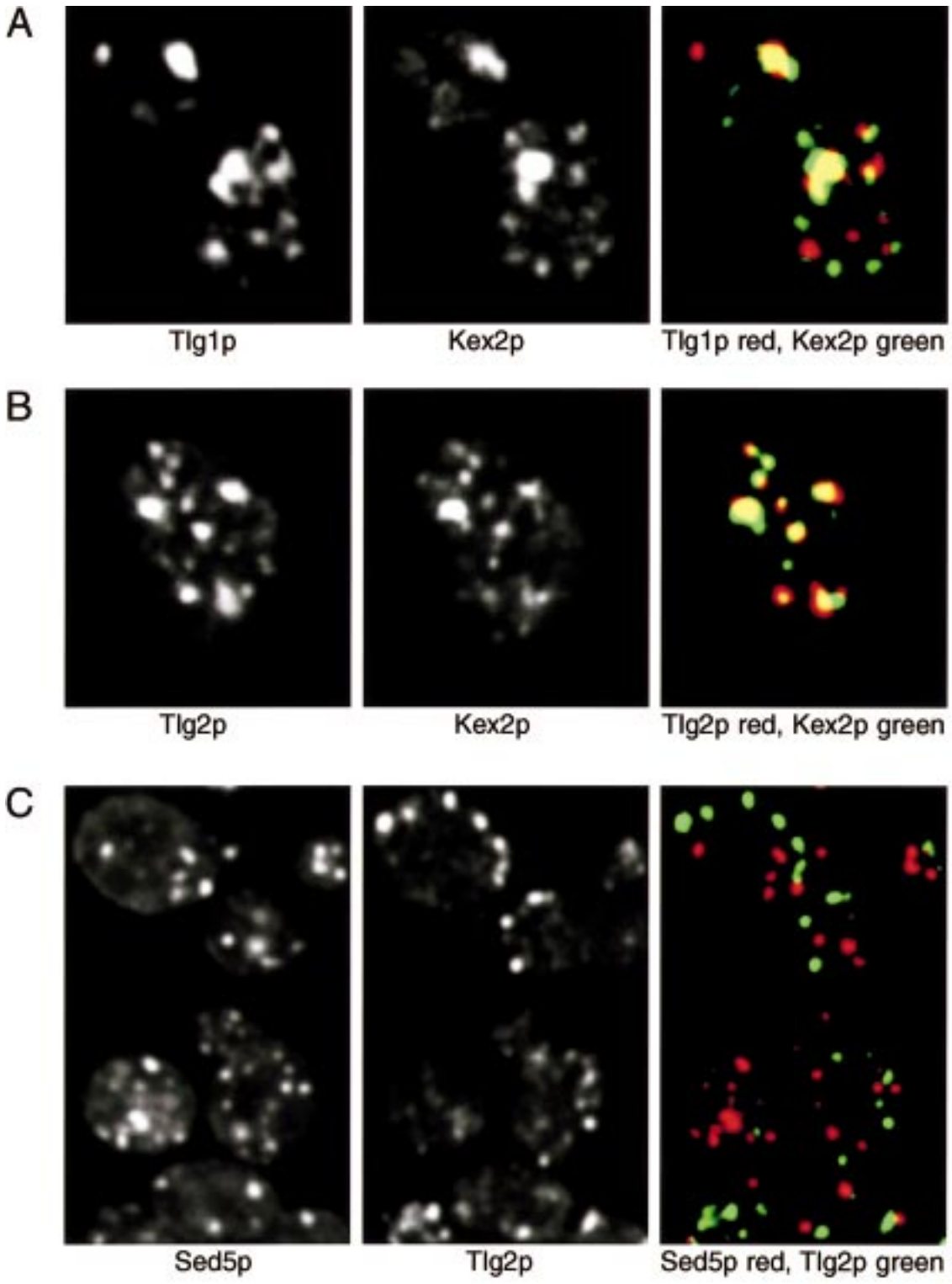

Fig. 2. Immunofluorescent localization of Tlg1p and Tlg2p. In (**A**) and (**B**), cells expressing myc-tagged Tlg1p or Tlg2p from a CEN vector, and Kex2p from a multicopy vector, were double-labelled with a monoclonal antibody to the myc epitope (9E10) and a polyclonal to Kex2p. (A) shows two cells, (B) a single cell. In (**C**), HA-tagged Tlg2p expressed from the endogenous gene was labelled with a monoclonal antibody, and endogenous Sed5p was detected with a specific antiserum. Five cells and their buds are visible, at slightly lower magnification than (A) and (B). Cells were imaged in one plane by confocal microscopy. The colour image was produced by combining high-contrast images of the fluorescent signals, to allow their positions to be compared. The precision of this comparison is limited by differences in relative intensity, and slight differences in depth of focus between the channels.

the chromosomal *TLG2* gene, thus ensuring normal levels of expression of Tlg2p. Both proteins gave the expected punctate staining pattern, but they were clearly present in different structures (Figure 2C).

To assess by an independent method the locations of endogenous Tlg1p and Tlg2p, we prepared specific antisera and used them to follow the distribution of the proteins during subcellular fractionation. As shown in Figure

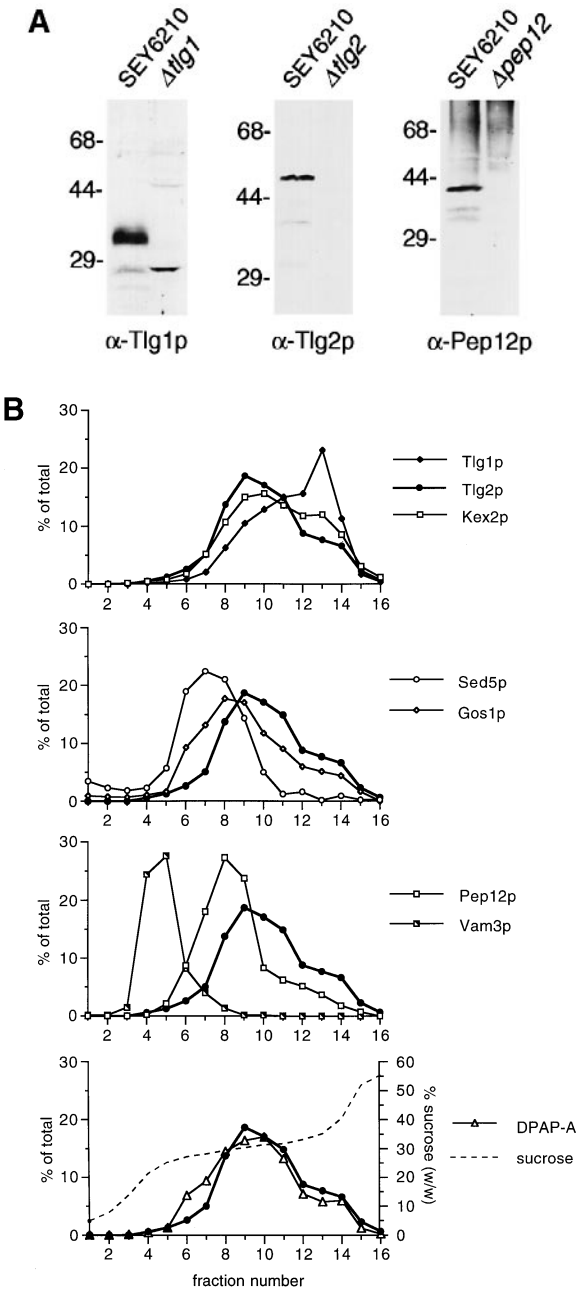

Fig. 3. Subcellular fractionation of Tlg1p and Tlg2p. (**A**) Antibody detection of Tlg1p, Tlg2p and Pep12p. Equal amounts of total cell extracts prepared from parental (SEY6210) and appropriate deletion strains were resolved on an SDS–polyacrylamide gel, blotted and probed with polyclonal antibodies raised against recombinant Tlg1p, Tlg2p and Pep12p. The positions of size markers (kDa) are indicated. (**B**) Sucrose gradient fractionation of membranes. A high-speed membrane pellet (100 000 *g*) from SEY6210 cells was fractionated on a sucrose density gradient as described in Materials and methods. Fractions were assayed for Kex2p enzyme activity and, by immunoblotting, for Tlg1p, Tlg2p, Pep12p, Sed5p, Gos1p and Vam3p. Quantitation was by densitometric scanning of fluorograms produced from the immunoblots. The same fractions were analysed in each panel, the profile of Tlg2p being shown in each to aid comparison.

3A, each antiserum detected a single major band on immunoblots, and this band was absent in strains from which the corresponding genes had been deleted (see below). Figure 3B shows an example of the fractionation of wild-type cells on an equilibrium sucrose density

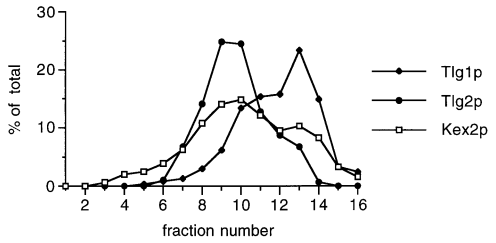

Fig. 4. Subcellular fractionation of Tlg1p and Tlg2p in Δ*pep12* cells. A high-speed membrane pellet (100 000 *g*) from Δ*pep12* cells was fractionated on a sucrose density gradient identical to that shown in Figure 3. Fractions were assayed for Kex2p enzyme activity and, by immunoblotting, for Tlg1p and Tlg2p. Quantitation was by densitometric scanning of fluorograms produced from the immunoblots.

gradient, which was adjusted to give optimal resolution of the Tlg-containing membranes. All the data shown are from a single set of gradient fractions, but for clarity the results are presented in four panels, each containing the profile of Tlg2p.

Figure 3B (top panel) shows that Tlg1p and Tlg2p were at least partially separated and peaked at different densities. However, the distributions overlapped and we reproducibly observed a shoulder on the Tlg2p profile in the position of the Tlg1p peak. Endogenous Kex2p activity was present in a major peak coincident with Tlg2p (fractions 9 and 10) together with a minor peak in the position of Tlg1p (fraction 13). Immunoblotting revealed that a second TGN marker, DPAP A (Nothwehr *et al.*, 1993), coincided closely with the Tlg2p profile (Figure 3B, bottom panel).

As expected from the immunofluorescence studies, Tlg2p did not co-fractionate with Sed5p (Figure 3B). We also examined the distribution of the Golgi v-SNARE Gos1p, which binds Sed5p. Gos1p has not previously been localized, but its mammalian homologue GOS28 is found in the medial/trans region of the Golgi apparatus (Nagahama *et al.*, 1996), and in agreement with this the yeast protein peaked at a density midway between that of Sed5p and Tlg2p. This profile is consistent with Gos1p being in both the Sed5p and Tlg2p compartments, or in membranes of intermediate density.

Tlg2p was separated well from the vacuolar t-SNARE Vam3p (Nichols *et al.*, 1997; Wada *et al.*, 1997), and at least partially from Pep12p, the t-SNARE for a late endosomal/prevacuolar compartment (Becherer *et al.*, 1996) (Figure 3B). Since TGN proteins are thought to recycle through late endosomes, we sought to address the possibility that some of the Tlg2p was present in the Pep12p compartment. Pep12p is not essential for growth, but in its absence transport of hydrolases to the vacuole is severely impaired (Becherer *et al.*, 1996), due to a failure of vesicles to fuse with the prevacuolar/late endosome compartment. One would therefore expect the removal of Pep12p to strongly affect the fractionation behaviour of this compartment. However, analysis of a *pep12* deletion strain revealed a distribution of Kex2p, Tlg1p and Tlg2p indistinguishable from that in wild-type cells (Figure 4). Thus, it seems unlikely that Tlg2p is present in the Pep12p compartment under normal conditions, or that passage through this compartment is required for Kex2p or Tlg1p to achieve their usual steady-state distribution.

The combination of subcellular fractionation and

immunofluorescence strongly suggests that Tlg2p is predominantly associated with the TGN. In contrast, Tlg1p did not co-fractionate precisely with any of the markers tested. It was clearly separable from the bulk of the TGN, earlier Golgi markers, late endosomes and the vacuole, as well as the plasma membrane (which is found in a sharp peak at fraction 15 in these gradients). It did coincide with the minor Kex2p peak but, as suggested by the immunofluorescence data, much of the Kex2p was elsewhere. Hence, Tlg1p seems to be concentrated in a previously undefined compartment, which may be accessible to Kex2p.

Phenotypes of tlg deletion mutants

To investigate the functions of Tlg1p and Tlg2p, we prepared strains in which the corresponding open reading frames were completely removed. Neither gene proved essential. The *tlg2* disruptants were superficially normal and grew only slightly slower than the parental strain, with a doubling time at 25°C of 2.0 versus 1.9 h, and at 37°C of 2.0 versus 1.6 h. The *tlg1* disruptants grew well at 25°C (2.0 h doubling time), but could barely grow at 37°C. At semi-permissive temperatures they tended to produce large misshapen cells, and showed inefficient separation of daughters from mothers. Strains lacking both genes had a phenotype only slightly more exaggerated than that of the Δ*tlg1* mutants (2.2 h doubling time at 25°C). The viability of these mutant strains contrasts strongly with the lethal effects of removing the early Golgi t-SNARE, Sed5p, or the Golgi v-SNARE Sft1p (Hardwick and Pelham, 1992; Banfield *et al.*, 1995).

Electron microscopy of Δ*tlg1* and Δ*tlg1* Δ*tlg2* cells grown at 30°C confirmed that they were much larger than normal and frequently misshapen, with abnormal bud scars (Figure 5C and D). The vacuoles were also rather more fragmented than usual, a common phenotype of mutations affecting the secretory or endocytic pathways. In addition, the cells accumulated some darkly staining circular structures (indicated by arrowheads in Figure 5). These differed from the characteristic cup-shaped Golgi membranes seen in an *sft1* mutant (Banfield *et al.*, 1995) and their identity is unknown. Incubation of the cells at 37°C for 2 h before fixation did not change their EM phenotype substantially (data not shown). By comparison with Δ*tlg1* cells, the Δ*tlg2* mutant showed few abnormalities other than some vacuolar fragmentation and the presence of a few of the darkly staining structures (Figure 5B).

To search for possible genetic interactions, we also prepared all possible double deletions of *TLG1*, *TLG2* and genes encoding the other non-essential syntaxins in the endocytic pathway, namely *PEP12* and *VAM3*. All pairwise combinations were viable except for the *pep12 tlg1* double mutant. This strain could be sustained with a plasmid bearing one of the deleted genes and the *URA3* marker, but fluoro-orotic acid (FOA) selection for loss of the plasmid yielded only extremely rare colonies, which presumably contain suppressor mutations (data not shown). It thus appears that the functions of Pep12p and Tlg1p are in some way related.

Loss of TGN proteins

Since Tlg2p, and perhaps to some extent Tlg1p, appear to reside in compartments containing Kex2p, we investigated the fate of Kex2p when they were removed. Strikingly, the level of Kex2p activity was reduced by 75% in Δ*tlg1* cells (Figure 6, left). Substantial reductions were also observed in Δ*tlg2* cells and in the double mutant. This loss was evidently due to degradation by vacuolar proteases, because additional deletion of the *PEP4* gene restored the activity in the double mutant cells to normal (Figure 6, centre). Immunoblotting showed that DPAP A was also depleted in the *tlg* mutants, though less dramatically than Kex2p (Figure 6, right). Since degradation is the normal fate of Kex2p and DPAP A mutants that lack a specific retrieval signal (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993), we infer that the *tlg* mutants have a defect in the retrieval pathway to the TGN, and that TGN proteins are consequently lost to the vacuole.

Endocytosis defects

Loss of Kex2p is a phenotype common to a subset of mutants that have endocytosis defects (Luo and Chang, 1997). We therefore examined endocytosis in the *tlg* mutants. A ready test is provided by the internalization and subsequent degradation in the vacuole of the alpha-factor receptor, a process that is triggered by the addition of the alpha-factor pheromone (Schandel and Jenness, 1994; Hicke and Riezman, 1996). As shown in Figure 7, the receptor is normally degraded rapidly. Cells lacking either Tlg1p or Tlg2p showed significantly reduced kinetics of receptor degradation, the effect being greatest in Δ*tlg1* cells. Hence, efficient delivery of the receptor to the vacuole requires the action of both Tlg2p and Tlg1p.

Secretory pathway function

The TGN receives traffic both from the earlier part of the Golgi apparatus and from the endocytic pathway, and a t-SNARE present in the TGN could be involved in both processes. However, the viability of the *tlg* mutants, and the lack of any clear accumulation of Golgi membranes in EM images suggests that neither Tlg1p nor Tlg2p is essential for secretion. As a more sensitive test, we examined the maturation of the vacuolar protease carboxypeptidase Y (CPY). CPY appears as a p1 precursor in the ER, is modified to a slightly larger p2 form in the Golgi apparatus, and then passes through the TGN to late endosomes and eventually the vacuole, where it is proteolytically processed to its mature form (reviewed by Stack *et al.*, 1995). Pulse–chase analysis showed that CPY maturation in the Δ*tlg1* Δ*tlg2* double mutant was essentially normal, though slightly slower than in the parental cells (Figure 8A). Each single mutant showed at most a slight delay (Figure 8B), and incubation of Δ*tlg1* cells at 37°C did not increase the severity of the phenotype. Hence, forward traffic of CPY through the Golgi complex was largely unaffected by removal of Tlg1p and Tlg2p.

The efficient processing of CPY in the mutants indicated that there was little mis-sorting to the cell surface, and indeed we failed to detect any labelled CPY released from the cells. This was confirmed by a colony blotting assay (Figure 8C). This assay detected slightly more CPY released by Δ*tlg1* and the double mutant than by wild-type cells, but the effect was insignificant compared with the mis-sorting in Δ*pep12* cells. It seems that diversion of CPY from the secretory pathway, a process that depends on the shuttling of a receptor between late endosomes and

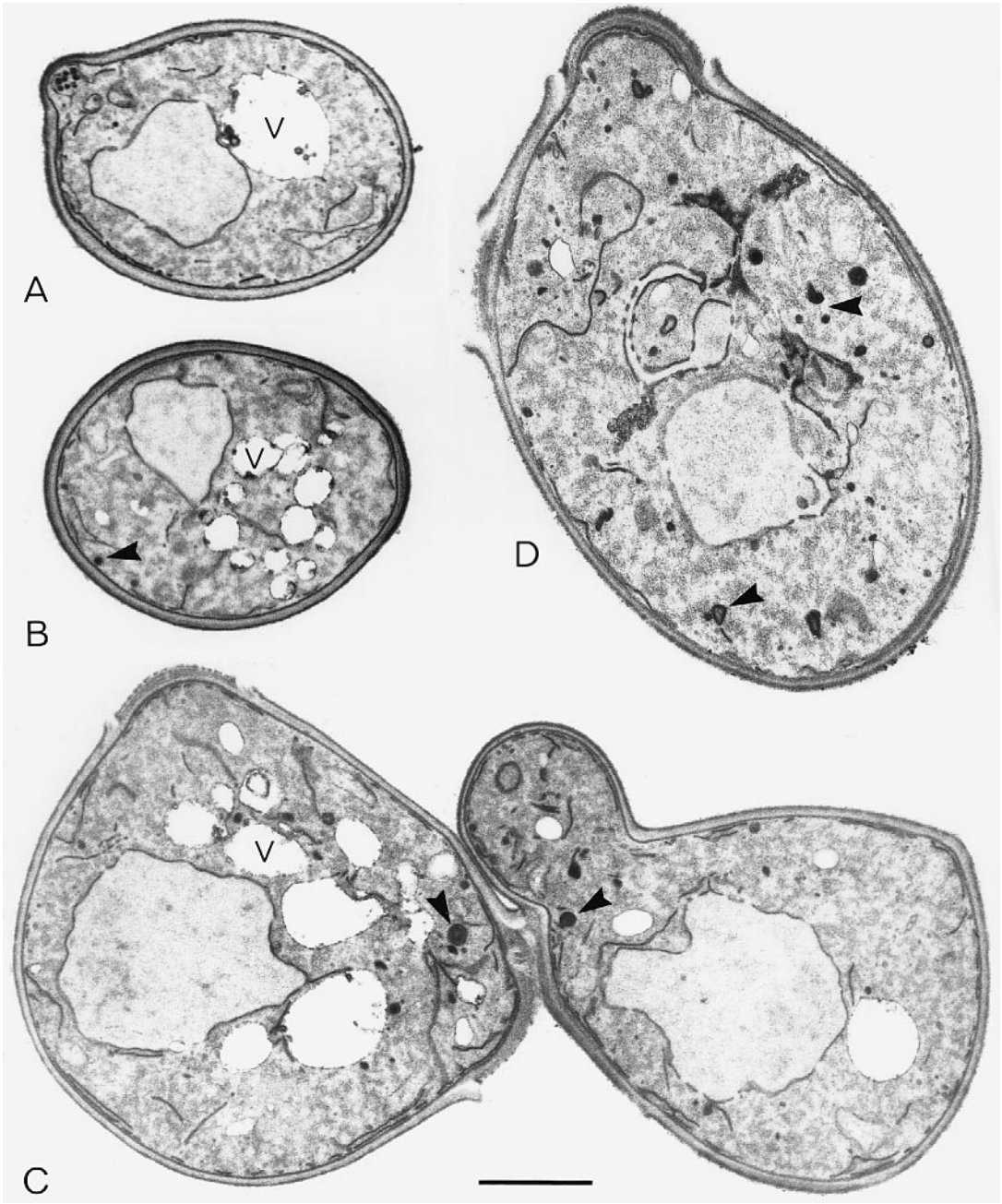

Fig. 5. Electron micrographs of parental SEY6210 (**A**), Δ*tlg2* (**B**), Δ*tlg1* (**C**) and Δ*tlg1* Δ*tlg2* (**D**) cells. Note the 100 nm exocytic vesicles in the bud tip in (A), abnormal membranous structures [rare in (B), more common in (C) and (D); arrowheads] and fragmented vacuoles (V) in the mutants. The abnormal membranes are heterogeneous, with some circular structures displaying a double membrane, reminiscent of Berkeley bodies, while other structures are smaller and uniformly densely stained, often with irregular outlines. Bar, 1 μm.

the Golgi complex (Cooper and Stevens, 1996), does not require the Tlg proteins.

We next assayed the secretion of the periplasmic enzyme invertase. Since our standard strains lack the chromosomal invertase gene, we transformed the Δ*tlg1* and Δ*tlg2* mutants with a plasmid expressing high levels of the secreted form of invertase. Assay of internal and total invertase activity showed that more than 80% of the invertase was secreted in each case. Examination of the low levels of internal invertase by immunoblotting showed that there was no significant accumulation of glycosylated precursors in the mutants, even when Δ*tlg1* cells were incubated at 37°C (Figure 9A). This indicates that there is no block in transport at any point in the pathway from the ER to the cell surface in the mutants. However, ~10% of the total invertase synthesized accumulated as an unglycosylated precursor which had failed to enter the ER. We have observed a similar phenomenon with a variety of strains overexpressing invertase, apparently because the translocation capacity of the ER is easily saturated. The immunoblot also revealed that the Δ*tlg1* cells made invertase with slightly shorter outer polysaccharide chains than normal (Figure 9A). This suggests some reduction in the levels of mannosyl transferases in the Golgi apparatus, reminiscent of the dramatic loss of Kex2p from this strain.

As a more general test of glycoprotein secretion we

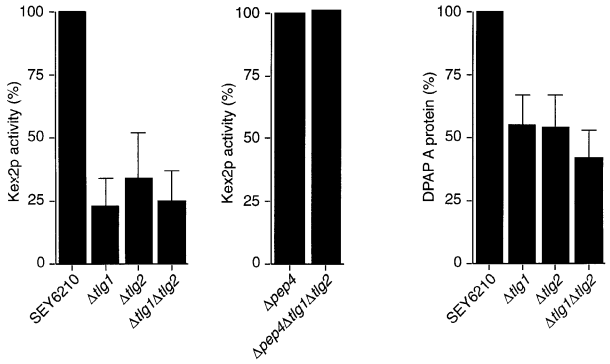

Fig. 6. Loss of TGN proteins in *tlg* deletion strains. Kex2p activity and DPAP A protein levels in total cell lysates of the deletion strains are expressed as a percentage of those measured in the appropriate parental strains (SEY6210 or Δ*pep4*). Deletion of *PEP4* had a negligible effect on Kex2p activity. The data shown are means (± standard deviation) of at least four independent experiments

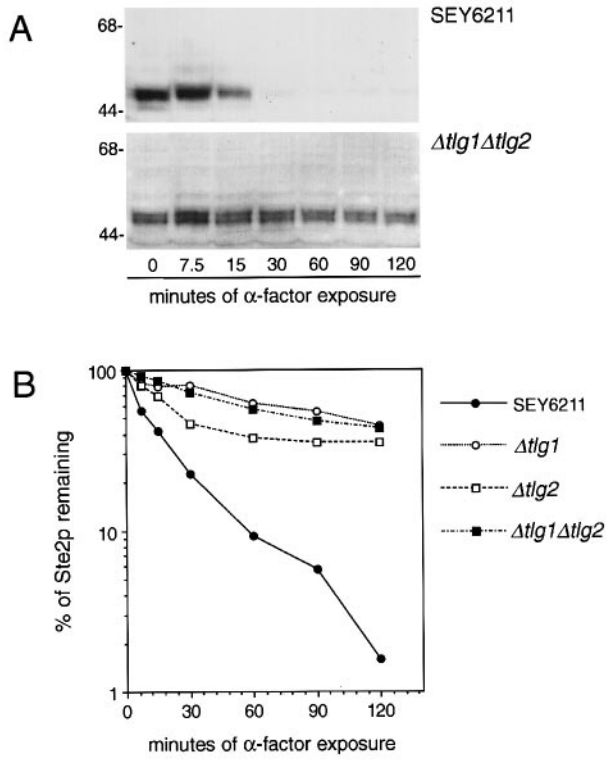

Fig. 7. Impairment of ligand-induced degradation of α-factor receptor in *tlg* deletion strains. (**A**) Immunoblot of Ste2p (α-factor receptor) from extracts of parental (SEY6211) and Δ*tlg1* Δ*tlg2* cells prepared before (0 min) or after exposure to α-factor. Ste2p migrates as a doublet of ~47 kDa. (**B**) Ste2p levels in parental cells (SEY6211) and *tlg* mutants before and after exposure to α-factor. Ste2p levels in cell extracts were quantified by immunoblotting and densitometric scanning of fluorographs, and expressed as % of receptor present before α-factor exposure (0 min). The results shown are the average of three independent experiments.

labelled spheroplasts of the single and double deletion strains with ^{35}S-labelled amino acids and monitored the appearance of conA-binding glycoproteins in the medium. Figure 9B shows that a number of glycoproteins were secreted whose sizes, relative abundance and kinetics of secretion were similar in all strains. The main exception was a large protein (indicated by an asterisk in Figure 9B) which appeared to be expressed at a lower level (or

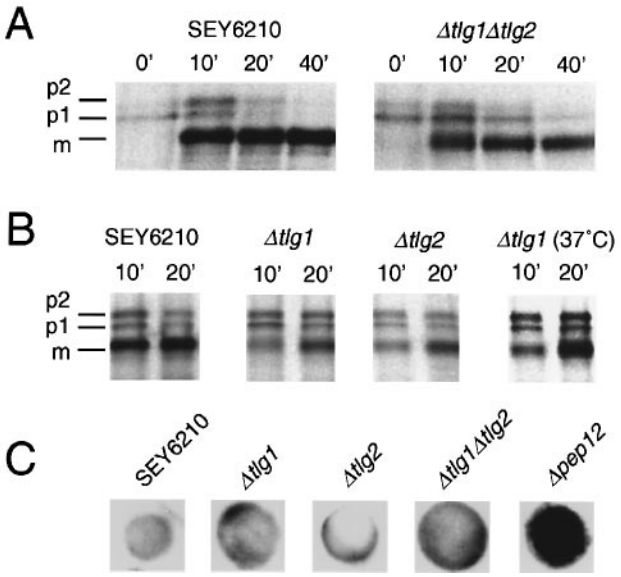

Fig. 8. CPY sorting and processing in *tlg* deletion strains. (**A**) Pulse–chase analysis of parental (SEY6210) and Δ*tlg1*Δ*tlg2* strains. Cells were labelled for 4 min at 30°C and chased for the times indicated (min), before immunoprecipitation of CPY. The ER (p1), Golgi (p2) and mature, vacuolar (m) forms of CPY are indicated. (**B**) Rate of CPY processing in single *tlg* deletion strains. Pulse–chase analysis was carried out as in (A), but one batch of Δ*tlg1* cells was preincubated for 2 h at 37°C, and pulse–chased at 37°C. Comparison of the amounts of p1 and p2 relative to mature CPY after 10 and 20 min of chase reveals only a slight decrease in the processing rate in each mutant compared with the parental strain, even at 37°C. (**C**) CPY secretion from parental (SEY6210), Δ*tlg1*, Δ*tlg2*, Δ*tlg1*Δ*tlg2* and Δ*pep12* strains. Cells were grown overnight at 30°C in contact with nitrocellulose and secreted CPY detected by immunostaining. While Δ*tlg1* and Δ*tlg1*Δ*tlg2* cells secreted slightly more CPY than the parental strain, far more was secreted by cells lacking the pre-vacuolar t-SNARE Pep12p.

degraded intracellularly) in the *tlg* mutants. These results confirm the lack of any substantial secretory defect in cells lacking Tlg1p and/or Tlg2p.

Binding of v-SNAREs to Tlg1p and Tlg2p

The Tlg proteins were identified as t-SNAREs by sequence homology, an assignment which can be tested biochemically. Thus, they should form complexes with Sec17p/SNAP and one or more v-SNAREs, which should be disrupted by Sec18p/NSF in an ATP-dependent manner (Söllner *et al.*, 1993a). It has been reported that SNARE complexes can only be observed after inactivation of Sec18p *in vivo* by a temperature-sensitive mutation (Søgaard *et al.*, 1994), but we and others have found that complexes can be identified in extracts of wild-type yeast cells (Protopopov *et al.*, 1993; Lewis *et al.*, 1997), and they can also readily be isolated from mammalian cells without deliberate inactivation of NSF (e.g. Hay *et al.*, 1997). Recent analysis of vacuolar SNAREs has resolved this discrepancy by showing that the complexes exist in normal cells, but are disrupted in a Sec18p-dependent manner when the cells are lysed in the presence of ATP. Under the conditions used, complexes did not form efficiently in the cell lysates (Ungermann *et al.*, 1998).

With this in mind, we examined the associations of Tlg1p and Tlg2p by immunoprecipitating the proteins from extracts of *sec18* cells that had been incubated at permissive (25°C) or non-permissive temperature (37°C),

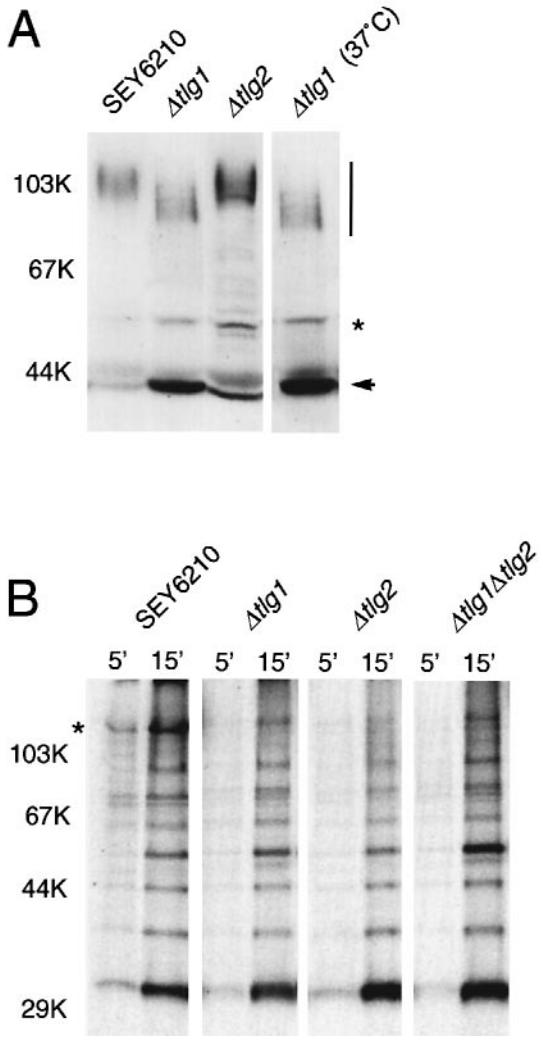

Fig. 9. Biosynthesis and secretion of glycoproteins by *tlg* deletion strains. (**A**) Intracellular invertase in *tlg* deletion strains. An immunoblot of myc-tagged intracellular invertase in parental strain (SEY6210), Δ*tlg1* and Δ*tlg2* strains were grown at 25°C and, where shown, preincubated at 37°C for 2 h before lysis. The positions of size markers (kDa) are shown. Golgi-modified invertase is indicated with a vertical bar. The invertase in the Δ*tlg1* strain migrates more rapidly than the normal mature form, but is still considerably larger than the ER precursor (~80 kDa). The asterisk marks a background band that is seen also in cells not expressing the myc-tagged invertase, and the arrow indicates the non-glycosylated cytoplasmic form of invertase. The Δ*tlg2* sample contained slightly more protein than the others in this experiment. (**B**) Radiolabelled glycoprotein secreted by *tlg* deletion strains. Cells were labelled with Tran^{35}S-label for 5 and 15 min, and extracellular glycoproteins were isolated as described in Materials and methods. Labelled protein was detected by autoradiography. An asterisk marks a glycoprotein secreted inefficiently in the *tlg* deletion strains, as discussed in the text.

and lysed with or without ATP. As a control, we also examined Sed5p, whose interactions are well documented (Søgaard *et al.*, 1994). The immunoprecipitations were highly efficient (Figure 10A), and immunoblotting of the precipitates showed that each t-SNARE was associated with Sec17p if cells grown at the permissive temperature were lysed in the absence of ATP, but not if ATP was present (Figure 10B).

Several v-SNAREs have been identified that are involved in intra-Golgi, Golgi–endosome and Golgi–plasma membrane traffic, and we tested whether any of these could be found in association with Tlg1p or Tlg2p. The putative v-SNARE Vti1p has been reported to be present in late endosomes and also in a TGN-like compartment. It binds Pep12p and Sed5p, but not the plasma membrane t-SNARE Sso1p, and is required both for sorting of CPY to the vacuole and for an additional function involving Sed5p (Fischer von Mollard *et al.*, 1997). We found that Vti1p was bound to both Tlg1p and Tlg2p, in complexes that could be disrupted by Sec18p in an ATP-dependent manner (Figure 10C). The apparent promiscuity of Vti1p binding prompted us to test the two remaining syntaxins, using in this experiment a myc-tagged version of Vti1p. We found that Vti1p bound to Vam3p, but not to the ER t-SNARE Ufe1p (Figure 10D). The Vam3p binding was confirmed using antibody to endogenous Vti1p (data not shown). Thus, remarkably, Vti1p binds to five different syntaxins, and we estimate that at least 50% of it can be recovered in one or other of these complexes.

Snc1p is one of two closely related v-SNAREs implicated in transport to the plasma membrane, and it binds the plasma membrane t-SNARE Sso1p (Protopopov *et al.*, 1993). A myc-tagged version of the protein was associated with Tlg1p, but only low background levels of binding were observed with Sed5p and Tlg2p (Figure 10C). The proportion of the myc-Snc1p bound to Tlg1p was quite small (~2%), but it showed the expected ATP and Sec18p dependence. The low abundance of Tlg1p/Snc1p complexes was not unexpected because it has been shown that in growing cells the vast majority of tagged Snc1p is present on the plasma membrane rather than in an internal compartment (Protopopov *et al.*, 1993) and thus cannot be bound to Tlg1p.

We next tested the two intra-Golgi v-SNAREs, Sft1p and Gos1p. Gos1p showed the expected binding to Sed5p, but did not co-precipitate with Tlg1p or Tlg2p (Figure 10C). Myc-tagged Sft1p also failed to bind to the Tlg proteins, but did associate with Sed5p (Figure 10D). A tagged version of Sft1p was used because the available antibodies to endogenous Sft1p produced only weak signals on blots. As a control, we also assayed the ER–Golgi v-SNARE Sec22p. As previously described (Søgaard *et al.*, 1994), ~10% of the total Sec22p was associated with Sed5p, but we could detect no binding to Tlg1p or Tlg2p (Figure 10C).

These results show that the Tlg proteins have the expected characteristics of SNAREs. They can be found in complexes containing Sec17p and v-SNAREs implicated in traffic between the Golgi apparatus, endosomes and the plasma membrane. However, they do not seem to provide a target for Sft1p or Gos1p, v-SNAREs with a role in intra-Golgi traffic.

Discussion

Two new syntaxins on the late side of the Golgi complex

This work completes the initial characterization of the eight syntaxins in yeast. These SNAREs have been hypothesized to define the identity of individual membrane compartments by determining which transport vesicles are able to fuse with them (Hardwick and Pelham, 1992; Söllner *et al.*, 1993b), and members of the family have

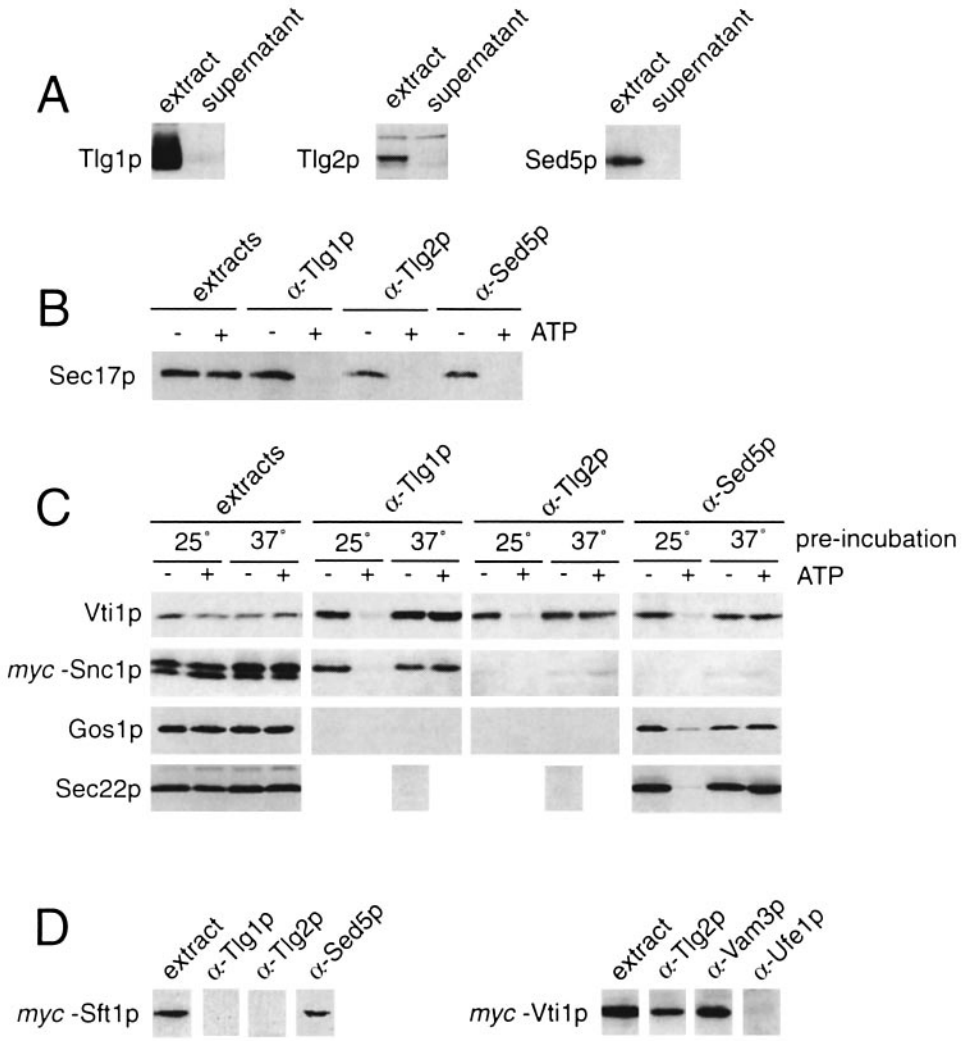

Fig. 10. Co-precipitation of SNAREs. (**A**) Immunoprecipitation of Tlg1p, Tlg2p and Sed5p. Specific antisera were used to precipitate, and subsequently detect by immunoblotting, the SNARE proteins. Precipitation was >90% efficient, as evidenced by removal of protein from the post-precipitation supernatant. (**B**) Co-precipitation of Sec17p with Tlg1p, Tlg2p and Sed5p. Extracts from *sec18-1* cells grown at a permissive temperature were prepared in the presence or absence of ATP and incubated with antibodies to Tlg1p, Tlg2p or Sed5p coupled to Protein A–Sepharose beads. Both extracts and precipitates were immunoblotted with antibodies to Sec17p. The extract samples correspond to 5% of the precipitated material. (**C**) Co-precipitation of putative v-SNAREs with Tlg1p, Tlg2p and Sed5p. *sec18-1* cells were incubated at permissive (25°C) or non-permissive (37°C) temperatures before lysis, and lysed in the presence or absence of ATP, as shown. The resultant extracts were incubated with antibodies to Tlg1p, Tlg2p or Sed5p coupled to Protein A–Sepharose beads, and the precipitates analysed by immunoblotting with antibodies to Vti1p, Gos1p and Sec22p. Snc1p in the precipitates was detected using the same procedure, but extracts were prepared from *sec18-1* cells expressing myc-tagged Snc1p from a multicopy vector, and immunoblotting was with a polyclonal anti-myc antibody. The extract samples again correspond to 5% of the precipitated material. (**D**) Co-precipitation of Sft1p and Vti1p with putative t-SNAREs. SEY6210 cells expressing myc-tagged Sft1p or Vti1p from multi-copy plasmids were lysed in the absence of ATP and incubated with antibody beads as shown. Precipitates were analysed as in (C), using a polyclonal anti-myc antibody.

previously been detected on the ER, early Golgi, plasma membrane, late endosome and vacuole.

Both immunofluorescence and density gradient fractionation indicate that Tlg2p co-localizes with the TGN marker Kex2p, and it also co-fractionates with a second TGN marker, DPAP A. We therefore consider Tlg2p to be located primarily in the yeast equivalent of the TGN. That this is largely distinct from the early Golgi compartment containing Sed5p was shown both by density gradient fractionation and by double-label immunofluorescence of Tlg2p and Sed5p.

The location of Tlg1p is more intriguing. It showed at most partial co-localization with Tlg2p, Kex2p and DPAP A, and peaked on gradients at a density distinct from that of earlier Golgi compartments, late endosomes, vacuoles and plasma membrane. However, Δ*tlg1* mutants showed significant loss of TGN proteins to the vacuole and a severe block to endocytosis. Since all known TGN proteins in yeast are thought to cycle through an endosomal compartment, we suggest that these data can best be explained if Tlg1p is present in this recycling compartment. Indeed, Tlg1p was found at the position of a minor Kex2p peak on density gradients. Other unpublished observations made during the course of this study are also consistent with this interpretation. Thus, we noticed that highly overexpressed Tlg2p accumulated in the Tlg1p fraction on gradients, and co-localized with it by immunofluorescence, whereas overexpressed Tlg1p was found mostly at its

normal position, with some in the late endosome/vacuole region. Since overexpression tends to saturate retrieval mechanisms, these observations support the idea of a pathway from Tlg2p to Tlg1p, then to late endosomes.

We thus tentatively identify the Tlg1p compartment as an early endosome, presumably accessible from both the TGN and the plasma membrane. The size and shape abnormalities of Δ*tlg1* cells could be explained by inefficient endocytosis from the plasma membrane, and transfer to the TGN, of components required for the targeting of TGN-derived secretory vesicles to the bud. In fact, Δ*tlg1* cells show some residual endocytosis, which may be required to sustain growth; complete elimination of this might account for the synthetic lethality of Δ*pep12* and Δ*tlg1*.

A role for Tlg1p in endocytosis also provides an explanation for its association with Snc1p: just as the v-SNARE Sec22p is required for both ER–Golgi and Golgi–ER traffic (Lewis *et al.*, 1997), so Snc1p could be involved in both exocytosis and endocytosis. Interestingly, mammalian syntaxin 6, which shows some similarity to Tlg1p, has been reported to bind the Snc1p homologue cellubrevin (Bock *et al.*, 1997). Syntaxin 6 is found both in the TGN and in vesicles and endocytic structures, and has been postulated to cycle between TGN and endosomes.

Perhaps surprisingly, endocytosis was also inhibited in a Δ*tlg2* mutant, though less strongly than in Δ*tlg1* cells. This suggests a functional link between TGN and endosomes. A functional TGN might be important for the removal of material from endosomes, or for the recycling to the cell surface of some component required for endocytosis. Indeed, we have recently found that in a Δ*tlg2* strain Tlg1p fractionates at a lower density than normal, implying either that Tlg1p shifts its location, or that the composition of the Tlg1p compartment is affected in some other way by the absence of Tlg2p.

Evidence for an early endosomal compartment in yeast has been presented previously (Hicke *et al.*, 1997), and we have tried to relate these earlier observations to ours. However, we have so far been unable to detect internalized alpha-factor receptor in early endosomal intermediates using our fractionation procedure, possibly due to the short half-life of these intermediates. As an alternative approach, we have expressed a GFP-tagged version of Tlg1p and observed cells during uptake of the endocytic tracer dye FM4-64 (Vida and Emr, 1995). At early times there was considerable coincidence of GFP and FM4-64 fluorescence, but also some tendency of the GFP-tagged protein to be mislocalized, which made identification of the fluorescent spots uncertain. Hence, while it seems very likely that Tlg1p is on the endocytic pathway, proof of this must await more detailed uptake studies.

Organization of the intracellular membrane system

Figure 11 illustrates our working model for the organization of the yeast endomembrane system, with the locations of the eight syntaxins indicated. The principal exocytic and endocytic pathways, as currently envisaged, are marked by double arrows, and single arrows indicate some likely vesicular transport routes between them. So far the only candidate v-SNARE to mediate these connections is Vti1p. Remarkably, this and previous work (Fischer von Mollard *et al.*, 1997) indicate that Vti1p binds to five of the eight

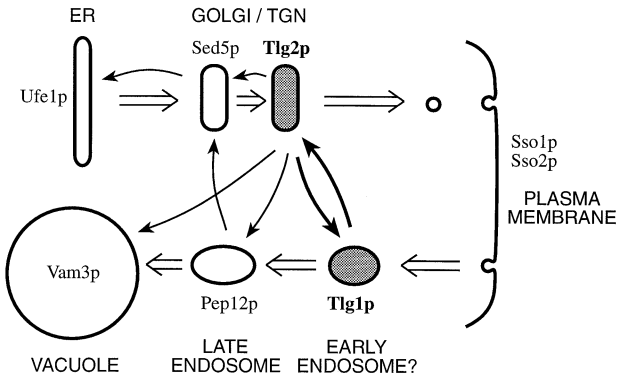

Fig. 11. Model for the distribution and function of all syntaxins within the yeast cell. Double arrows indicate the main exocytic and endocytic pathways; single arrows indicate presumed vesicular steps as discussed in the text. The Tlg1p and Tlg2p compartments and the likely connections between them are highlighted by shading and bold arrows. Note that additional pathways, for example from early endosomes to the plasma membrane, are also possible.

syntaxins, namely Sed5p, Tlg1p, Tlg2p, Pep12p and Vam3p. It may thus be involved in numerous transport pathways. Clearly other factors must contribute to the specificity of the docking and fusion events, but one consequence of the repeated use of Vti1p is that it may result in alternative targets being selected if the correct one is unavailable.

As discussed above, TGN proteins are likely to pass to the Tlg1p compartment, probably directly since they do not normally reach the cell surface (Wilcox *et al.*, 1992). In the absence of Tlg2p, Kex2p tends to follow the endocytic pathway to the vacuole, presumably because the return pathway to the TGN is impaired. However, in Δ*tlg1* cells Kex2p is also degraded in the vacuole, implying that passage through the Tlg1p compartment is not obligatory for transport from Golgi to late endosomes (or to the vacuole). Late endosomes apparently remain functional in the absence of Tlg1p, because sorting of CPY continues, in contrast to its secretion in a *pep12* mutant. Hence, the inefficient retrieval of Kex2p in Δ*tlg1* cells argues against the previous assumption that all TGN proteins recycle from late endosomes (Bryant and Stevens, 1997). A recent report describes two other genes, *SOP2* and *SOP6*, which are required to maintain Kex2p levels but not for CPY sorting (Luo and Chang, 1997). A possible explanation is that Kex2p passes preferentially from the TGN to the Tlg1p compartment, and returns from there, whereas the CPY sorting receptor travels directly to and from late endosomes. A precedent for such selective alternative pathways is provided by the recent demonstration that the membrane protein alkaline phosphatase carries a sorting signal that allows it to be transported from the Golgi to the vacuole, bypassing late endosomes (Cowles *et al.*, 1997; Piper *et al.*, 1997).

There are also likely to be multiple return paths from the endocytic pathway to the Golgi complex. The continued sorting of CPY in a Δ*tlg2* mutant (and in the Δ*tlg1* Δ*tlg2* double mutant) implies that the CPY receptor can return to the Golgi complex by a Tlg2p-independent route. As pointed out by Fischer von Mollard *et al.* (1997), this could occur via the Sed5p compartment. Though such alternative routes are likely to be at least partially selective,

with coat proteins recognizing particular sorting motifs, their inherent redundancy may help to explain the viability of *tlg1*, *tlg2* and *pep12* mutants.

Implications for intra-Golgi transport

It was surprising to find that removal of Tlg2p, or even of both Tlg2p and Tlg1p, had remarkably little effect on forward traffic through the Golgi complex as measured by the secretion of invertase and other glycoproteins, the maturation of CPY, and the lack of accumulation of Golgi membranes. Furthermore, neither Tlg protein was associated with the known intra-Golgi v-SNAREs Sft1p and Gos1p. Thus, although the TGN contains a syntaxin, it does not appear to be needed for transport from the early Golgi to the TGN. Golgi function evidently requires only a single syntaxin, Sed5p.

This result does not obviously fit a simple model for intra-Golgi traffic based on SNARE-mediated anterograde vesicular transport between stable compartments (Rothman and Warren, 1994). A different t-SNARE might exist for forward transport to the TGN, but this could not be syntaxin-like since all the syntaxins have been accounted for. This step would thus differ from other known vesicular transport steps in yeast, each of which uses a syntaxin as an essential component. Alternatively, the Golgi complex might function, in the absence of Tlg2p, as a single compartment. However, the ability to do so seems incompatible with the essential requirement for Sft1p, a SNARE whose only known role is in intra-Golgi traffic. Possibly, different compartments could each contain Sed5p, but these would require additional targeting mechanisms to maintain their separate identities.

Our results seem more compatible with a cisternal maturation model for Golgi transport, a concept which has received considerable attention recently (Bannykh and Balch, 1997; Mironov *et al.*, 1997; Schekman and Mellman, 1997; Pelham, 1998). This model assumes that new Golgi compartments are formed by the fusion of ER-derived transport vesicles and then 'mature' by receiving Golgi enzymes by vesicular transport from older cisternae, followed by TGN proteins recycled from endosomes. At any one time, membranes at all stages of maturation are present. Eventually, the cisternae break up entirely into sets of vesicles destined for the plasma membrane, endosomes, or younger Golgi compartments.

In this scenario the only roles for Sft1p and Gos1p would be in the retrograde vesicular transport of Golgi proteins, and thus they would need to bind only Sed5p, as observed, and not Tlg2p. Tlg2p would act merely as a target for vesicles from the endocytic pathway and thus not be needed for forward transport *per se*. Furthermore, the need to recycle TGN proteins, including Tlg2p, can be seen as an inevitable consequence of the limited life of any individual Golgi compartment, making the distinction between TGN and endosomal proteins a subtle one. The situation may be even more fluid: as with the Golgi complex, vesicular and maturation models for the endocytic pathway have been much discussed, and the early/late endosome transition in animal cells is thought to involve at least an element of maturation (Gruenberg and Maxfield, 1995). Thus, the Tlg1p compartment may not be stable, and Tlg1p itself may have to recycle.

In conclusion, we now have a relatively simple picture of the yeast endomembrane system consisting of seven different membrane-bound organelles defined by eight syntaxins. Despite the uncertainty about the precise ways in which proteins and lipids move between these, the syntaxins should provide useful landmarks for future studies.

Materials and methods

Plasmids

N-terminally myc-tagged constructs containing the open reading frames of *SNC1*, *GOS1 TLG1*, *TLG2* and *VTI1* were generated by PCR amplification of yeast genomic DNA using Pfu polymerase (Stratagene) and subsequent ligation into the vectors JS209 (2μ, *URA3*; Semenza *et al.*, 1990) and pRS316 (CEN, *URA3*; Sikorski and Hieter, 1989) behind the *TPI* promoter. The CEN vectors contained three copies of the myc epitope. PCR-generated constructs were checked by dideoxy sequencing. Myc-tagged invertase and SFT1-expressing plasmids used in this study have been described previously (Banfield *et al.*, 1995). All enzymes for manipulation of DNA were from New England Biolabs.

Yeast strains

Unless indicated, all yeast strains were grown at 30°C in yeast extract–peptone–dextrose medium (YEPD) or synthetic dextrose medium (SD) as appropriate. Standard yeast genetic techniques for sporulation, tetrad analysis and gene disruption were employed as described by Guthrie and Fink (1991). Yeast transformations were carried out as described by Elble (1992).

Immunofluorescence was performed in diploid cells obtained by crossing the strains SEY6210 (*MAT*α *ura3-52 his3-*Δ*200 leu2-3, -112 trp1-*Δ*901 suc2-*Δ*9 lys2-801*) and SEY6211 (*MAT***a** *ura3-52 his3-*Δ*200 leu2-3, -112 trp1-*Δ*901 suc2-*Δ*9 ade2-101*). Deletion phenotypes were characterized in SEY6210 or in haploid progeny of SEY6210/6211 diploids; deletions of *TLG1* and *TLG2* in strain K699 (from K.Nasmyth) were also viable. Precipitation of the SNARE complexes was performed in strain RSY271 (*MAT*α *sec18-1 ura3-52 his4-619*).

Three copies of the HA epitope were inserted at the C-terminus of endogenously expressed Tlg2p in SEY6210 by recombination with a PCR-generated cassette containing appropriate homologous regions, the epitope tag, and the *HIS5* gene of *Schizosaccharomyces pombe*. Gene disruption was performed using PCR for replacement of the entire coding region with selectable markers. The PCR primers contained 40–65 bases of identity to the regions flanking the open reading frame. *TLG1* was disrupted with *TRP1*, and the resultant Δ*tlg1* strain (JHY016, *MAT***a**) was used throughout this study. *TLG2* was disrupted with a PCR-generated construct containing the *HIS5* gene of *S.pombe* flanked by similarly oriented coliphage *loxP* sites, generating a Δ*tlg2* strain (JHY002, *MAT*α). The *HIS5* gene was subsequently removed by excisive recombination at the *loxP* sites. This was accomplished by transforming JHY002 with a plasmid harbouring the coliphage *Cre* gene under control of the *GAL1* promoter, thus allowing transient expression of *Cre* recombinase (Sauer, 1987). The resulting Δ*tlg2* His$^-$ strain (JHY004, *MAT*α) was crossed to SEY6211, transformed with the *TRP1*-based *TLG1* disruption construct, and sporulated. Tetrad dissection and genotypic characterization of Trp$^+$ spores led to the isolation of a haploid Δ*tlg1* Δ*tlg2* strain (JHY014, *MAT***a**). This procedure also led to the isolation of a haploid Δ*tlg2* His$^-$ strain with a mating type opposite to JHY004 (JHY021, *MAT***a**). The JHY021 strain was used in the Ste2p degradation assay (see below), and JH004 for all other experiments described. For disruption of the *PEP12* gene, 500 base pairs homologous to the regions flanking the ORF were amplified by PCR from yeast genomic DNA and cloned into restriction sites located on either site of a *loxP–HIS3–loxP* cassette which was ligated into the *Eco*RI and *Spe*I sites of the pBluescript KS$^-$ vector (Stratagene; the *loxP–HIS3–loxP* plasmid was a generous gift of T.Levine, MRC, Cambridge). Transformation of the linearized *PEP12* disruption construct into SEY6210 cells allowed the isolation of a Δ*pep12* strain (JHY005, *MAT*α). The Δ*pep4* (JHY024, *MAT*α) and Δ*tlg1* Δ*tlg2* Δ*pep4* (JHY027, *MAT***a**) strains were generated by transforming SEY6210 and JHY014 cells with an *Eco*RI–*Xba*I fragment containing the *PEP4* gene with *HIS3* inserted between the *Bam*HI and *Hin*dIII sites (gift of R.Chapman, MRC, Cambridge). All gene disruptions were verified by PCR and/or immunoblot analysis using antibodies raised against the encoded gene products.

Antibodies and immunoblotting

To raise polyclonal antibodies against Tlg1p, Tlg2p, Gos1p and Pep12p, truncated versions of these proteins lacking transmembrane domains and with C- or N-terminal hexahistidine tails were expressed in *Escherichia coli* BL21 cells. For Tlg1p and Pep12p this was done by PCR amplification of plasmid using oligonucleotides that introduce *Nde*I and *Xho*I sites immediately upstream and downstream of the cytoplasmic domain. Subsequent cloning of PCR products into the *Nde*I and *Xho*I sites of the bacterial expression plasmid pET-24a (Novagen) allowed the production of C-terminally His_6-tagged protein (plasmid pTlg1HISC1 for Tlg1p, pP12HISC1 for Pep12p). To produce N-terminally His_6-tagged versions of Tlg2p and Gos1p, *Bam*HI and *Xho*I sites were introduced immediately upstream and downstream of their cytoplasmic domains by PCR and the amplified products cloned into the *Bam*HI and *Xho*I sites of the bacterial expression plasmid pTrcHisA (Invitrogen; plasmid pTLG2HisN1 for Tlg2p; pP28HisN3 for Gos1p). After purification by Ni^{2+}-NTA agarose affinity chromatography, the His_6-tagged proteins were used to immunize rabbits. The resulting antisera (Tlg1p: MRC03; Tlg2p: MRC20; Gos1p: MRC22; Pep12p: MRC01) were used for immunoblot analysis at a dilution of 1:2000.

Rabbit polyclonal antibodies to Sed5p, CPY, Sft1p, Ufe1p and Vam3p were described previously (Hardwick and Pelham, 1992; Banfield *et al.*, 1995; Lewis *et al.*, 1997; Nichols *et al.*, 1997). Rabbit antibodies to Ste2p were provided by J.Konopka (State University of New York, Stony Brook). Antibody to Sec17p was from W.Wickner (Dartmouth College, NH) and antibodies to Vti1p and DPAP A were from T.Stevens (University of Oregon). The human *c-myc* epitope was detected with mouse monoclonal antibody 9E10 or with rabbit polyclonal antibodies (Santa Cruz). Rabbit polyclonal and rat monoclonal antibodies recognizing the HA epitope were from Santa Cruz and Boehringer-Mannheim respectively.

For immunoblotting, all antibody incubations were carried out in phosphate-buffered saline (PBS) containing 5% dried milk and 0.2% Tween-20. After incubation with peroxidase-conjugated secondary antibodies (BioRad), detection was performed using enhanced chemiluminescence (ECL kit, Amersham) and bands quantified on fluorograms using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA) with Imagequant software.

Immunofluorescence

Cells were fixed and mounted on slides as described previously by Hardwick and Pelham (1992). Antibody incubations were carried out in PBS supplemented with 2% dried milk for 2 h at room temperature. Fluorescein and Cy3-conjugated secondary antibodies (Amersham) were used for visualization. Dual images were obtained with an MRC-600 confocal laser scanning microscope (BioRad) using separate excitation at the appropriate wavelengths to avoid bleed-through.

Subcellular fractionation

Fractionation studies were carried out essentially as described by Becherer *et al.* (1996). Cells were grown in 500 ml SD medium to mid-logarithmic phase (OD_{600} of 0.6–1.0), harvested and resuspended in 50 ml 200 mM Tris–HCl, pH 8.0, 20 mM EDTA, 1% β-mercaptoethanol, 5 mM NaN_3 and 5 mM NaF. After incubation at 30°C for 10 min, cells were harvested and resuspended in 50 ml synthetic medium containing 1 M sorbitol, 5 mM NaN_3 and 5 mM NaF (spheroplast medium) supplemented with 0.3 mg/ml zymolyase-100T (ICN). Cells were incubated at 30°C for 20–30 min, cooled on ice and washed twice in ice-cold spheroplast medium. All subsequent steps were carried out at 4°C. Spheroplasts were lysed in 4–6 ml ice-cold hypo-osmotic buffer (50 mM Tris–HCl, pH 7.5, 200 mM sorbitol, 1 mM EDTA) containing freshly added protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml antipain, 1 mM benzamidine and 1 mM PMSF) using 10–15 strokes in a Dounce homogenizer. The cell lysate was centrifuged sequentially at 500 *g* (5 min), 13 000 *g* (10 min) and 100 000 *g* (60 min). The 100 000 *g* membrane pellet was resuspended in 2 ml of hypo-osmotic lysis buffer containing protease inhibitors and loaded on top of a 22–60% sucrose step gradient. All sucrose solutions were made (w/w, %) with ultra-pure sucrose (Gibco-BRL) in 10 mM HEPES–KOH, pH 7.6, 1 mM EDTA and the gradient was generated using the following steps: 1.5 ml 60%, 1.0 ml 37%, 1.5 ml 34%, 2.0 ml 32%, 2.0 ml 29%, 1.5 ml 27% and 1.0 ml 22%. After centrifugation in a Beckman SW40Ti rotor at 170 000 *g* for 17–18 h, 16×0.78-ml fractions were collected from the top and the sucrose concentration was determined by refractive index. Equal volumes per fraction were used to assay for Kex2p endoprotease activity (Cunningham and Wickner, 1989), and for immunoblotting.

Electron microscopy

Wild-type (SEY6210) cells and *tlg* mutants were grown in YEPD medium and harvested at an OD_{600} of 1. Permanganate fixation, dehydration and embedding in Spurr's resin (Agar Scientific, Stansted, UK) was carried out as described by Kaiser and Sheckman (1990). Sections were stained with 5% uranyl acetate for 10 min at 60°C followed by 5 min in Reynold's lead citrate at room temperature.

TGN protein assays

Cells were spheroplasted as described in the section on immunoprecipitation experiments, resuspended in 50 mM Tris–HCl, pH 7.5, 200 mM sorbitol, 1 mM EDTA, and then lysed by vortexing with glass beads. Following centrifugation for 10 min at 25°C, supernatants were assayed for total protein and their volume adjusted to give equivalent protein concentrations. The samples were either assayed for Kex2p activity as described (Cunningham and Wickner, 1989) or immunoblotted with a specific antibody to DPAP A. Quantitation of immunoblots was by densitometric scanning of fluorograms.

Ste2p degradation assay

Ligand-induced degradation of Ste2p was analysed essentially as described by Hicke and Riezman (1996). In brief, cells were grown in 100 ml YEPD medium to mid-logarithmic phase (OD_{600} of 1.0) before addition of 10 μg/ml cycloheximide. After a 10 min incubation, a 10 ml aliquot of cells was removed to a tube on ice containing NaN_3/NaF (20 mM final concentration) and α-factor was added to the culture to a final concentration of 1 μM. At various times, 10 ml aliquots of cells were removed from the culture and transferred to tubes on ice as before. Cells were collected by centrifugation, washed in 5 ml of ice-cold TEPI (50 mM Tris–HCl, pH 7.5, 5 mM EDTA and protease inhibitors) and lysed by mechanical agitation with 0.4 g of glass beads in 350 μl TEPI and 5 mM NEM with five, 1-min bursts on an Ika Vibrax VXR at 4°C. Next, 350 μl urea/SDS buffer (40 mM Tris–HCl, pH 6.8, 0.1 mM EDTA, 9 M urea, 5% SDS) and 5 mM NEM was added and the lysate heated to 50°C for 10 min. After clearing the lysates by centrifugation (13 000 *g*, 15 min), equal aliquots were analysed by immunoblotting. Ste2p was identified as a doublet band of ~47 kDa which was absent in total cell extracts prepared from a Δ*ste2* strain (a kind gift of J.Konopka, State University of New York, Stony Brook).

Pulse–chase analysis of CPY and secreted glycoproteins

Pulse–chase analysis was carried out exactly as described by Harris and Waters (1996), although pulse labelling was for 4 rather than 2 min. In order to analyse total secreted glycoprotein, appropriate strains were grown in low-sulfate medium (Harris and Waters, 1996) and spheroplasted as described for immunoprecipitation experiments. Spheroplasts were resuspended at 3 OD_{600} per ml, and 50 μCi Tran^{35}S-label (ICN) per ml was added. At the time points indicated, 1 ml of spheroplasts was placed at 0°C and 1% NaN_3 was added. At the end of the experiment, intracellular and extracellular fractions were separated by centrifugation, and the cellular fraction was resuspended in SDS–PAGE sample buffer. The extracellular fraction was precipitated using methanol:chloroform and resuspended in SDS–PAGE sample buffer. Both fractions were analysed by SDS–PAGE and autoradiography. In order specifically to visualize labelled glycoproteins, the samples were diluted 10-fold in PBS and incubated for 1 h with Concanavalin A-coupled Sepharose beads (Pharmacia). The beads were washed twice in PBS containing 0.2% SDS and bound glycoproteins eluted by boiling in SDS–PAGE sample buffer.

Co-immunoprecipitation

Antisera were bound to Protein A–Sepharose (Pharmacia) and cross-linked with dimethyl pimelimidate (Harlow and Lane, 1988). 300 OD_{600} equivalents of cells grown to an OD_{600} of 3 were harvested by centrifugation, incubated for 10 min at 30°C in 25 ml 100 mM PIPES, pH 9.6, 10 mM DTT, re-isolated, spheroplasted in 25 ml YEPD containing 1 M sorbitol, 1 mM DTT and 5 mg zymolyase-100T for 30 min at 30°C, re-isolated, and washed in YEPD containing 1 M sorbitol. Where the effect of inactivating Sec18p by incubation of a *sec18-1* strain at non-permissive temperatures was to be studied the spheroplasts were resuspended in YEPD–sorbitol and incubated with gentle shaking for 30 min at either 25°C or 37°C. Spheroplasts were then harvested by centrifugation and resuspended in lysis buffer (PBS, 1.5% Triton X-100, 10 mM β-mercaptoethanol, and protease inhibitors), at 4°C. In some experiments the lysis buffer also included an ATP-regenerating system. The spheroplasts were lysed by Dounce homogenization, and clarified by centrifugation for 1 h at 20 000 *g*. Following removal of lipid droplets

and pelleted debris, detergent extracts were diluted to 2 mg/ml protein and typically 1 ml of extract was precipitated overnight at 4°C with 25 µl of antibody-coupled beads. Immunoprecipitates were washed consecutively with lysis buffer, lysis buffer containing 800 mM NaCl, and lysis buffer. Complexes were eluted from the beads with glycine–HCl, pH 2.4, precipitated with methanol:chloroform, and analysed by immunoblotting.

Acknowledgements

We thank Douglas Kershaw for help with electron microscopy, and Sean Munro, Tim Levine, Rob Arkowitz, Mike Lewis and Julian Rayner for valuable reagents and advice. We are grateful to Tom Stevens, Gabriele Fischer von Mollard and Nia Bryant for communicating unpublished data on Vti1p, and for generously providing antibodies to Vti1p and DPAP A. J.C.M.H. was supported by a training fellowship from the European Commission and S.D. by a Herchel Smith fellowship.

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Received July 3, 1997; revised September 24, 1997;
accepted October 14, 1997