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Got1p and Sft2p: membrane proteins involved in traffic to the Golgi complex

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Traffic through the yeast Golgi complex depends on a member of the syntaxin family of SNARE proteins, Sed5p, present in early Golgi cisternae. Sft2p is a non-essential tetra-spanning membrane protein, found mostly in the late Golgi, that can suppress some *sed5* alleles. We screened for mutations that show synthetic lethality with *sft2* and found one that affects a previously uncharacterized membrane protein, Got1p, as well as new alleles of *sed5* and *vps3*. Got1p is an evolutionarily conserved non-essential protein with a membrane topology similar to that of Sft2p. Immunofluorescence and subcellular fractionation indicate that it is present in early Golgi cisternae. *got1* mutants, but not *sft2* mutants, show a defect in an *in vitro* assay for ER–Golgi transport at a step after vesicle tethering to Golgi membranes. *In vivo*, inactivation of both Got1p and Sft2p results in phenotypes ascribable to a defect in endosome–Golgi traffic, while their complete removal results in an ER–Golgi transport defect. Thus the presence of either Got1p or Sft2p is required for vesicle fusion with the Golgi complex *in vivo*. We suggest that Got1p normally facilitates Sed5p-dependent fusion events, while Sft2p performs a related function in the late Golgi.

Keywords: Golgi/SNARE/syntaxin/yeast

Introduction

Traffic of proteins through the secretory and endocytic pathways of eukaryotic cells involves the budding and fusion of transport vesicles. Membrane fusion is highly specific, and typically involves the docking of a vesicle with a larger structure such as a Golgi cisterna, followed by the bilayer fusion event itself. These events depend on integral membrane proteins termed SNAREs which form tight helical bundles with each other, and in so doing help to force membranes together (Rothman, 1994; Hay and Scheller, 1997; Nichols and Pelham, 1998; Sutton *et al*., 1998). Disruption of these bundles prior to (or after) fusion is mediated by proteins termed SNAP (Sec17p in yeast) and NSF (Sec18p), and is necessary to allow formation of complexes which span the fusing membranes (Söllner *et al*., 1993; Ungermann *et al*., 1998a). Of particular importance are the members of the syntaxin family of SNAREs, a member of this family having been implicated in every fusion event that has been studied so far. Yeast has eight identifiable syntaxins distributed amongst the various organelles of the cell together with a dozen or so other SNAREs, and extensive localization and functional studies have resulted in a route-map of the trafficking pathways along and between the exocytic and endocytic pathways (Pelham, 1999).

Despite the importance of the SNAREs, it has become apparent that they are not sufficient for efficient membrane fusion, and that mutual recognition between them cannot entirely account for the specificity of membrane fusion events. Additional peripheral membrane proteins, some recruited by small GTPases of the rab/ypt family, can mediate specific tethering of membranes to each other prior to SNARE engagement, and may contribute significantly to the selectivity of the process (Simonsen *et al*., 1998; Sonnichsen *et al*., 1998; Ungermann *et al*., 1998b). In yeast Uso1p, a protein containing extensive coiled-coil motifs is recruited to membranes by Ypt1p and can mediate the docking of ER-derived vesicles with Golgi membranes *in vitro*, even when SNAREs are inactivated (Cao *et al*., 1998). Other proteins that are required for transport to and through the Golgi complex include Sec35p (VanRheenan *et al*., 1998) and the TRAPP complex (Sacher *et al*., 1998). How these proteins are bound to membranes is not known. Other than the SNAREs, few integral membrane proteins with a transport function are known, although one has recently been described: the Ypt1p-interacting integral membrane protein Yip1p (Yang *et al*., 1998).

In yeast, Golgi function shows an absolute requirement for only one syntaxin, Sed5p, which cycles through the ER but is abundant in early Golgi cisternae (Hardwick and Pelham, 1992; Holthuis *et al*., 1998b; Wooding and Pelham, 1998). Sed5p interacts with at least seven other SNAREs, forming at least three distinct complexes (Nichols and Pelham, 1998). One of these, characterized by the presence of the SNARE Bos1p, is involved in the fusion of ER-derived vesicles with early Golgi membranes, and/or the homotypic fusion of such vesicles to form new cisternae. The other two, which contain Gos1p and Vti1p, respectively, are probably formed when retrograde vesicles originating from later Golgi cisternae and from the endosomal system fuse with early cisternae. Two more syntaxins, Tlg1p and Tlg2p, seem to be at least partly associated with the Golgi complex, though they are probably also in early endosomes (Abeliovich *et al*., 1998; Holthuis *et al*., 1998b; Seron *et al*., 1998). They co-localize with late Golgi markers such as Kex2p and are required for the retrieval of proteins from the endocytic pathway to the yeast equivalent of the *trans*-Golgi network (Holthuis *et al*., 1998a,b).

A screen for genes which, when overexpressed, could suppress the growth phenotype of a *sed5* temperature-

sensitive mutation yielded two genes termed *sft1* and *sft2* (Banfield *et al*., 1995). Sft1p is a SNARE that binds to Sed5p but is mostly present in late Golgi membranes which lack Sed5p; it is required for transport of resident Golgi proteins from late to early Golgi cisternae (Banfield *et al*., 1995; Wooding and Pelham, 1998). Sft2p is a conserved protein with four putative transmembrane domains which is also located in a late Golgi compartment, but enters presumptive retrograde intra-Golgi vesicles whose fusion depends on Sft1p and Sed5p (Wooding and Pelham, 1998). Its genetic interaction with Sed5p suggests that Sft2p may be an additional membrane component involved in the docking or fusion process, but surprisingly strains lacking *SFT2* grow normally and show no obvious secretory defect, and hence the normal role of this gene has remained unknown.

We sought clues to the function of *SFT2* by isolating mutant yeast strains in which it is essential. Mutations that show synthetic lethality with *sft2* include a new allele of *sed5*, and a disruption of a previously uncharacterized gene we term *GOT1*. Got1p is a functionally conserved protein with four putative transmembrane domains, which partially co-localizes with Sed5p. It is not essential for growth, but its removal significantly reduces the efficiency of ER–Golgi transport *in vitro*, hindering not the initial tethering of vesicles, but their subsequent SNARE-dependent fusion. *In vivo*, simultaneous removal of both Sft2p and Got1p affects both ER–Golgi transport and the retrieval of proteins from the endocytic pathway. Thus these two integral membrane proteins seem to have partially redundant roles in the fusion of vesicles with Golgi membranes. We suggest that Got1p normally facilitates Sed5p-dependent fusion events, while Sft2p provides an equivalent function in the late Golgi.

Results

Synthetic lethal screen for isolation of mutants that require Sft2p function

To gain insight into the function of Sft2p, we undertook a genetic approach. A synthetic lethal screen was used to identify other genes whose mutation renders Sft2p essential for survival. The screen was based on a colony sectoring assay (Koshland *et al*., 1985; Kranz and Holm, 1990). Strains of each mating type with mutations in *ade2*, *ade3* and *sft2* were transformed with a centromeric plasmid carrying *URA3*, *ADE3* and *SFT2*. The presence of the *ADE3* gene on the plasmid caused these strains to form red colonies, but under non-selective conditions spontaneous loss of the plasmid generated white sectors. After UV mutagenesis, colonies that no longer sectored were selected for further analysis. From a total of ~100 000 colonies screened, three mutants with a clear synthetic lethal phenotype were identified. The corresponding mutant genes were identified by complementation of the sectoring phenotype after transformation with a yeast genomic DNA library. For each mutant, one or two complementing plasmids were obtained and sequences determined from each end of the genomic DNA inserts.

The DNA fragment able to complement the first mutant consisted of a region of chromosome XII which contains the complete *SED5* gene, and transformation of a plasmid containing only the *SED5* gene was sufficient to restore sectoring. The mutated allele of *sed5* was isolated by PCR and sequenced, which revealed a single point mutation Y311→D. This amino acid is nine residues from the transmembrane domain, and the mutation introduces a charge on the predominantly hydrophobic face of the coiled-coil domain of the protein. Hence, it would be expected to hinder the formation or function of Sed5p-containing SNARE complexes. The synthetic lethality of this mutation with *sft2* mirrors the suppression of *sed5-1* by *SFT2* overexpression, and further emphasizes the link between these two proteins.

The sectoring phenotype of the second mutant was restored by a region of chromosome IV containing the *VPS3* gene. *VPS3* encodes a large, hydrophilic, membrane-associated protein which is required for efficient sorting of carboxypeptidase Y (CPY) to the vacuole; its precise function is unknown. Our isolation of this gene suggests a possible involvement of *SFT2* in traffic between the endocytic and exocytic pathways, but we did not analyse the mutant further.

The final mutant was complemented by an 8 kb genomic fragment from chromosome XIII. After restriction and subcloning, the complementing activity was localized to the previously uncharacterized open reading frame YMR292w. Sequencing of this region from the mutant revealed a single point mutation, G73→S. To confirm that mutation of YMR292w was responsible for the synthetic lethality with *sft2*, we disrupted this gene in our wild-type strain (SEY6210). The disruptant was viable and had no obvious growth or secretory defect. However, when the cells were mated with *sft2* null cells and the diploids sporulated, no viable haploids carrying both disruptions could be recovered. This establishes YMR292w as a non-essential gene that is required for growth in the absence of *SFT2*. In the light of subsequent experiments we named this gene *GOT1*, for Golgi transport, and the G73→S allele *got1-1*.

Got1p is a member of an evolutionarily conserved family of proteins

The predicted protein sequence encoded by *GOT1* is shown in Figure 1A. Got1p is a 138-residue protein with a calculated mass of 15.4 kDa. It is likely to be an integral membrane protein since its sequence contains at least three and probably four hydrophobic stretches of sufficient length to be transmembrane domains (Figure 1B).

Database searches revealed that Got1p is a member of a phylogenetically conserved family of proteins. Homologues were identified in *Caenorhabditis elegans* (39.8% identity), *Plasmodium falciparum* (33% identity) and in mammals. None of these homologues had previously been characterized. As shown in Figure 1A, two related gene sequences were also identified in the human expressed sequence tag (EST) database. We designated these hGot1a and hGot1b, with 38.4% and 41.3% identity to Got1p, respectively. The sequence of hGot1a was constructed entirely from overlapping ESTs. The sequence of hGot1b was not completely represented in ESTs, but was obtained from the corresponding cDNA clone.

To confirm the functional relevance of these homologies we expressed the hGot1b sequence from a centromere-based yeast vector, and found that it could support growth of a *got1 sft2* double null strain at a rate comparable with

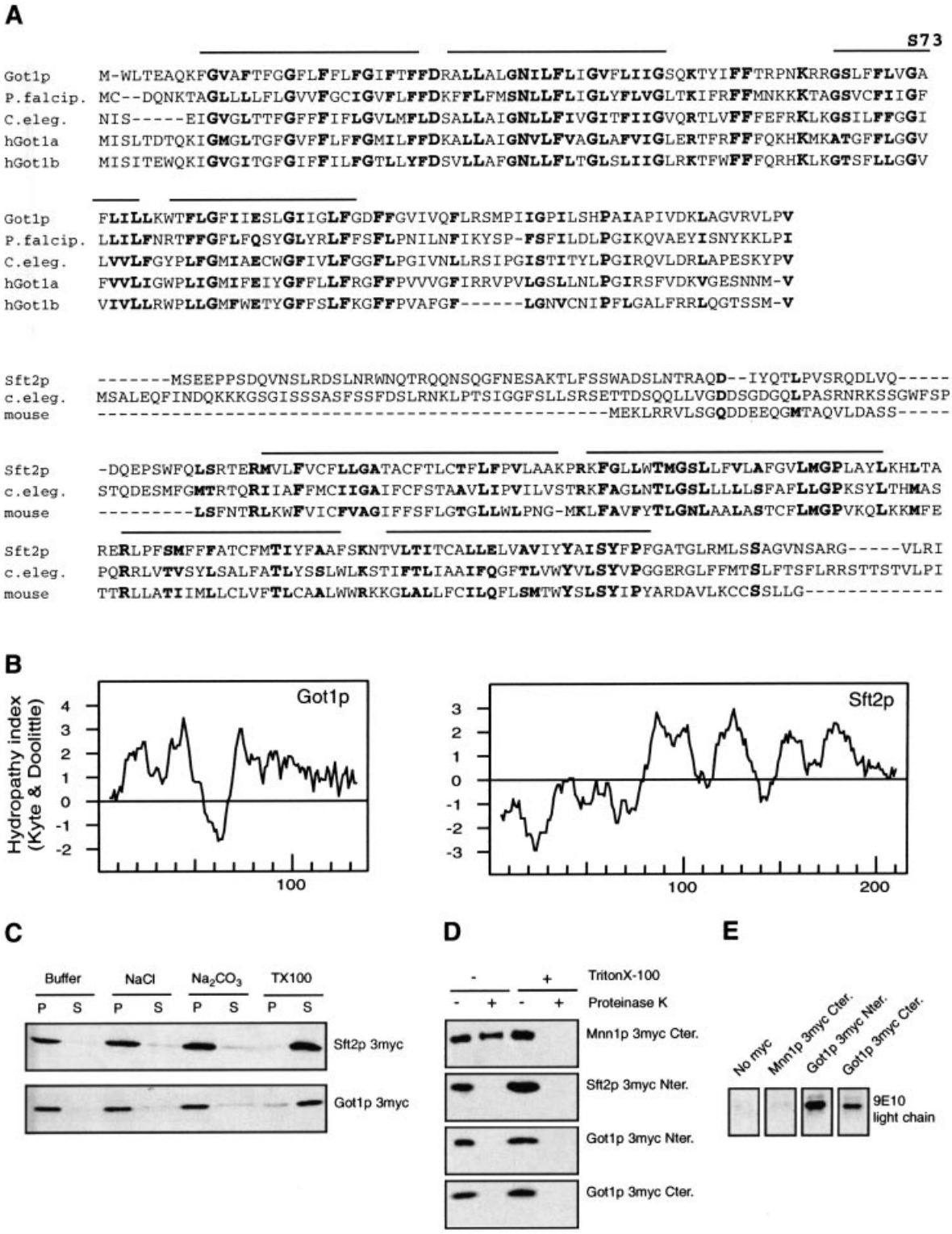


Fig. 1. Structure and membrane association of Got1p and Sft2p. (**A**) Predicted amino acid sequences of Got1p and Sft2p aligned with those of homologues identified for Got1p in *P.falciparum* (accession number AL010285), *C.elegans* (accession number U23521) and humans (hGot1a and hGot1b), and for Sft2p in *C.elegans* (accession number CAA93859) and mouse (accession number AA790425). Residues identical in all species are shown large and bold, similar residues in bold. Lines indicate the hydrophobic potential transmembrane domains. The position of the single amino acid change in *got1-1*, G73→S, is indicated. Note that the exon containing the N-terminus of the *C.elegans* Got1p sequence could not be unequivocally identified. (**B**) Hydropathy profiles of Got1p and Sft2p. (**C**) Membrane association of myc-tagged proteins expressed in yeast. Myc-tagged proteins were expressed in yeast, 100 000 *g* membrane pellets prepared and extracted with buffer, sodium chloride, sodium carbonate or Triton X-100 as indicated before recentrifugation and analysis of the pellets (P) and supernatants (S) by immunoblotting. (**D**) Protease protection assays. Membrane preparations containing the indicated proteins tagged at either the N- or C-terminus, as indicated, were digested with proteinase K in the presence or absence of detergent, and then analysed by immunoblotting. (**E**) Binding of anti-myc mAb 9E10 to unpermeabilized microsomes. Membranes from cells expressing the indicated myc-tagged proteins, or none (no myc), were incubated with antibody, pelleted and bound antibody light chains detected by immunoblotting.

that of the wild-type strain (see Materials and methods for details). Thus Got1p evidently provides a function that is common to evolutionarily distant eukaryotes.

For comparison, Figure 1A also shows the sequence of Sft2p, and related sequences from *C.elegans* and mouse (the latter a partial sequence from an EST). Though not closely related to Got1p, Sft2p also has a compact region of four putative transmembrane domains, and it is these that show most evolutionary conservation.

Got1p and Sft2p are membrane proteins

To investigate the membrane location of Got1p, the molecular clone was modified to encode three myc epitopes at the C-terminus of the protein. Yeast cells expressing this tagged protein produced a single product detectable on immunoblots with anti-myc antibodies, with an apparent size of ~25 kDa. Expression of the tagged protein from a centromere vector was sufficient to allow sectoring (i.e. loss of untagged Got1p) of the original *got1-1 sft2*Δ mutant strain, and also supported growth of the *got1*Δ *sft2*Δ double disruptant, indicating that the tagged Got1p is functional. A version of Sft2p tagged with three myc epitopes at the N-terminus has previously been described (Wooding and Pelham, 1998).

Both Sft2p and Got1p were efficiently pelleted from cell homogenates by centrifugation at 100 000 *g*, and Figure 1C shows that they could not be extracted from these pellets with sodium chloride or sodium carbonate, but could be solubilized by detergent, confirming a membrane location for both proteins.

Previous work indicated that the N-terminus of Sft2p was likely to be cytoplasmic (Wooding and Pelham, 1998), and this was confirmed by protease digestion of the membranes (Figure 1D). Whether or not detergent was added, the N-terminal myc tag was completely digested by proteinase K, no protected fragments being detectable. As a control, the Golgi enzyme Mnn1p, myc-tagged at its (lumenal) C-terminus, was subjected to the same procedure and found to be substantially protease resistant in the absence of detergent (Figure 1D).

The Myc-tagged C-terminus of Got1p was found to be fully accessible to protease. Furthermore, a version of the protein bearing three myc tags at the N-terminus was similarly protease sensitive, and again no smaller fragments could be detected (Figure 1D). This suggests that the protein has an even number of transmembrane domains, with both termini cytoplasmically exposed. This was confirmed by an independent assay in which anti-myc antibodies were bound to and co-sedimented with unpermeabilized microsomes bearing Got1p with myc tags at either the N- or C-terminus (Figure 1E). The cytoplasmic location of the C-terminus was also confirmed for hGot1a (see below).

Based on these results and the hydropathy profiles, we conclude that both Got1p and Sft2p are likely to be tetraspanning membrane proteins with their termini on the cytoplasmic side of the membrane.

Got1p is an early Golgi protein

Visualization of myc-tagged Got1p by immunofluorescence revealed a punctate pattern, characteristic of the yeast Golgi apparatus. As shown in Figure 2, some but not all of the structures containing Got1–myc also

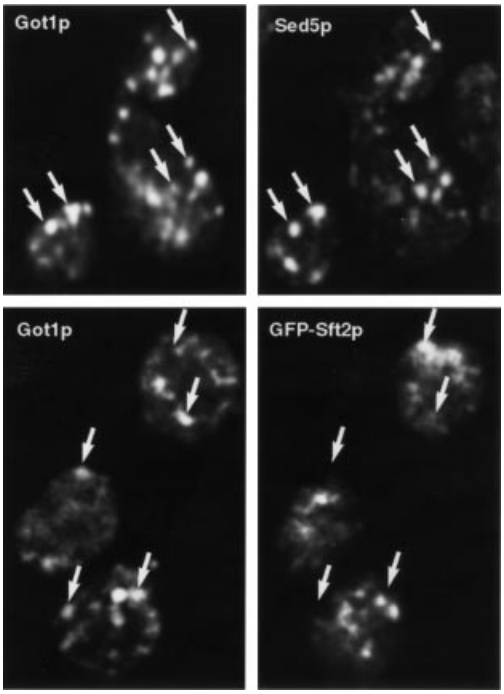


Fig. 2. Immunofluorescent localization of Got1p. Myc-tagged Got1p was compared with endogenous Sed5p by double-label immunofluorescence (top panels) or with a co-expressed GFP–Sft2p fusion protein. Arrows indicate identical positions in each pair of images.

contained the early Golgi t-SNARE Sed5p. Such partial co-localization has often been seen for proteins that reside in the same region of the Golgi, presumably reflecting differences in the kinetics of their delivery to and removal from individual cisternae (Rayner and Munro, 1998; Wooding and Pelham, 1998). In contrast, there was much less obvious overlap with GFP–Sft2p, which has previously been shown to be functional and localized to a later Golgi compartment than Sed5p (Wooding and Pelham, 1998).

To investigate further the localization of Got1p a strain expressing the tagged protein was analysed by subcellular fractionation. A 100 000 *g* pellet was fractionated on an equilibrium sucrose-density gradient (Figure 3), and the distribution of Got1–myc compared with that of other markers: Vam3p (vacuoles), Pep12p (endosomes), Kex2p, Tlg1p and Tlg2p (late Golgi/early endosomes) and Sed5p. All the data shown are from a single set of gradient fractions, but for clarity the results are shown in two panels, each containing the profile of Got1p. The distributions of the different markers were similar to those previously described (Holthuis *et al*., 1998b). Under these conditions, Got1–myc was separated from the TGN, endosomal and vacuolar markers, but most of it was found in fractions containing Sed5p. As expected from the immunofluorescence observations, the Sed5p and Got1p profiles were similar but not completely identical. We conclude that Got1p resides mostly in early Golgi cisternae.

Further evidence for the Golgi location of Got1p was provided by expression and immunolocalization of a C-terminally myc-tagged version of hGot1a in COS cells. Figure 4 shows co-staining of cells for hGot1a and β′-COP (mainly found in the *cis* Golgi), γ-adaptin and TGN46 (TGN markers). Though all proteins gave Golgi-like

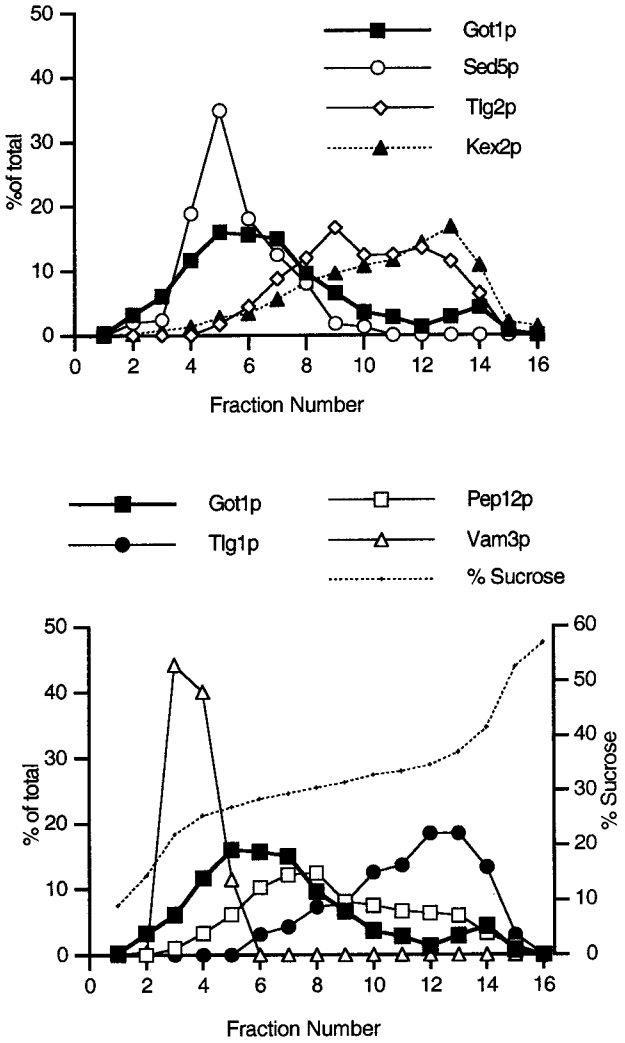


Fig. 3. Subcellular fractionation of cells expressing myc-tagged Got1p. Membranes from a 100 000 *g* pellet were separated on a sucrose-density gradient, and fractions probed by immunoblotting (or, in the case of Kex2p, by enzyme assay). All data are from a single gradient, plotted in two parts for clarity. Note that the Got1p profile most closely resembles that of Sed5p.

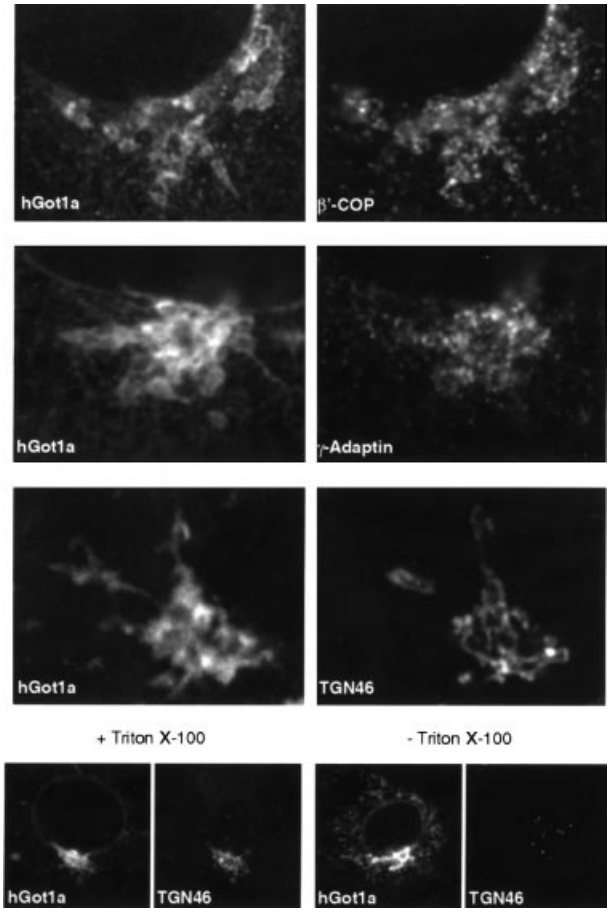


Fig. 4. Location of hGot1a expressed in COS cells. Cells expressing myc-tagged protein were analysed by double-label immuno-fluorescence, using antibodies to β′-COP, γ-adaptin or KDEL-containing ER proteins. The hGot1a is concentrated primarily in the Golgi region, though some faint ER-like staining is also visible. The lower panels show cells that were freeze–thawed to rupture the plasma membrane alone, or subsequently treated with detergent to permeabilize internal membranes. Note that the C-terminal tag on hGot1a is accessible to antibodies with or without detergent treatment, whereas the lumenal domain of TGN46 can be detected in the Golgi region only after detergent treatment.

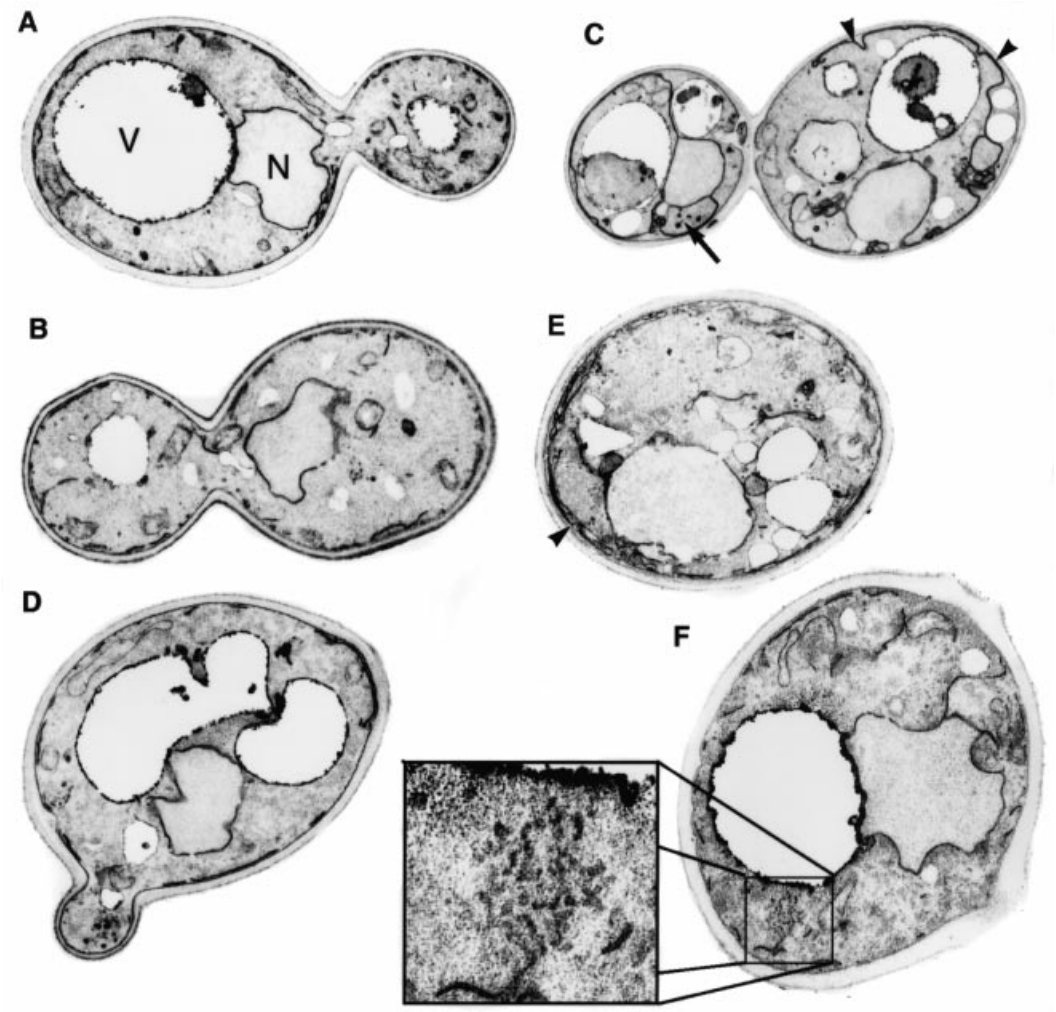
patterns, we consistently observed better co-localization of hGot1a with β′-COP than with the TGN markers. This suggests that hGot1a is not restricted to the *trans*/TGN region, and may be preferentially localized in *cis* cisternae. We also observed some staining of the nuclear envelope and ER (Figure 4). When cells were lysed by freeze–thawing, without detergent, the C-terminal myc tag on hGot1a was clearly accessible to antibodies. In contrast, an antibody recognizing the lumenal domain of TGN46 stained the Golgi region only after detergent treatment. Thus, hGot1a has a membrane orientation and distribution in mammalian cells comparable with that observed for Got1p in yeast.

Isolation and properties of conditional alleles of got1 and sft2

Deletion of either *GOT1* or *SFT2* alone did not affect cell growth, and preliminary studies showed no obvious defect in the processing or sorting of CPY in these mutants. We therefore sought clues to their function by analysing the phenotypes of cells lacking both proteins.

We first transformed the non-sectoring *got1-1 sft2*Δ strain with a plasmid containing the *SFT2* cDNA downstream of a *GAL1* inducible promoter. These cells grew normally in galactose-containing medium and were able to lose the *SFT2*-containing plasmid which had been supporting their growth. Subsequent transfer of the cells to glucose-containing medium resulted in growth arrest after 12–15 h. Electron microscopy (EM) samples were prepared of these cells after 14 h in either galactose or glucose. As shown in Figure 5B, C and G, the repression of Sft2p synthesis resulted in a significant accumulation of ER membranes. This suggests that the presence of either Sft2p or Got1p is required, directly or indirectly, for the maintenance of efficient ER–Golgi transport. In addition, we observed a moderate number of darkly staining circular structures similar to those previously seen in cells lacking the TGN/early endosomal SNARE Tlg1p (Holthuis *et al.*, 1998b).

We then used PCR mutagenesis to prepare thermosensitive (ts) alleles of both *GOT1* and *SFT2*, selecting them for their ability to maintain growth of the *got1*Δ *sft2*Δ



G % of cell sections showing indicated amount of ER membrane

	fraction of plasma membrane circumference				
Strain	0-0.25	0.25-0.5	0.5-0.75	0.75-1.0	>1.0
SEY6210	43	43	14	-	-
sft2Δ got1-2, 37°	18	54	19	9	-
got1Δ sft2-1, 37°	5	42	38	15	-
got1-1 GAL-SFT2, Glc	-	5	27	36	32

Fig. 5. Electron micrographs of *got1* and *sft2* mutants. Cells were permanganate-fixed to accentuate membranes. (**A**) *sft2*Δ, with *SFT2* on a plasmid (SCY11); indistinguishable from wild type. (**B**) *sft2*Δ *got1-1* with p*GAL1-SFT2*, grown on galactose. (**C**) As (B), but after transfer to glucose for 14 h. Note accumulation of ER (arrowheads) and some small darkly staining structures (arrow). (**D**) *got1*Δ *sft2-1ts*, grown at 25°C. (**E**) and (**F**) As (D), but after incubation at 37°C for 3 h. Note some accumulation of ER in (E) (arrowhead), and a cluster of vesicles or tubules, enlarged in (F). N, nucleus; V, vacuole. (**G**) Quantitation of the ER accumulation in various strains. Random fields of cells were analysed, and each cell section scored for the proportion of the circumference that was occupied by peripheral ER.

strain at low but not high temperature. One ts allele of each gene was selected for further study. The *got1*Δ *sft2-1* strain had the strongest growth phenotype: it grew slightly more slowly than an *SFT2* control at 25°C and upon shifting to 37°C ceased growth altogether after a lag of 2 h. The *sft2*Δ *got1-2* strain grew normally at 25°C but took longer to stop growing at 37°C, inhibition being apparent by 2 h but complete only after 6 h.

EM samples of these strains were prepared and their morphologies at 25 and 37°C were compared. The *sft2*Δ *got1-2* ts strain showed no obvious abnormality at 25°C, and at 37°C accumulated only a small amount of ER membrane (Figure 5G). The *got1*Δ *sft2-1* strain also appeared normal at low temperature; when it was shifted to the non-permissive temperature there was some accumulation of ER membranes, and clusters of small vesicles were frequently seen (Figure 5D–G). It appears, therefore, that the ts alleles have a less severe phenotype than the complete depletion of both Got1p and Sft2p; even under conditions where their growth was inhibited, their morphology did not suggest a tight block to secretion.

As a further test of secretory pathway function, the maturation of CPY was examined. In pulse–chase experiments (Figure 6A) CPY appears as a p1 precursor in the ER, is then modified to a larger form, p2, in the Golgi and passes through endosomes to vacuoles where it is processed to its mature form (m) by proteolysis. The *got1-1 GAL–SFT2* strain, when grown on galactose to

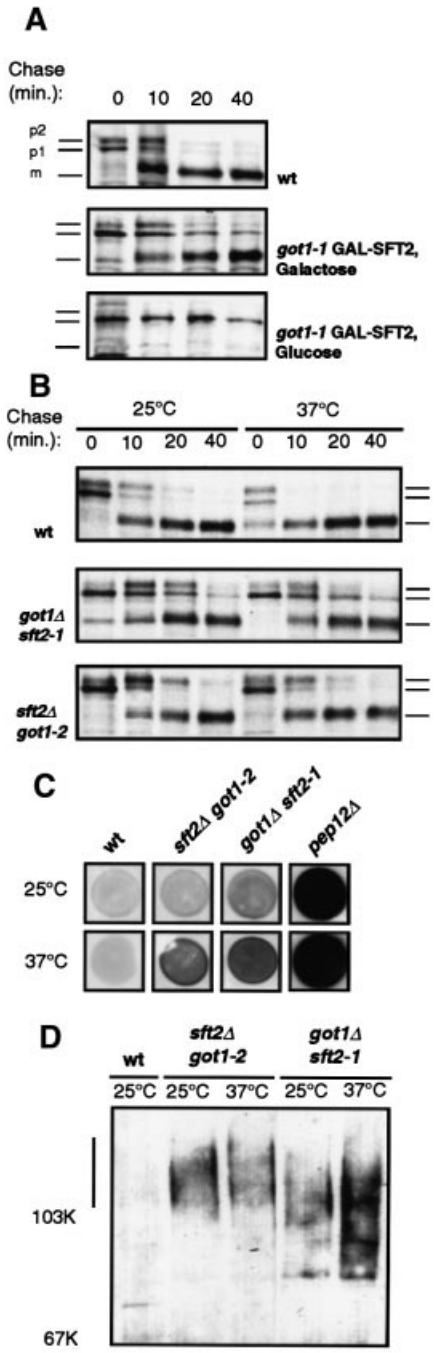


Fig. 6. Processing of CPY and invertase in *got1 sft2* mutants. (**A**) Pulse–chase analysis of CPY in strains containing and lacking Got1p and Sft2p. Wild-type cells, or cells of the *got1-1 GAL–SFT2* strain grown in galactose or transferred to glucose medium for 14 h were labelled for 10 min, and chased for the times indicated at 30°C before lysis and immunoprecipitation of CPY. The positions of the ER precursor (p1), the Golgi-modified precursor (p2) and the mature vacuolar enzyme (m) are indicated. (**B**) Pulse–chase analysis of CPY in the temperature-sensitive mutants. Cells were labelled and chased at 25°C, or preincubated at 37°C for 2.5 h and then labelled and chased at this temperature. (**C**) Analysis of secreted CPY. Patches containing similar numbers of cells were grown in contact with a nitrocellulose filter at either 25°C or 37°C for 3 h, and the secreted CPY detected on the filter with antibodies. (**D**) Immunoblotting of intracellular myc-tagged invertase precursors. The indicated strains were incubated at 25°C, or for 2.5 h at 37°C prior to analysis. The positions of molecular weight markers, and of the heterodisperse mature forms of invertase (line) are indicated.

maintain levels of Sft2p, was able to process CPY but showed some delay, with a noticeable persistence of the p1 form. After 14 h on glucose, to repress Sft2p synthesis, CPY processing was completely blocked at the p1 stage, in agreement with the EM data showing accumulation of ER under these conditions.

As with the EM analysis, the CPY processing phenotype of the ts alleles was much milder than in cells depleted of both proteins. In the *got1*Δ *sft2-1* ts strain CPY maturation was slower than normal, with a persistence of the p1 precursor form, but maturation was not completely blocked even at 37°C. In the *sft2*Δ *got1-2* strain processing was only slightly slowed, even at 37°C (Figure 6B). In this experiment a portion of the p2 form of CPY was secreted into the medium by the mutants, but this amounted to no more than 10–15% of the total (not shown). This was also apparent when secreted CPY was adsorbed on to nitrocellulose and detected by immunoblotting (Figure 6C). Both ts mutants showed slightly enhanced secretion of CPY at the non-permissive temperature, but this effect was mild compared with the level of secretion from *pep12*Δ cells, which have a strong mis-sorting phenotype.

We also examined the secretion of periplasmic invertase from the ts mutants (Figure 6D). Since our standard strains lack the chromosomal invertase gene, they were transformed with a plasmid which expresses a myc-tagged version of the secreted form of invertase. Examination of the intracellular invertase by immunoblotting showed that there was a slight increase in the hyperglycosylated forms of the enzyme in the mutant strains, suggesting a reduced rate of transport through the Golgi to the cell surface. In addition, the invertase present in the *got1*Δ *sft2-1* strain had slightly shorter polysaccharide chains than normal, consistent with a reduction in mannosyltransferase activity in the Golgi apparatus of this strain.

Total glycoprotein secretion was also examined in these strains by incubating spheroplasts with ^{35}S-labelled amino acids and monitoring the appearance of labelled concanavalin A-binding glycoproteins in the medium (as described in Holthuis *et al*., 1998b). No significant quantitative or qualitative abnormalities were observed (data not shown).

Together, these results show that Got1p and Sft2p affect ER–Golgi transport, as shown by the accumulation of ER when both are absent or depleted, but that the growth defect of the ts alleles cannot be accounted for entirely by a block to this process, or indeed to any severe block to the exocytic pathway. Thus, Got1p and Sft2p must also contribute to some other function that is required for growth.

Evidence for defects in endosome–Golgi traffic

The Golgi complex receives traffic not only from the ER, but also from the endocytic pathway. This is necessary both for the retrieval of TGN proteins from endosomes and for the recycling of at least some plasma membrane proteins. Defects in this process are revealed by a loss of the Kex2p activity. Kex2p, a processing protease, usually recycles between the TGN and an endosomal compartment, but if retrieval is blocked it passes instead to the vacuole, where it is degraded (e.g. Holthuis *et al*., 1998b). Figure 7 shows that in both ts strains, a shift to the non-permissive

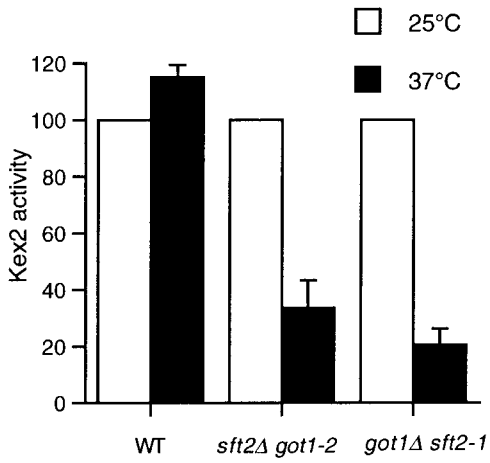


Fig. 7. Loss of Kex2p activity from ts mutants. Cells of the strains indicated were incubated at 37°C for 4 h, then 100 000 *g* membrane pellets were prepared and assayed for Kex2p activity. The percentage activity remaining was estimated relative to that in the same strains maintained at 25°C (set to 100%).

temperature resulted in loss of Kex2p activity, whereas no such loss was apparent in wild-type cells.

Another way to study endosome–Golgi traffic is to examine the distribution of a plasma membrane protein that normally recycles by this route (Holthuis *et al*., 1998a). One example is provided by a GFP-tagged version of Snc1p, a v-SNARE that mediates vesicle fusion with the plasma membrane. This is found mostly on the cell surface, but is continually undergoing endocytosis; when retrieval from endosomes is blocked, for example in a *tlg1* mutant, GFP–Snc1p accumulates in late endosomes (op. cit. in Holthuis *et al*., 1998a; M.Lewis and H.Pelham, unpublished observations). We therefore examined the distribution of GFP–Snc1p in *sft2* and *got1* mutants, to see whether it had a similar fate in these strains.

GFP–Snc1p was expressed in five different strains: wild-type, *got1*Δ, *sft2*Δ, *got1*Δ *sft2-1* and *sft2*Δ *got1-2*. In the parental strain and the single *got1*Δ and *sft2*Δ mutants GFP–Snc1p was found primarily at the plasma membrane at 25°C, though some could be observed in internal structures as well (Figure 8A–C). Incubating these three strains at 37°C increased the amount of internal GFP–Snc1p, but it remained clearly visible at the cell surface. Strikingly, however, both the *sft2*Δ *got1-2* and *got1*Δ *sft2-1* strains showed no GFP–Snc1p at the cell surface at 37°C; instead it was present in punctate structures and in an intracellular haze which may correspond to small vesicles. As shown above, these strains are not defective in secretion, and we confirmed that a mutant form of GFP–Snc1p that is defective for endocytosis could reach the surface at 37°C (M.Lewis and S.Conchon, unpublished observations). We conclude that the *sft2 got1* mutants can endocytose the normal form of GFP–Snc1p, but fail to retrieve it from the endocytic pathway to the Golgi.

Role of Got1p in ER–Golgi transport

The most dramatic phenotype that we observed *in vivo* was the block to ER–Golgi transport when both Sft2p and Got1p were absent. To examine this defect directly, we made use of an *in vitro* assay in which ER-derived vesicles containing radiolabelled pro-alpha factor fused with Golgi

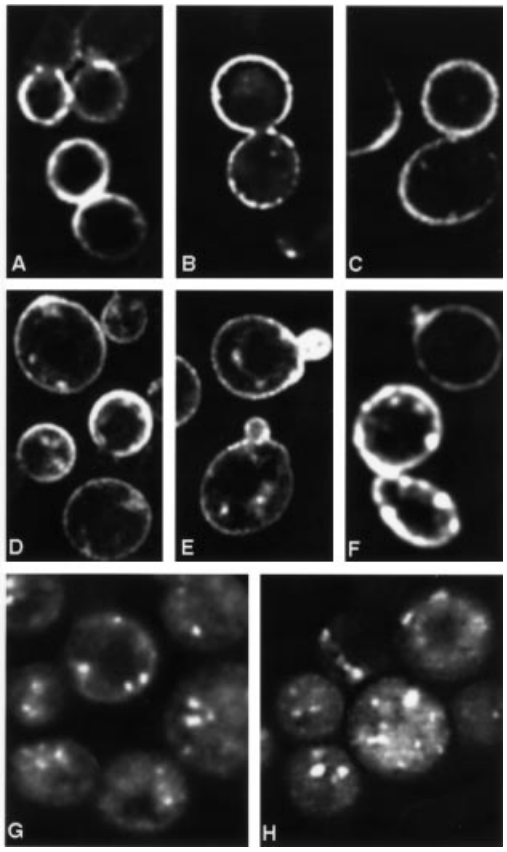


Fig. 8. Distribution of GFP–Snc1p in *got1* and *sft2* mutants. Confocal images of unfixed cells grown at 25°C or incubated for 4 h at 37°C are shown. (**A**) Wild-type, 25°C; (**B**) *got1*Δ, 25°C; (**C**) *sft2*Δ, 25°C; (**D**) wild-type, 37°C; (**E**) *got1*Δ, 37°C; (**F**) *sft2*Δ, 37°C; (**G**) *sft2*Δ *got1-2*, 37°C; (**H**) *got1*Δ *sft2-1*, 37°C. Note the absence of plasma membrane staining in (G) and (H).

membranes, allowing the alpha factor precursor to acquire Golgi-specific carbohydrate modifications. This assay has been characterized in detail, and steps corresponding to the budding of vesicles (dependent on added COPII coat components), their initial tethering to Golgi acceptor membranes (which requires addition of Uso1p) and subsequent SNARE-dependent fusion can each be followed (Barlowe, 1997; Cao *et al*., 1998). As shown in Figure 9, *sft2*Δ cells showed no defect in this assay—in fact, they consistently showed a higher efficiency of *in vitro* transport than wild-type cells. However, *got1*Δ cells showed a very substantial reduction in transport efficiency (Figure 9A). Further investigation showed that vesicle budding, measured by the appearance of labelled pro-alpha factor in slowly sedimenting structures, was not affected, nor was the initial Uso1p-dependent tethering of these vesicles to membranes (Figure 9B). This provides direct evidence that Got1p is involved in vesicular transport, and suggests that it acts at or close to the Sed5p-dependent fusion step which follows tethering (Cao *et al*., 1998).

In a further experiment, the membrane in which Got1p acts was defined more precisely. ER-derived vesicles were prepared *in vitro* from both wild-type and *got1*Δ cells, and mixed with Golgi acceptor membranes which were similarly either wild-type or mutant. The results (Figure 10) show that vesicles lacking Got1p could fuse well with a wild-type Golgi acceptor, but that Golgi

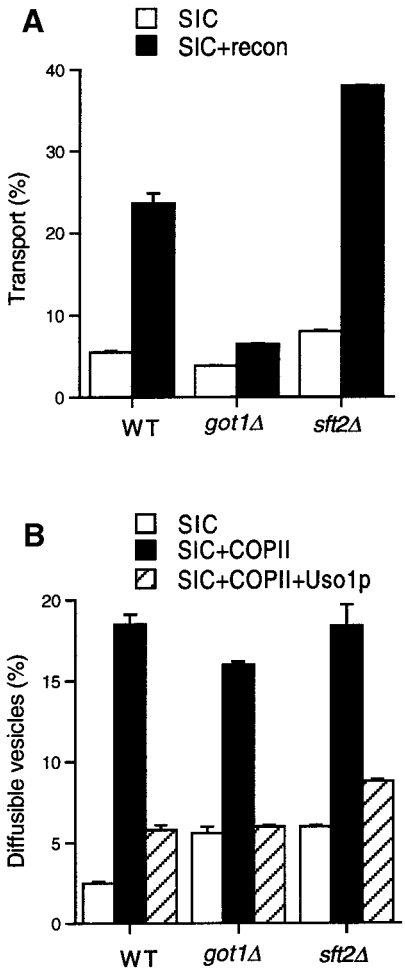


Fig. 9. Influence of *got1*Δ and *sft2*Δ mutations on ER–Golgi transport. (**A**) Overall transport of [^{35}S]gp-α-factor to the Golgi complex in semi-intact cell membranes (SIC) prepared from wild-type, *got1*Δ or *sft2*Δ strains. Membranes were incubated alone (open bars) or with the reconstitution proteins (solid bars) consisting of COPII, Uso1p and LMA1. The percentage transport was quantified after precipitation of outer-chain modified forms of [^{35}S]gp-α-factor. In this experiment, maximal transport represents ~4000 c.p.m. Note that *got1*Δ membranes display a defect in overall transport. (**B**) Vesicle budding and tethering in wild-type and mutant strains. The level of freely diffusible vesicles in cell-free reactions containing wild-type, *got1*Δ or *sft2*Δ semi-intact cell membranes alone (open bars) or in the presence of COPII proteins (solid bars) or COPII proteins plus the tethering factor Uso1p (hatched bars).

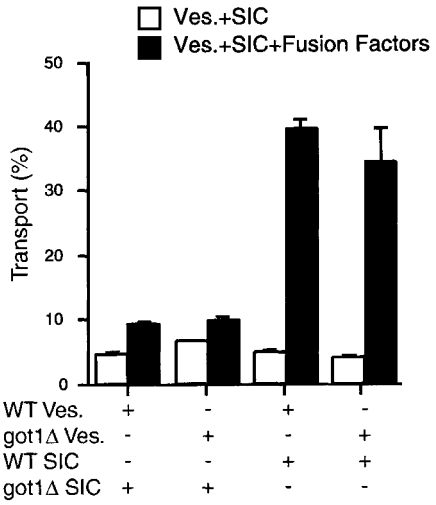


Fig. 10. Got1p function is required on acceptor membranes and not on ER-derived vesicles. COPII vesicles containing [^{35}S]gp-α-factor were isolated from wild-type and *got1*Δ semi-intact cell membranes. A second stage incubation was then performed mixing vesicles with acceptor membranes prepared from wild-type or *got1*Δ cells in the absence (open bars) or presence (solid bars) of purified fusion factors (Uso1p and LMA1). The percentage transport reflects the amount of outer-chain modified [^{35}S]gp-α-factor divided by the total amount of [^{35}S]gp-α-factor. In this experiment, maximal transport represents 850 c.p.m.

membranes from *got1*Δ cells were severely impaired in their ability to fuse with vesicles from wild-type cells. Hence, Got1p appears to function, in this assay at least, in Golgi membranes.

Though *got1*Δ cells grew well, we were concerned that the continuous absence of Got1p might have indirect effects, such as a reduction in the levels of Golgi glycosyltransferases, that could influence the *in vitro* assay in a trivial way. To address this, we repeated the assay using the temperature-sensitive *sft2*Δ *got1-2* strain. This strain showed little defect in ER–Golgi traffic *in vivo*, even at 37°C, but we reasoned that the mutant Got1p might show at least some temperature sensitivity *in vitro*. Indeed, while the transport efficiency of this strain was high at 23°C, it was halved when the assay was performed at 29°C (Golgi modification falling from 32.9 to 16.9%). In contrast, wild-type cells retained 80% of their *in vitro* transport activity at this temperature. Though modest, this difference in temperature sensitivity between mutant and wild-type was observed reproducibly. It strongly suggests that Got1p is directly involved in the ER–Golgi transport step itself.

Discussion

Sft2p was originally identified because its overexpression can compensate for the temperature-sensitive growth phenotype of several *sed5* alleles (Banfield *et al*., 1995). This property suggested an involvement of Sft2p in SNARE-dependent membrane traffic, but because deletion of *SFT2* has no obvious phenotype its role has been unclear. In the present study we have identified mutations which make Sft2p essential for growth. One of these is in *sed5*, underlining the connection between Sft2p and Sed5p, but we also found that deletion of a novel gene *GOT1*, whilst not itself affecting growth, made cells dependent on Sft2p. This suggests that Got1p and Sft2p might have related functions, and we have sought to determine what the role of Got1p might be. The results are likely to be of general significance since both proteins have homologues in other species and human Got1p will even function in yeast.

If Got1p and Sft2p share similar functions, one might expect to see some similarity between the two proteins. Indeed, although they show little direct sequence similarity, both appear to be tetra-spanning membrane proteins with the same orientation, and the transmembrane domains are contained in regions of similar length (~120 residues). In both proteins it is the membrane-associated region that seems to be important: it is the most evolutionarily conserved, and a single Gly→Ser mutation within one of

the predicted transmembrane domains of Got1p is sufficient to abolish function.

A Golgi membrane protein required for transport from the ER

Both Got1p and Sft2p are located predominantly in the Golgi apparatus, but whereas Got1p shows substantial co-localization with Sed5p in early cisternae, Sft2p is found mainly in later cisternae (Wooding and Pelham, 1998). *In vivo*, removal of both proteins has a profound inhibitory effect on ER–Golgi transport, as demonstrated by an accumulation of ER membranes and a block to the maturation of p1 CPY. Careful examination of the data reveals that a modest effect on CPY transport can be observed even when Sft2p is still present, for example in a *got1*Δ *GAL–SFT2* strain grown on galactose, or in a *got1*Δ *sft2ts* strain at the permissive temperature (Figure 6). This effect is not seen when residual Got1p activity remains, for example in a *sft2*Δ *got1ts* strain.

A more dramatic and specific effect can be observed *in vitro*: even in the presence of Sft2p there is a severe impairment in the ability of ER-derived vesicles to fuse with Golgi membranes lacking Got1p, though budding from the ER is unaffected. Furthermore, a ts allele of *got1* shows temperature-sensitive transport *in vitro*. The stronger phenotype of *got1*Δ mutations *in vitro* than *in vivo* is not without precedent: for example, the LMA1 proteins are needed for ER–Golgi traffic *in vitro* (Barlowe, 1997), but are not essential *in vivo*. Taken as a whole, the evidence is consistent with an important role for Got1p in ER–Golgi transport, albeit one that can be partially bypassed *in vivo*.

What is this role? Two-stage transport assays indicate that Golgi membranes from a *got1*Δ strain are defective, whereas ER-derived vesicles isolated from this same strain are fully functional, implying that Got1p acts on the acceptor membranes. Similarly, *in vitro* experiments with a temperature-sensitive *sed5* strain indicate a requirement in this assay for Sed5p function on acceptor membranes but not on transport vesicles (X.Cao and C.Barlowe, unpublished observations). Like Sed5p, Got1p is required for a step that follows vesicle tethering. Together with the genetic interactions, these results suggest that the function of Got1p is closely connected to that of Sed5p, which in turn is intimately concerned with membrane fusion.

How exactly Got1p contributes to vesicle fusion remains a question for the future. Several possibilities can be imagined, but the overall structure of the protein is reminiscent of that of an ion channel subunit and we have preliminary evidence that it is oligomeric, which would be consistent with this. Interestingly, ER–Golgi transport is known to involve a Ca^{2+}-dependent step that follows vesicle docking (Rexach and Schekman, 1991) and it has recently been reported that vacuolar fusion, which is also Ca^{2+} dependent, is accompanied by the release of calcium ions from the organelles at the time of fusion (Peters and Mayer, 1998). It has also been suggested, based on studies with neuroendocrine cells, that calcium ions facilitate SNARE complex formation (Chen *et al*., 1999). By analogy, it is conceivable that Ca^{2+} is released from Golgi membranes, that this aids Sed5p function and that Got1p is somehow involved in this process.

Role of Sft2p

In contrast to Got1p, Sft2p does not co-localize extensively with Sed5p, and *sft2*Δ cells do not show a defect in ER–Golgi transport *in vitro*. Nevertheless, Sft2p has been conserved during evolution. It follows that Sft2p is likely to have an important function that is distinct from that of Got1p. A clue comes from a second kind of defect exhibited *in vivo* by cells deficient for both Sft2p and Got1p activity. With the temperature-sensitive alleles in particular, the phenotypes are strikingly similar to those previously characterized in mutants lacking the late Golgi/endosomal SNAREs Tlg1p and Tlg2p (which like Sed5p are members of the syntaxin family). Like the *got1 sft2* double mutant, *tlg1*Δ cells show reduced size of invertase precursors, a mild CPY sorting defect, loss of Kex2p and a failure to recycle GFP–Snc1p from endosomes to the cell surface (Holthuis *et al*., 1998a,b). All these effects can be explained by a defect in the retrieval of proteins from early endosomes to the Golgi.

A role for Sft2p (and Got1p) in this step could explain another apparent mystery, namely that a defect in ER–Golgi transport does not seem to account for growth inhibition in the temperature-sensitive *got1* and *sft2* mutants. It seems likely that the ts mutations do not completely inactivate the proteins; it is notoriously difficult to obtain such alleles for hydrophobic proteins buried in membranes. Low residual activity could be sufficient to sustain ER–Golgi transport, but inefficient endosome–Golgi transport may eventually block growth. This is plausible since other mutations that affect such traffic, such as *tlg1*, also inhibit growth at high temperature without affecting secretion (Holthuis *et al*., 1998b).

Though their functions are clearly not identical, the genetic interactions between *GOT1* and *SFT2* are striking. It is not simply that defects in late Golgi traffic are intrinsically deleterious to a *got1* mutant. Since direct testing of one such mutant, *tlg2*, showed that it was not lethal in combination with *got1*. In the light of all the data, we suggest the following hypothesis: that Sft2p performs a function in vesicular transport analogous to that of Got1p, but acts primarily in later Golgi compartments. Its role would be to facilitate fusion of endosome-derived vesicles with the Golgi, perhaps in combination with one or both of the late Golgi syntaxins, Tlg1p and Tlg2p. The redundancy of Sft2p and Got1p functions *in vivo* could then be simply explained. Their distributions are likely to overlap—Sft2p at least recycles through early Golgi compartments (Wooding and Pelham 1998)—and each protein may thus be able to act throughout the Golgi and partially substitute for the other. When the activity of Sed5p is limiting they evidently co-operate to sustain ER–Golgi traffic, explaining why *sft2* shows synthetic lethality with *sed5*. Functional redundancy in endosome–Golgi transport could arise either by direct substitution of Got1p for Sft2p, or because the traffic routes are flexible, vesicles being able to fuse with either late or early Golgi cisternae (Fischer von Mollard *et al*., 1997; Holthuis *et al*., 1998b; Nichols and Pelham, 1998; Nichols *et al*., 1998).

Just how similar the roles of Sft2p and Got1p are will only become apparent when their biochemical properties are better understood. For Got1p, at least, the *in vitro* assay will allow detailed analysis of its key features, its interactions with other proteins and its effects on vesicular transport.

Table I. Strains used in this study

Strain No.	Genotype
IAY11	*MAT*α *ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 ade2-101 ade3-Δ853*
IAY25	*MAT***a** *ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 ade2-101 ade3-Δ853*
SCY11	*MAT*α *sft2Δ::HIS3 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 ade2-101 ade3-Δ853* containing pADE3SFT2 (*CEN6, URA3, ADE3, SFT2*)
SCY25	*MAT***a** *sft2Δ::HIS3 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 ade2-101 ade3-Δ853* containing pADE3SFT2 (*CEN6, URA3, ADE3, SFT2*)
SEY6210	*MAT*α *ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 lys2-801*
SEY6211	*MAT***a** *ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY01	*MAT*α *sft2Δ::HIS3 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 lys2-801*
SCY02	*MAT***a** *got1Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY03	*MAT*α *sft2Δ::HIS3 got1Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101* containing p316GOT1 (*CEN6, URA3, GOT1*).
SCY04	*MAT*α *LEU2::sft2-1 sft2Δ::HIS3 got1Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY05	*MAT*α *LEU2::got1-2 sft2Δ::HIS3 got1Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY07	*MAT*α *LEU2::GFP–SFT2 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 lys2-801* containing p316GOT3m (*CEN6, URA3, GOT1-3myc*)
SCY08	*MAT*α *URA3::GFP–SNC1 sft2Δ::HIS3 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 lys2-801*
SCY09	*MAT***a** *URA3::GFP–SNC1 got1Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY12	*MAT*α *URA3::GFP–SNC1 LEU2::sft2-1 sft2Δ::HIS3 got1 Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*
SCY13	*MAT*α *URA3::GFP–SNC1 LEU2::got1-2 sft2Δ::HIS3 got1 Δ::TRP1 ura3-52 his3-Δ200 leu2-3, –112 trp1-Δ901 suc2-Δ9 ade2-101*

Materials and methods

Plasmids

Constructs containing the *SFT2* and *GOT1* open reading frames were generated by PCR amplification of yeast genomic DNA using Pfu polymerase (Stratagene) and subsequent ligation into the vectors pRS315, pRS316 or pRS414 (all CEN-based, *LEU2*, *URA3* or *TRP1*, respectively, Sikorski and Hieter, 1989), behind the TPI or the *GAL1* inducible promoter. They were designated by vector and gene name (e.g. p315SFT2), the promoter region being TPI unless otherwise stated. The plasmid used for the synthetic lethal screen (pADE3SFT2) was created by inserting the *SFT2* sequence behind the TPI promoter into a pRS316 derivative carrying *ADE3* (kindly provided by I.Adams, MRC, Cambridge). C-terminally myc-tagged *GOT1* was expressed from the TPI promoter in a pRS414 or pRS316 vector, the terminal sequence being changed from RVLPV* to RVLPVFEMEQKLISEEDLNSR-EQKLISEEDLMEQKLISEEDLN*, where the myc epitope is underlined. The GFP–SFT2 and myc-tagged invertase constructs have been described previously (Banfield *et al*., 1995; Wooding and Pelham, 1998). The pRS406 vector which contains the GFP–SNC1 construct was a generous gift from M.Lewis and will be described elsewhere.

The cDNA clones 0428529 and 0267460 which contain the *GOT1* human homologues were obtained from the I.M.A.G.E. Consortium. The inserts were cloned by PCR and sequenced. The myc-tagged version of hGot1a (tagged as described above for *GOT1*) was inserted into a SMH5-derived vector (Levine and Munro, 1998) for transfection into COS cells.

Yeast strains and growth conditions

Yeast strains are described in Table I. These were constructed by standard methods and grown in yeast extract–peptone–dextrose or synthetic dextrose (SD) medium as appropriate. Standard yeast techniques for sporulation, tetrad analysis and gene disruption were employed as described in Guthrie and Fink (1991). Yeast transformations were carried out as described by Elble (1992).

IAY11 and IAY25 were kindly provided by I.Adams and J.Kilmartin. SCY11, SCY25 and SCY01 were prepared from IAY11, IAY25 and SEY6210, respectively, by replacing the entire coding region of *SFT2* with *HIS3*. For *GOT1* disruption the entire coding sequence was replaced with *TRP1* in SEY6210/11 diploid cells which were then sporulated to yield SCY02. This was transformed with p316GOT1, crossed with SCY01 cells and sporulated to give SCY03. This strain was unable to grow on 5-fluoro-orotic acid (5-FOA) plates (to counter-select the plasmid). It was used to test the functionality of proteins such as Got1p-3 myc, by transformation of the corresponding plasmid and analysis of the ability to survive on 5-FOA.

Synthetic lethality screen

Mutants synthetically lethal with *sft2* were isolated using the *ade2/ade3*, red/white sectoring system (Kranz and Holm, 1990). The SCY11 and SCY25 strains were red but give white sectors under non-selective conditions due to plasmid loss. They were mutagenized with ultraviolet light and 20 non-sectoring colonies identified among 100 000 screened. Five of them regained their sectoring ability after transformation with a *LEU2*-based vector carrying *SFT2* (p315SFT2). They were transformed with a TRP1/CEN-based yeast genomic library and three strains gave transformants that sectored and carried plasmids that did not contain *SFT2*. The complementing genes were identified by sequencing the ends of the inserts, testing smaller restriction fragments and finally by expression of individual ORFs prepared by PCR.

Generation of ts alleles of SFT2 and GOT1

The plasmids p315SFT2 and p315GOT1 were used as templates for PCR-mediated mutagenesis and gap repair as described by Muhlard *et al*. (1992). The PCR products and gapped plasmid (pRS315) were cotransformed into SCY03. Following selection on 5-FOA (to remove the wild-type *GOT1* plasmid), colonies were selected that were viable at 25°C but not at 37°C. The corresponding plasmids were rescued (Ward, 1990), the ts alleles subcloned into a pRS405 vector behind a TPI promoter, and integrated at *LEU2* in SCY03. The rescuing plasmid p316GOT1 was then eliminated on 5-FOA plates. One ts allele of each gene (strains SCY04 and SCY05) was further characterized.

Antibodies and immunoblotting

Rabbit polyclonal antibodies to Sed5p, CPY, Tlg1p, Tlg2p, Pep12p and Vam3p were described previously (Hardwick and Pelham, 1992; Banfield *et al*., 1995; Nichols *et al*., 1997; Holthuis *et al*., 1998b). The human c-*myc* epitope was detected with mouse mAb 9E10 or with rabbit polyclonal antibody (Santa Cruz). Monoclonal antibodies used in COS cells were: 23C, anti-β′-COP (Harrison-Lavoie *et al*., 1993); 100/3, anti-adaptin (Ahle *et al*., 1988) and anti-TGN46 (Prescott *et al*., 1997). For immunoblotting, all antibody incubations were carried out in phosphate-buffered saline (PBS) containing 5% dried milk and 0.5% Tween-20. After incubation with peroxidase-conjugated secondary antibodies (Bio-Rad), detection was performed using enhanced chemiluminescence (ECL kit, Amersham).

Confocal microscopy

Indirect immunofluorescence was performed as described previously (Hardwick and Pelham, 1992). Both primary and secondary antibody incubation were carried out in PBS supplemented with 2% dried milk for 2 h at room temperature. Primary antibody to Sed5p was used at a dilution of 1:2000. Fluorescein or Cy3-conjugated secondary antibodies (Amersham) were used for visualization. Dual images were obtained with an MRC-600 confocal laser scanning microscope (Bio-Rad) using separate excitation at the appropriate wavelengths to avoid bleed-through.

Cells expressing GFP–Snc1p fusion protein were grown in SD medium at 25°C to early log phase (0.3 OD_{600} per ml) and then split into two, and further incubated for 3–4 h at 25 or 37°C. They were placed on to a slide, a coverslip was added and they were immediately examined under the MRC-600 confocal microscope.

COS cells transfection and protein localization

COS cells were transfected using DEAE–dextran as described previously (Munro and Pelham, 1987). After 24 h, cells were split on to eight-well slides [C.A.Hendley (Essex) Ltd] and on the following day, were fixed in 2% formaldehyde, 0.1% glutaraldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS and immunofluorescence performed as described by Munro (1995). For freeze–thaw permeabilization, cells on

polylysine-coated slides were washed once in 25 mM HEPES, 125 mM KOAc, 5.5 mM glucose, 2.5 mM MgOAc, pH 7, then frozen on dry ice. The cells were quickly thawed by adding the same buffer, then fixed as above.

Electron microscopy

Cells were grown in SD medium and harvested at a density of ~1 OD_{600} per ml. Permanganate fixation, dehydration and embedding in Spurr's resin (Agar Scientific, Stansted, UK) were carried out as described by Kaiser and Schekman (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. Sections were observed using a Philips CM10 transmission electron microscope.

Membrane association and protease protection assays

A p100 fraction was prepared and resuspended as described by Holthuis *et al*. (1998b). Aliquots were incubated in buffer alone or with 0.5 M NaCl or 0.1 M Na_2CO_3 (pH 11) or 1% Triton X-100 for 20 min at 4°C. Samples were centrifuged at 100 000 *g* for 45 min and equivalent amounts of supernatant and pellet fractions analysed by immunoblotting. For protease treatment, a 13 000 *g* supernatant (Holthuis *et al*., 1998b) was split into aliquots that were incubated with or without proteinase K (5 μg/ml) and with or without Triton X-100 (2%) for 20 min at room temperature. Proteinase K was inhibited by adding PMSF to a final concentration of 10 mM before gel analysis. Binding of anti-myc mAb (9E10) to the membrane fraction was determined by centrifugation at 100 000 *g* through a sucrose cushion, bound antibody being detected by immunoblotting.

Subcellular fractionation and Kex2p activity measurement

Preparation of the samples to be loaded on the sucrose gradients was carried out as described by Holthuis *et al*. (1998b) except that the 100 000 *g* membrane pellet was resuspended in a final volume of 1 ml of buffer (50 mM Tris–HCl pH 7.5, 200 mM sorbitol, 1 mM EDTA) that was loaded on to a 22–60% sucrose step gradient which was generated using the following steps [all sucrose solutions were made (w/w, %) with ultra-pure sucrose (Gibco-BRL)]: 1.5 ml 60%, 1.0 ml 40%, 1.0 ml 37%, 1.5 ml 34%, 2 ml 32%, 2 ml 29%, 1.5 ml 27% and 1.0 ml 22%. After 17–18 h centrifugation in a Beckman SW40Ti rotor at 170 000 *g*, 16 fractions of 0.78 ml were collected. Kex2p endoprotease activity was determined in each of these fractions (Cunningham and Wickner, 1989) and in membrane pellets prepared from 100 000 *g* centrifugation following a comparable procedure.

Analysis of CPY and invertase processing

Pulse–chase analysis of carboxypeptidase Y was performed as described by Stepp *et al*. (1997), although the incubation temperatures were either 25 or 37°C. Intracellular invertase was analysed as described by Holthuis *et al*. (1998b).

In vitro transport assays

Yeast semi-intact cells from wild-type, *got1*Δ or *sft2*Δ strains were prepared from log phase cultures grown at 25°C and stored frozen at –70°C. Prior to assays, cells were quickly thawed, washed with buffer to remove cytosol and [^{35}S]prepro-α-factor was post-translationally translocated into ER membranes at 10°C (Baker *et al*., 1988). Vesicle budding, tethering and fusion assays were performed as described (Cao *et al*., 1998). Briefly, the addition of purified COPII proteins buds ER-derived vesicles from semi-intact cells that are freely diffusible and remain in the supernatant fraction after centrifugation at 12 000 *g*. Protease protected [^{35}S]glyco-pro-(gp)-α-factor contained in budded vesicles was quantified after solubilization and precipitation with concanavalin A–Sepharose. For transport assays, COPII proteins in addition to purified Uso1p and LMA1 were added as indicated in figure legends and the amount of Golgi-modified [^{35}S]gp-α-factor was measured by immunoprecipitation with α1,6-mannose specific antibodies. For two-stage vesicle fusion experiments, COPII vesicles that contained [^{35}S]gp-α-factor were generated from wild-type and *got1*Δ membranes under standard budding conditions (Cao *et al*., 1998). Freely diffusible vesicles (10 μl) were mixed with washed semi-intact cells from indicated strains in a total volume of 25 μl. The same membrane, protein and nucleotide concentrations as in one-stage transport reactions were maintained in these reactions. The extent of fusion with Golgi membranes was measured after incubation at 25°C by precipitation of Golgi modified [^{35}S]gp-α-factor. The data plotted in these experiments are the average of duplicate determinations and the error bars represent the range.

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