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Sgf1p, a New Component of the Sec34p/Sec35p Complex

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Here we report the identification of *SGF1* as a high-copy suppressor of the *sec35–1* mutant. *SGF1* encodes an essential hydrophilic protein of ~100 kDa. Using the yeast two-hybrid system and coprecipitation studies, we demonstrate that Sgf1p is a new subunit of the multiprotein Sec34p/Sec35p complex. Reduced levels of Sgf1p lead to the accumulation of a variety of membranes as well as a kinetic block in endoplasmic reticulum to Golgi traffic. Immunofluorescence studies demonstrate that Sec34p is found throughout the Golgi, with a high concentration on early Golgi. Although an earlier study suggested that Sec34p (Grd20p) is not required for protein secretion, we show here that the *sec34–2* and *sec35–1* mutations lead to a pleiotropic block in the secretion of all proteins into the growth medium.

Key words: membrane traffic, secretion, vesicle tethering

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The composition of organelles is maintained by the proper targeting of different classes of vesicles to their correct destination. Vesicle targeting reactions involve recognition of the vesicle by the target membrane followed by two different types of membrane interactions. The first, called tethering, does not bring the vesicle and acceptor membrane in close enough proximity to allow for membrane fusion. The second reaction, referred to as docking, leads to a more intimate interaction between the two membranes so that fusion can occur.

Tethering reactions at different stages of the exocytic and endocytic pathways appear to be effected by multicomponent complexes that are peripherally associated with the target membrane (1), while the docking reactions are mediated by a family of integral membrane proteins called the SNAREs. SNAREs are related to proteins found on the synaptic vesicle or the neuronal plasma membrane. They cycle between the donor and acceptor compartments and are highly conserved from yeast to man (2). Unlike the tethering complexes, which do not resemble each other, all SNAREs are homologous to either synaptobrevin/VAMP, syntaxin or SNAP-25. The pairing of a SNARE on the vesicle with its cognate partner on the target membrane brings the vesicle in direct contact with the acceptor compartment, allowing these two membranes to fuse (3).

ER to Golgi transport is unusual in that two large tethering complexes have been implicated in the tethering of COP II vesicles to the Golgi, TRAPP I (4,5) and the Sec34p/Sec35p complex (6,7). Surprisingly, it was recently reported that *GRD20*, which is identical to *SEC34*, is required for protein sorting in the trans-Golgi/endosomal system, but not protein secretion (8). Mutations in *grd20* exhibited only a modest reduction in the secretion of invertase, while the vacuolar protease carboxypeptidase Y (CPY) was secreted (missorted) into the medium. In light of these findings, we have re-evaluated the role of Sec34p and Sec35p in membrane traffic. Using an assay that measures the transport of all proteins secreted into the growth medium, we show here that the *sec34–2* and *sec35–1* mutants pleiotropically block secretion. Previous studies have shown that the entire cellular pool of Sec34p is in a complex with Sec35p (6). Interestingly, immunofluorescence studies have revealed a gradient of Sec34p throughout the Golgi with the majority concentrated on early Golgi. In an effort to identify additional members of this complex, we have performed a high-copy suppressor screen with the *sec35–1* mutant. This screen has led to the identification of Sgf1p, a new component of the complex. Reduced levels of Sgf1p lead to a kinetic delay in ER to Golgi transport and the accumulation of a variety of membranes. The implications of these findings are discussed.

Results

The sec34–2 and sec35–1 mutants pleiotropically block secretion

As a previous study suggested that Sec34p is not required for secretion (8), we re-evaluated the role of Sec34p in the secretory pathway using a different type of assay. A specific set of proteins are secreted into the medium in wild-type cells (see Figure 1, lane 1) (9), and mutations in genes whose products are directly involved in anterograde transport block the secretion of these proteins. In contrast, mutations in genes whose products are indirectly involved in secretion may block the traffic of some, but not all, proteins secreted by wild-type cells. For example, in *sec21–3*, which blocks retrograde Golgi to ER transport, a subset of proteins (CPY

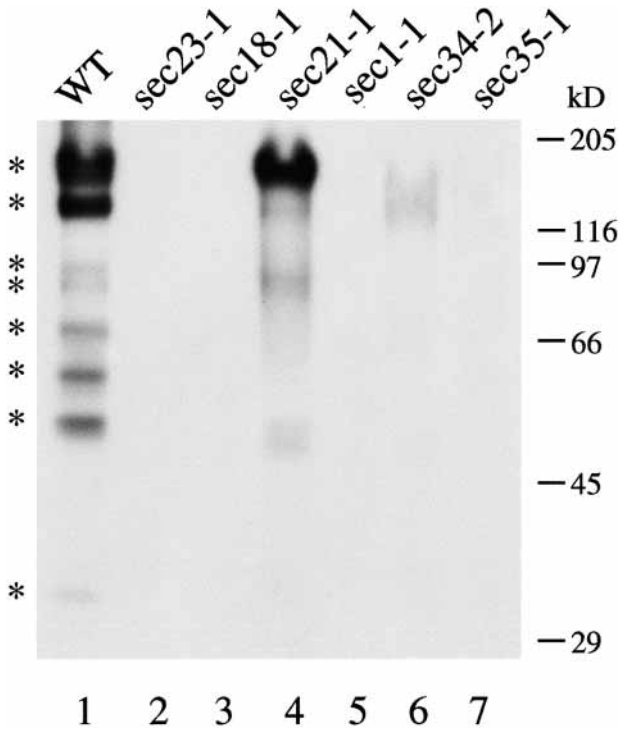

Figure 1: Sec34p and Sec35p are required for secretion. Two OD_{599} units of cells were pulse-labeled with 150 μCi of [^{35}S] ProMix in 0.4 ml of minimal media for 15 min at 37 °C before the cells were pelleted by centrifugation. Proteins secreted into the media were precipitated with TCA and visualized by SDS-PAGE and autofluorography. Molecular markers are shown to the right. The bands secreted into the medium are marked with asterisks.

and α-factor) are completely blocked in the ER, whereas other proteins (invertase and HSP150) are secreted normally (10). The block in CPY transport in *sec21–3* may be due to the limited retrieval of transport factors such as cargo receptors that are required for the packaging of certain cargo into anterograde ER to Golgi COP II vesicles (10).

To assay for a general defect in secretion, *sec34–2* and *sec35–1* as well as *sec21–1*, *sec23–1, sec18–1 and sec1–1*, were preshifted to 37 °C for 20 min and pulse-labeled for 15 min. Cells and media were separated by centrifugation and proteins in the medium were precipitated with TCA and resolved by SDS-PAGE. In wild-type, eight protein bands were visible in the medium (Figure 1, lane 1), while few were apparent in *sec21–1* (Figure 1, lane 4). No proteins were detected in the medium of three different mutants that block anterograde transport, *sec23–1, sec18–1* and *sec1–1* (Figure 1, lanes 2, 3 and 5). Interestingly, in *sec34–2* and *sec35–1* most secretory proteins failed to be transported into the medium (Figure 1, lanes 6 and 7). A faint protein band was seen in *sec34–2* (Figure 1, lane 6), which may be due to an incomplete block in secretion in this mutant at 37 °C. These findings clearly demonstrate that mutations in subunits of the Sec34p/Sec35p complex lead to a robust block in secretion.

Sec34p resides primarily on an early Golgi compartment

Previous localization studies, using a Sec34p-HA construct, have shown that Sec34p resides in the cytosol and on the Golgi (8). Immunofluorescence studies revealed that 51% of the Golgi-localized Sec34p colocalized with the trans-Golgi marker A-ALP. A-ALP contains the cytoplasmic domain of dipeptidyl aminopeptidase fused to the transmembrane and lumenal domains of alkaline phosphatase (8). The localization of over half the Sec34p to the trans-Golgi seemed counterintuitive to its proposed role in ER to Golgi tethering (7). To re-examine the distribution of Sec34p, we localized Sec34p-*myc* in strains expressing the *cis*-Golgi marker Och1p-HA, and a different late Golgi marker, Sec7p-GFP (11,12). We found that 79% of the Golgi puncta staining for Sec34p-*myc* colocalized with puncta staining for Och1p-HA (Figure 2A), whereas only 28% colocalized with puncta staining for Sec7p-GFP (Figure 2B). Looking at the reverse colocalizations, we found that 73% of the Golgi puncta staining for Och1p-HA and 27% of the puncta staining for Sec7p-GFP also stained for Sec34p-*myc*. Hence, although Sec34p-*myc* can be found throughout the Golgi, it predominantly localizes to an early Golgi compartment. Interestingly, a human ortholog of Sec34p has recently been reported to colocalize well with *cis*/medial Golgi markers and partially with a trans-Golgi marker (13).

Identification of high-copy suppressors of the sec35–1 mutation

The Sec34p/Sec35p complex contains several putative members (6). To identify new subunits or genes whose products may interact with the Sec34p/Sec35p complex, we transformed the *sec35–1* mutant with a 2-μm yeast genomic library and screened for genes that suppress the growth defect of *sec35–1* at 37 °C. A total of 81 suppressors were isolated from 77 000 transformants and each showed plasmid-dependent suppression. Restriction analysis and DNA sequencing revealed that these plasmids could be divided into five groups, each with a different region of genomic DNA. The *SEC35* and *SEC34* structural genes comprised two of the groups. The third group was found to contain the *SBP1* and *RPL8A* genes. *SBP1* encodes a single-stranded nucleic acid-binding protein, whereas *RPL8A* encodes the ribosomal protein L8A. This group was also found to suppress *sec34–2* (6). Previous studies suggested that this suppression was indirect, as members of this group were found to suppress mutations that block membrane traffic at all stages of the exocytic pathway. The insert in the fourth group was subcloned to 3.75 kb (data not shown). It contained a hypothetical open reading frame (ORF) (YPR105C) that conferred suppression. YPR105C encodes a hydrophilic protein of 861 amino acids with a predicted molecular weight of ~98 kDa. It lacks a signal sequence as well as transmembrane domains, and does not have any known motifs that facilitate membrane attachment. Thus, this ORF is predicted to encode a cytosolic or peripheral membrane protein that we have named *SGF1* (suppressor gene of *sec35*). A search of the database revealed homologs of *SGF1* in *S. pombe* (CAA19344) and humans (AL050101). The insert in

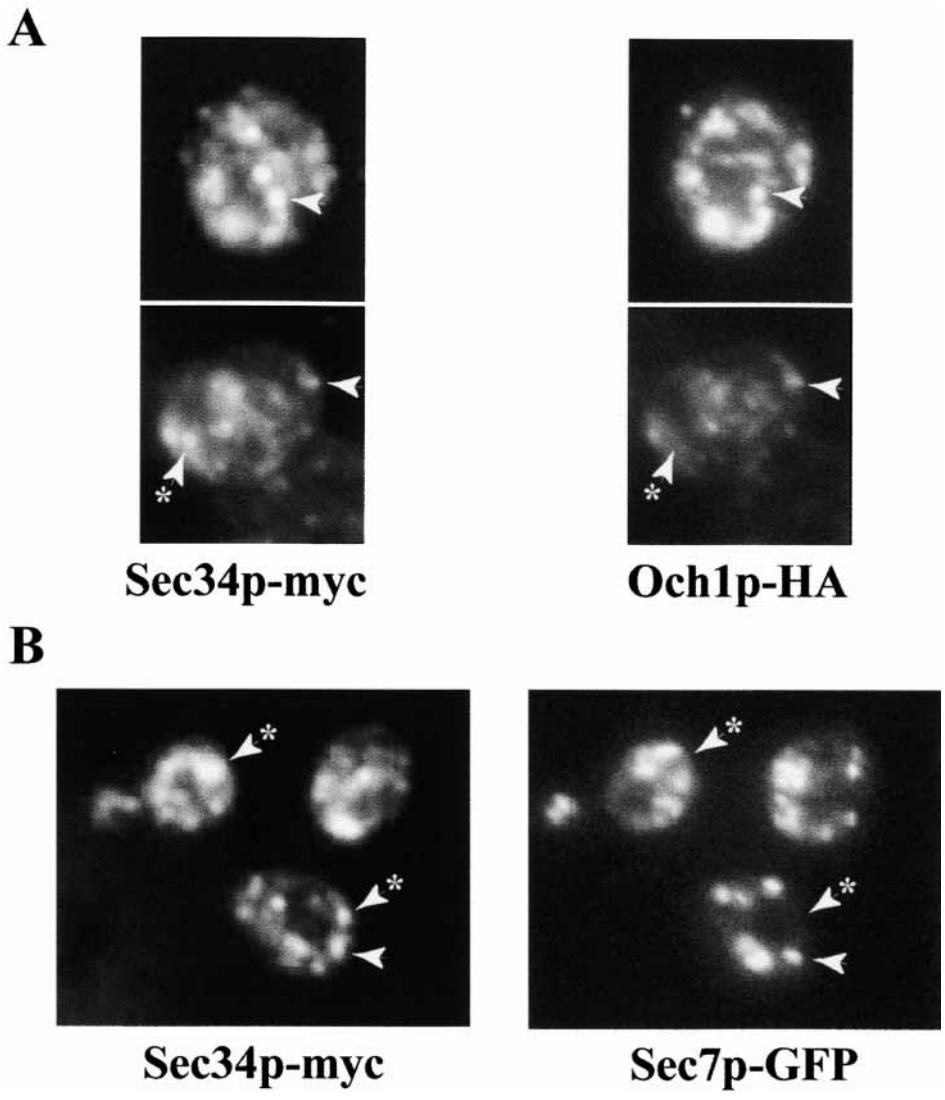

Figure 2: The majority of Sec34p-*myc* colocalizes with the early Golgi marker Och1p-HA. (A) Subcellular localization of Sec34p-*myc* and Och1p-HA. A strain carrying integrated Sec34p-*myc* and plasmid-encoded Och1p-HA was fixed. The cell wall was removed and the sample was probed with mouse anti-*myc* and rabbit anti-HA antibodies. Cy3- (anti-mouse) and FITC- (anti-rabbit) conjugated secondary antibodies were used to localize the primary antibodies by fluorescence microscopy. Representative cells are shown. Plain arrowheads highlight colocalization and arrowheads with asterisks indicate a lack of colocalization. (B) Subcellular localization of Sec34p-*myc* and Sec7p-GFP. A strain containing both Sec34p-*myc* and Sec7p-GFP was fixed, converted to spheroplasts and probed with mouse anti-*myc* antibody. Cy3-conjugated secondary antibody was used to localize Sec34p-*myc* by fluorescence microscopy, whereas GFP marked Sec7p-GFP through its intrinsic fluorescence. Representative cells are shown. Plain arrowheads highlight colocalization and arrowheads with asterisks indicate a lack of colocalization.

the fifth group was subcloned to a 2.5-kb fragment that retained suppression activity (data not shown). This fragment contained a hypothetical ORF (YML068W) that encodes a protein with a molecular weight of ~54 kDa which is predicted to have at least two zinc finger domains. The N-terminus of this protein is also predicted to have a coiled-coil region and its human ortholog (AF060544) associates with a known transcription factor (14). This finding and the experimental results presented below have led us to conclude it suppresses *sec35–1* indirectly.

Sgf1p interacts with Sec34p and Sec35p in the yeast two-hybrid system

To begin to investigate whether Sgf1p physically interacts with Sec34p and Sec35p, we used the yeast two-hybrid system. This system, which takes advantage of the properties of the Gal4p transcriptional activator, is a genetic assay for detecting protein–protein interactions (15). Gal4p contains two functionally essential domains: a DNA-binding domain (BD) and a transcription activation domain (AD). The interaction of two proteins fused to Gal4-BD and Gal4-AD, respectively, results in the transcriptional activity of a reporter gene (here, β-Galactosidase). Full-length Sgf1p, YML068W-p, Sec34p and Sec35p were fused to Gal4-AD in a pACT2 fish vector or Gal4-BD in a pAS1-CYH2 bait vector (16). Activation of the reporter β-Galactosidase gene by the Gal4p transcriptional activator was assessed by the ability of yeast colonies to produce a blue color when X-gal was used as a substrate. We found, as expected, that Sec34p and Sec35p interacted with each other in this assay (Table 1). In addition, both Sec34p and Sec35p interacted with Sgf1p, but not with YML068W-p. Thus, Sgf1p, Sec34p and Sec35p specifically interact with each other in the yeast two-hybrid system.

Sgf1p is a new component of the Sec34p/Sec35p complex

The finding that Sgf1p interacts with Sec34p and Sec35p in the two-hybrid assay suggested that Sgf1p may be a mem-

Table 1: Sgf1p interacts with Sec34p and Sec35p by yeast two hybrid

AD construct	BD construct	Interactions
Sgf1p	YML068W-p	–
	Sec34p	+
YML068W-p	Sgf1p	–
	Sec34p	–
Sec34p	Sgf1p	+
	YML068W-p	–
Sec35p	Sgf1p	+
	YML068W-p	–
	Sec34p	+

ber of the Sec34p/Sec35p complex. To characterize this interaction further, we performed coprecipitation experiments. Lysates were prepared from yeast strains containing *myc*-tagged Sgf1p or YML068W and an untagged strain. The lysates were immunoprecipitated with anti-c-*myc* antibody, subjected to Western blot analysis and probed for the presence of Sec35p (Figure 3A). Sec35p was only detected in the immunoprecipitate from the Sgf1p-*myc* tagged lysate, but not the untagged lysate (Figure 3A, lanes 1 and 2). When the lysate from the strain containing *myc*-tagged Sgf1p was treated with 1% SDS prior to immunoprecipitation, only Sgf1p-*myc*, but not Sec35p, was detected (lane 3). These results indicate that Sec35p only coprecipitates with Sgf1p under nondenaturing conditions. In a similar experiment, *myc*-tagged Sec35p did not coprecipitate with YML068W (data not shown), indicating that there is no direct interaction between the two.

To analyze the protein that *SGF1* encodes, we raised polyclonal antibody to Sgf1p. Anti-Sgf1p antibody recognized a polypeptide of ~100 kDa (Figure 3B, lane 3) that was not detected by preimmune serum (lanes 1 and 2) and was overproduced in a strain that overexpresses *SGF1* (lane 4). This antibody was then used to probe Western blots in the reciprocal of the coprecipitation experiment described above. Sec35p-*myc* was precipitated from a lysate and Western blot analysis was used to analyze the precipitate for Sgf1p and Sec34p (Figure 3C). Sgf1p and Sec34p coprecipitated with Sec35p-*myc* under nondenaturing conditions, but not from untagged or Sec35p-*myc* tagged lysates under denaturing conditions (Figure 3C, compare lane 2 with lanes 1 and 3).

When wild-type lysates were analyzed on a Superdex-200 gel filtration column, all of the Sgf1p was found to cofractionate with Sec34p and Sec35p (Figure 4). Furthermore, unlike the TRAPP complex which is found in two forms (5), only one form of the Sec34p/Sec35p complex, migrating between TRAPP I (~300 kDa) and TRAPP II (~1000 kDa), was detected. Previous studies have shown that Sec34p and Sec35p quantitatively bind to a Mono Q anion exchange column and are eluted together as a complex (7). If the Sec34p, Sec35p and Sgf1p that cofractionate on the Superdex-200 column are components of the same complex, they should co-elute with each other on a Mono Q anion exchange column. Fractions 9 and 10 from the Superdex-200 column (see Figure 4A) were pooled and applied to a Mono Q column in the presence of 100 mM KCl and then eluted with a linear salt gradient (100–500 mM). Western blot analysis of the column fractions revealed the presence of Sec34p, Sec35p and Sgf1p in fractions 9 and 10 (see Figure 4B), supporting the

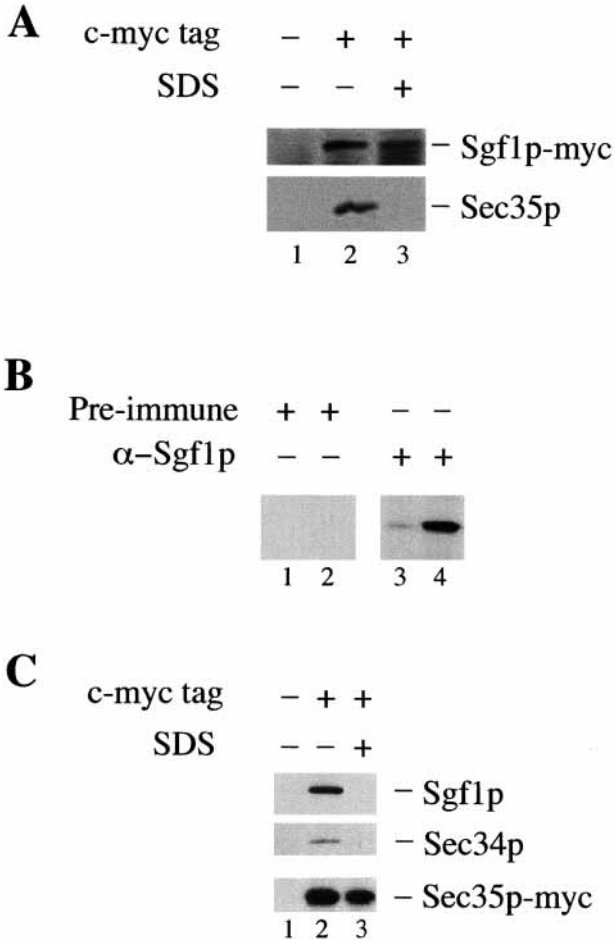

Figure 3: Sgf1p is a component of the Sec34p/Sec35p complex. (A) Sec35p coprecipitates with Sgf1p-*myc*. Lysates prepared from a strain containing tagged Sgf1p-*myc* (lane 2) and an untagged strain (lane 1) were incubated with anti-*c-myc* antibody and the immune complexes were collected onto protein A-Sepharose beads as described previously (6). One sample was pretreated with 1% SDS before the addition of antibody (lane 3). The beads were washed and treated with sample buffer. The solubilized precipitates were resolved by SDS-PAGE (10%) and subjected to Western blot analysis using anti-*c-myc* and anti-Sec35p antibodies. (B) Production of anti-Sgf1p antibody. Lysates prepared from wild-type without (lanes 1 and 3) or with *SGF1* on a high-copy vector (lanes 2 and 4) were resolved by SDS-PAGE (10%) and blotted with either preimmune serum (lanes 1 and 2) or anti-Sgf1p serum (lanes 3 and 4). (C) Sgf1p and Sec34p coprecipitate with Sec35p-*myc*. Lysates prepared from a strain containing tagged Sec35p-*myc* and an untagged strain were treated as in (A). Lane 1, untagged sample; lane 2, Sec35p-*myc*-tagged sample; lane 3, Sec35p-*myc*-tagged sample pretreated with 1% SDS.

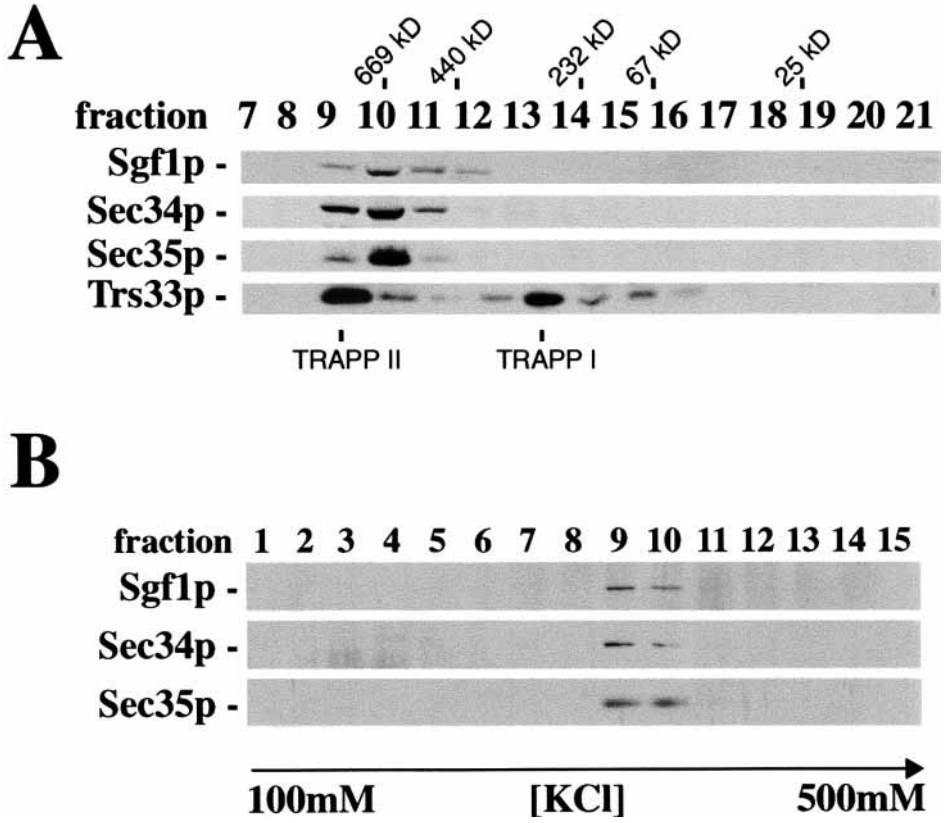

Figure 4: Sgf1p cofractionates with Sec34p and Sec35p. (A) Cytosol (5 mg of total protein), prepared from wild-type cells was fractionated on a Superdex-200 gel filtration column and 25 (1 ml) fractions were collected. Each fraction was probed with antibodies directed against Sgf1p, Sec34p, Sec35p or Trs33p (a subunit of TRAPP I and TRAPP II). Molecular size standards used to calibrate the column were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (67 kDa) and chymotrypsinogen A (25 kDa). (B) Fractions 9 and 10 from the Superdex-200 column were loaded onto a Mono Q column that was eluted with a linear salt gradient (100 mM to 500 mM KCl). Fractions (15 × 1 ml) were collected and analyzed for the presence of Sec34p, Sec35p and Sgf1p by Western blot analysis. Fractions 9–10 correspond to a salt concentration of ~325 mM.

hypothesis that Sgf1p is a member of the Sec34p/Sec35p complex.

Reduced levels of Sgf1p lead to a delay in CPY transport and the accumulation of membranes

To evaluate whether *SGF1* is essential, we constructed a diploid strain in which one allele of *SGF1* was replaced with the *S. pombe* his5$^+$ gene (17). This strain was sporulated and dissected, and the tetrads were incubated on YPD plates at 25 °C. After 5 days, the 12 tetrads examined clearly showed 2 + :2 – segregation for viability. The colonies that grew well were all his$^-$, indicating that they contained the wild-type copy of the gene. This finding demonstrates that *SGF1* is essential for growth.

Since previous work has implicated Sec34p and Sec35p in ER to Golgi traffic (7,18,19), we reasoned that Sgf1p, as a component of the Sec34p/Sec35p complex, may also play a role at this stage of the secretory pathway. To test this hypothesis, we constructed a strain (SFNY1031) in which the sole copy of *SGF1* was placed behind the glucose-repressible *GAL1* promoter. Since *SGF1* is essential, this strain should grow in galactose-containing medium, but not in the presence of glucose. Using this strain, we tested whether reduced levels of Sgf1p would result in a membrane traffic defect. To repress the expression of *SGF1*, cells that grew in galactose-containing medium were inoculated into glucose-containing medium. After ~10 h at 25 °C, the growth rate of SFNY1031 decreased compared to wild-type (Figure 5A).

CPY transport was analyzed in SFNY1031 and wild-type cells that were incubated for 12 h in glucose-containing medium. In wild-type, CPY is translocated into the ER where it is core-glycosylated (p1 form). Upon transit to the Golgi, outer chain carbohydrate is added to the core-glycosylated form to yield p2CPY. Finally, in the vacuole CPY is processed to the mature form (20). Cells were pulse-labeled for 4 min at 30 °C and chased for up to 30 min. Wild-type cells efficiently transported CPY to the vacuole during the 30-min chase (Figure 5C). In contrast, at early time points SFNY1031 showed a clear delay in processing p1 CPY. This delay in CPY transport correlated with a decrease in Sgf1p (Figure 5B). The amount of Bos1p, an ER membrane protein, remained unchanged under these conditions.

To characterize the morphological consequences of reduced levels of Sgf1p, thin-section electron microscopy of SFNY1031 (Figure 6B) was compared with that of wild-type (Figure 6A). In wild-type, tubules of ER were occasionally found at the periphery of the cell or in contact with the nuclear envelope. In contrast, SFNY1031 cells incubated for 12 h in glucose-containing medium accumulated membranes. The ER lumen and the nuclear envelope were modestly dilated in these cells, and small vesicles (50–60 nm in

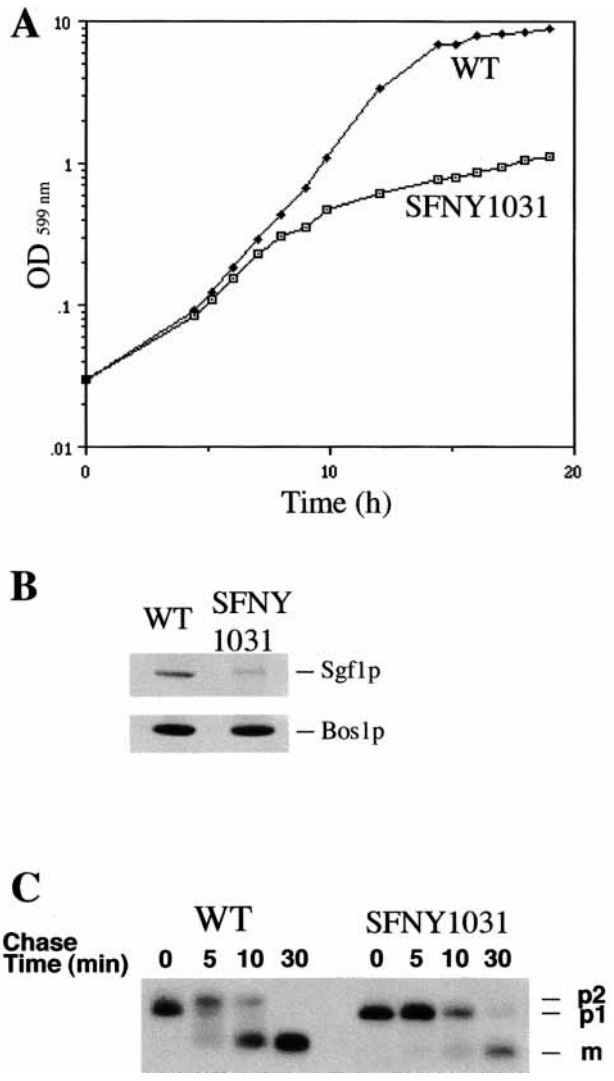

Figure 5: Reduced levels of Sgf1p lead to a delay in CPY transport. (A) Reduced levels of Sgf1p result in a growth defect in SFNY1031. Cells were grown at 25 °C in YP medium containing 0.5% galactose and 2% raffinose to an OD_{599} of 18 and then inoculated into YP medium containing 2% glucose (final $OD_{599} = 0.03$). The OD_{599} was measured at each time point. (B) The amount of Sgf1p in SFNY1031 was significantly reduced after a 12-h incubation in glucose-containing medium. Cell lysates prepared from wild-type and SFNY1031 were resolved on SDS-PAGE (10%) and subjected to immunoblot analysis using anti-Sgf1p and anti-Bos1p antibodies. (C) The p1 form of CPY accumulates in SFNY1031. After a 12-h incubation, cells were pelleted, washed and pulse-labeled with [^{35}S] ProMix at 30 °C in minimal medium containing 2% glucose.

size) that are comparable in size to ER to Golgi transport vesicles were also observed. Interestingly, large vesicles of the size seen in mutants blocked in post-Golgi transport and membrane structures that resembled aberrant Golgi (21) were sometimes observed, suggesting that Sgf1p might have a role at multiple stages of the secretory pathway. Thin-section electron micrographs of the *sec34–2* (Figure 6C) and *sec35–1* (Figure 6D) mutants have similar phenotypes.

sec34–2 displays synthetic lethal interactions with mutations that disrupt Golgi and post-Golgi traffic

Several lines of evidence suggest that the Sgf1p/Sec34p/Sec35p complex may function at more than one stage of the secretory pathway. First, the overexpression of *SEC34* has been reported to inhibit the growth of the *sec9–4* mutant, which is defective in post-Golgi secretion (6). Overexpression of *SNC2*, a post-Golgi v-SNARE, suppresses *sec35–1* (19). Second, the loss of Grd20p/Sec34p function results in the missorting of CPY and the mislocalization of a trans-Golgi membrane protein, Kex2p (8). Third, endocytosis of the a-factor receptor is defective in *grd20* mutants (8). Finally, mutants with defects in subunits of the Sgf1p/Sec34p/Sec35p complex accumulate a variety of membranes (Figure 6). This finding prompted us to examine whether *sec34–2* displays genetic interactions with other mutations that block protein transport at different stages of the secretory pathway (Table 2). Synthetic lethality or sickness of double mutants results when the effect of combining two mutations in the same haploid cell causes lethality or sickness under normally permissive conditions. Such interactions suggest that the proteins encoded by the mutated genes are functionally related (22,23). The *sec34–2* mutant was crossed to mutants that block vesicle budding (*sec13–1*, *sec23–1*, *sec24–1* and *sec31–1*), ER to Golgi traffic (*bet3–1*), retrograde transport from the Golgi to the ER (*sec21–1*, *ret2–1*, *sec27–1* and *arf1Δ*), intra-Golgi traffic (*trs130^{ts2}*) and post-Golgi transport (*sec2–41* and *sec4–8*) (5,21,23–26). For each cross, 12–24 tetrads were dissected and the viability of the segregants was investigated. In combinations of *sec34–2* with *sec23–1*, *sec24–1*, *sec21–1*, *ret2–1*, *sec27–1*, *sec4–8* and *bet3–1*, three viable colonies were found in the majority of tetrads, with some having two or four viable colonies at 25 °C or 30 °C. Inviable colonies were confirmed to have double mutations by scoring the viable segregants for temperature sensitivity and the appropriate auxotrophic marker. In crosses of *sec34–2* with *sec13–1*, *sec2–41* and *trs130^{ts2}*, but not with *sec31–1* or *arf1Δ*, the double mutants were sick. These genetic interactions, together with other results, support the hypothesis that the Sgf1p/Sec34p/Sec35p complex may act in more than one membrane traffic event.

Discussion

Previous studies have shown that Sec34p and Sec35p are members of a multiprotein complex (6). Here we show that Sgf1p is a high-copy suppressor of the *sec35–1* mutant. Sgf1p coprecipitates with Sec34p and Sec35p and interacts with both of these proteins in the yeast two-hybrid system. Sgf1p, which is approximately the same size as Sec34p (~100 kDa), is larger than the other putative members of this complex (91, 73, 68 and 51 kDa) that were identified in a precipitate of Sec35p-*myc* from a radiolabeled lysate (6). Interestingly, two different high-throughput two-hybrid screens have shown that Sec35p interacts with Sgf1p (27,28).

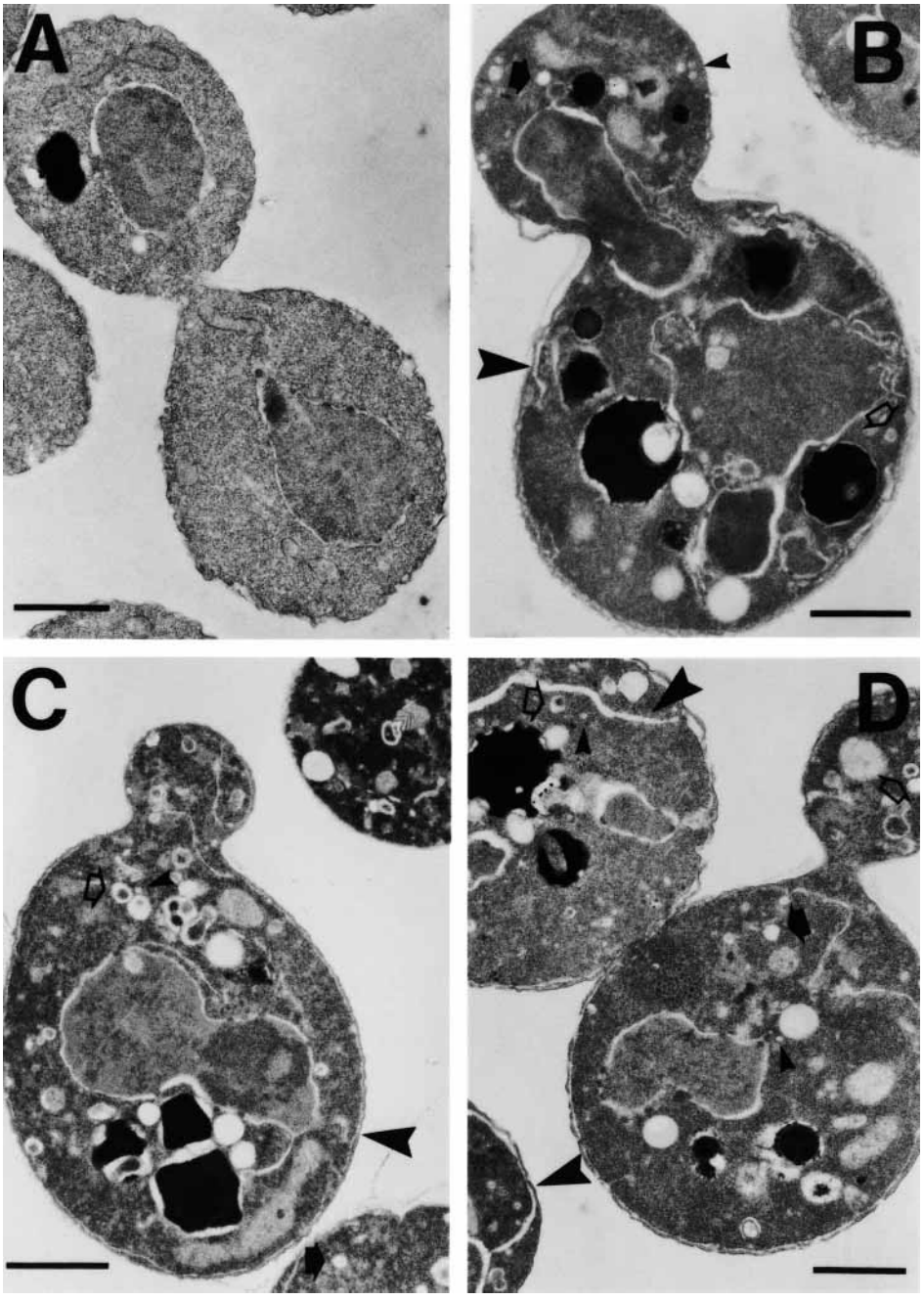

Figure 6: Reduced levels of Sgf1p lead to the accumulation of membranes. EM analysis of wild-type (panel A) and SFNY1031 (panel B) after a 12-h incubation at 25°C in glucose-containing medium, and *sec34–2* (panel C) and *sec35–1* (panel D) after a 2-h shift at 38°C. Samples were prepared for electron microscopy as described previously (34). ER (large arrowheads); small vesicles (small arrowheads); large vesicles (full arrows); aberrant membranes that resemble Golgi (open arrows). The bars in each panel represent 1 μm.

In vitro transport studies have implicated the Sec34p/Sec35p complex in the tethering of ER-derived COP II vesicles to the Golgi (7). Several other tethering factors have also been implicated in this process. These factors include TRAPP I, Uso1p, and the small GTP-binding protein Ypt1p [for reviews, see (1,29)]. TRAPP I specifically binds to COP II vesicles and stimulates guanine nucleotide exchange on Ypt1p (5,30). As the recruitment of Uso1p to membranes is dependent on Ypt1p (31), the activation of Ypt1p by TRAPP I (5) may be important for this event. Uso1p is a large protein with a globular head and a coiled-coil tail (32). Its participation in vesicle tethering may be a prerequisite to SNARE pairing as p115, the mammalian ortholog of Uso1p, has been shown to bind to the SNAREs (33).

A recent report, however, has questioned the role of Sec34p (also called Grd20p) in the trafficking of secretory proteins (8). This claim was prompted by the observation that mutations in *grd20* result in the mislocalization of resident trans-Golgi proteins, such as Kex2p, with only modest affects on the secretion of invertase. To address whether the Sec34p/Sec35p complex is required for the secretion of other pro-

Table 2: Synthetic growth defects between *sec34–2* and other mutations

		sec 34–2
COPII	*sec13–1*	+
	sec23–1	+
	sec24–1	+
	sec31–1	–
COPI	*sec21–1*	+
	ret2–1	+
	sec27–1	+
	arf1Δ	–
Post-Golgi	*sec2–41*	+
	sec4–8	+
Others	*bet3–1*	+
	trs130ts2	+

teins, we analyzed the secretion of all proteins into the growth medium. Mutants that block retrograde Golgi to ER traffic only block a subset of these proteins, while mutations in bona fide components of the anterograde secretory apparatus pleiotropically block the secretion of all these proteins (10). Our findings indicate that *sec34–2* and *sec35–1* pleiotropically block the secretion of this class of proteins into the medium. Consistent with these observations, a significant block in invertase secretion was previously reported for *sec34–2* (18).

The localization of Sec34p to the trans-Golgi and its proposed role in the localization of trans-Golgi proteins (8) seems to be in contradiction with a role in tethering ER-derived COP II vesicles. In an effort to resolve this apparent inconsistency, we localized Sec34p with respect to early (Och1p) and late (Sec7p) Golgi markers. Our findings revealed that most of the membrane-bound Sec34p is found on early Golgi. That Sec34p can be found throughout the Golgi together with the observation that CPY is missorted and Kex2p is mislocalized in *grd20* (*sec34*) mutants (8), suggests that Sec34p may be a general transport factor that mediates more than one vesicle trafficking event. Consistent with this hypothesis, we find genetic interactions between *sec34–2 and* mutations that block ER to Golgi, Golgi, and post-Golgi traffic.

Stage-specific mutants that block membrane traffic between the ER and Golgi complex accumulate an extensive network of dilated ER (5,21,24,34). In contrast, mutants that harbor defects in components of the Sec34p/Sec35p complex accumulate modest amounts of ER and the lumen does not appear to be as dilated as other known ER-accumulating mutants (21). Mutations in subunits of the Sec34p/Sec35p complex also lead to the accumulation of a variety of membranes including structures that resemble aberrant Golgi. Thus, it is possible that the morphological and kinetic affects observed on ER to Golgi traffic in *sec34, sec35, and sgf1* mutants may be an indirect consequence of blocking the flow of traffic through the Golgi. Similar phenotypes have been reported before for mutations in *vti1* (35). The yeast v-SNARE Vti1p mediates multiple transport events, including retrograde traffic to the *cis*-Golgi (35–37). Like *sec34*, some alleles of *vti1* do not block secretion.

Thus far only one complex, TRAPP II, has been implicated in the tethering of Golgi vesicles (5). As traffic through the Golgi involves multiple tethering events, it is likely that other tethers are involved in these events. An attractive possibility is that the Sec34p/Sec35p complex is one of these tethers. Interestingly, other mutants that disrupt Golgi traffic have been shown to display genetic interactions with mutations that block ER to Golgi, Golgi and post-Golgi transport (5,34,38).

An alternate explanation of the data is that Sec34p is present in two different complexes that tether different classes of transport vesicles. This has recently been reported for the TRAPP complex which is found in two forms, TRAPP I and TRAPP II (5). The two complexes share seven subunits (Bet5p, Trs20, Bet3p, Trs23p, Trs31p, Trs33p and Trs85p), while three subunits (Trs65p, Trs120p and Trs130p) are unique to TRAPP II. Mutational analysis and *in vitro* transport studies have revealed that these two complexes mediate different transport steps. The two forms of TRAPP can be separated by fractionating cytosol on a Superdex-200 column. However, using gel filtration conditions that have allowed us to separate TRAPP I from TRAPP II, and by anion exchange chromatography, we only detect one form of the Sec34p/Sec35p complex. Thus, this hypothesis seems unlikely but we cannot definitively rule it out. Additional experiments will be needed to determine if the Sec34p/Sec35p complex mediates multiple tethering events.

Materials and Methods

Strain constructions and growth conditions

The strains used in this study are listed in Table 3. SFNY1091 and SFNY1092 were constructed by transforming SFNY772 with the Och1HA plasmid (pOH) (39), or a plasmid containing Sec7p-GFP (pSSEC7-EGFPx3; a gift from Benjamin Glick). pSSEC7-EGFPx3 was first digested with *Spe*I and the linear fragment was transformed into SFNY772. SFNY1006 was constructed as previously described (6). The *SGF1* gene was disrupted by replacing the ORF (bp 1–2586) with the *S. pombe* his5$^+$ gene (17). Briefly, a hybrid sequence containing the his5$^+$ gene flanked by part of *SGF1* was amplified by polymerase chain reaction (PCR). This product was then transformed into wild-type diploid cells (NY1523). His$^+$ transformants (SFNY1033) were selected and the disruption was confirmed by PCR before the strain was subjected to tetrad analysis. SFNY1031 was constructed as follows: the amino terminal portion (1–348 bp) of the *SGF1* gene was amplified by PCR, digested with *Bam*HI and *Hind*III, and inserted into the same sites of pNB529, placing the amino terminal portion of *SGF1* behind the *GAL1* promoter. The plasmid was digested with *Msc*I, which cuts within the *SGF1* gene, and the linear fragment was transformed into NY604 to construct a strain in which full-length *SGF1* was placed under the control of the regulatable *GAL1* promoter. Leu$^+$ transformants were selected and the chromosomal fusion of *SGF1* to the *GAL* promoter was confirmed by PCR. Cells expressing Sgf1p from the *GAL1* promoter were grown to an OD_{599} of 18 in YP medium containing 0.5% galactose and 2% raffinose and then inoculated into YP medium containing 2% glucose.

Table 3: Yeast strains used in this study

Strain	Genotype
NY8	*MATα sec1–1 ura3–52*
NY413	*MATα sec13–1 ura3–52*
NY424	*MATα sec21–1 ura3–52*
NY604	*MATα GAL+ ura3–52 leu2–3, 112*
NY771	*MATa sec2–41 leu2–3, 112*
NY775	*MATa sec4–8 leu2–3, 112*
NY806	*MATa sec23–1 ura3–52*
NY807	*MATα sec23–1 leu2–3, 112*
NY808	*MATa sec23–1 leu2–3, 112*
NY1228	*MATα sec18–1 leu2–3, 112*
NY1523	*MATa/α GAL+/GAL+ ura3–52/ ura3–52 leu2–3, 112/leu2–3, 112 his3-Δ200/his3-Δ200*
SFNY26–3A	*MATa ura3–52*
SFNY26–12C	*MATα ura3–52*
SFNY313	*MATα bet3–1 leu2–3, 112*
SFNY351	*MATα ura3–52 leu2–3, 112 his3-Δ200 lys2–801 arf1Δ::URA3*
SFNY690	*MATa sec34–2 lys2–801*
SFNY691	*MATα sec34–2 ura3–52 leu2–3,112*
SFNY770	*MATα sec34–2 ura3–52*
SFNY772	*MATa ura3–52 SEC34 (with three c-myc tags)*
SFNY794	*MATa sec34–1 ura3–52*
SFNY816	*MATa sec35–1 ura3–52*
SFNY947	*MATa ura3–52 SEC35 (with three c-myc tags)*
SFNY969	*MATα ura3–52 leu2–3, 112 his3-Δ200 trs130ts2*
SFNY981	*MATα sec27–1 ura3–42 leu2–3112 trp1–901*
SFNY983	*MATa ret2–1 ura3–52 leu2–3, 112 his3-Δ200 lys2–801 suc2-Δ9*
SFNY1003	*MATα sec24–1 ura3–52 leu2–3, 112*
SFNY1005	*MATα sec31–1 ura3–52 leu2–3, 112*
SFNY1006	*MATa ura3–52 L-A-o SGF1 (with three c-myc tags)*
SFNY1007	*MATa ura3–52 YML068W (with three c-myc tags)*
SFNY1031	*MATα GAL+ ura3–52 leu2–3, 112 GAL1-SGF1*
SFNY1033	*MATa/α GAL+/GAL+ ura3–52/ ura3–52 leu2–3, 112/leu2–3, 112 his3-Δ200/his3-Δ200 SGF1/sgf1Δ::HIS3*
SFNY1091	*MATa ura3–52 SEC34 (with three c-myc tags) pOH(OCH1-HA URA3 CEN)*
SFNY1092	*MATa ura3–52 SEC34 (with three c-myc tags) SEC7-GFP URA3*

Cell labeling and immunoprecipitation

Secretion of [^{35}S] radiolabeled proteins was measured by a modification of the method of Gaynor and Emr (10). Cells grown at 25 °C to early exponential phase in minimal medium were harvested, resuspended in 400 μl of minimal medium and preshifted to 37 °C for 20 min before 2 OD_{599} units were radiolabeled for 15 min at 37 °C with 150 μCi of [^{35}S] ProMix (Amersham, England). The radiolabeled cells (380 μl) were transferred to an ice-cold tube containing 38 μl of 500 mM NaN_3/500 mM NaF. To separate medium from cells, cell suspensions were centrifuged at 14 000 g for 1 min. The supernatant (300 μl) was transferred to a tube containing 19.5 μl of 100% TCA and 1.5 μl of 2% deoxycholate, and incubated for at least 1 h on ice. The TCA-precipitated proteins were washed three times with ice-cold acetone, air-dried, resuspended in SDS sample buffer and resolved on an 8% SDS-polyacrylamide gel.

For pulse-chase analysis of CPY, wild-type and SFNY1031 were grown at 25 °C in YP medium containing 0.5% galactose and 2% raffinose to an OD_{599} of 18 and then inoculated into YP medium containing 2% glucose (final OD_{599} = 0.03). After a 12-h incubation at 25 °C, cells (10 OD_{599} units) were pelleted, washed and resuspended in 3.5 ml of minimal medium containing 2% glucose. The cells were preincubated for 20 min at 30 °C and then pulse-labeled with 250 μCi of [^{35}S] ProMix. Following a 4-min pulse labeling, 700 μl of cells were removed (0 min chase) and cold methionine and cysteine were added to a final concentration of 10 mM. After 5, 10 and 30 min, samples were transferred to a cold tube containing NaN_3 and NaF (final concentration of 10 mM) and CPY was immunoprecipitated as described previously (34).

The precipitation of *myc* tagged proteins was performed as described previously (6).

Immunofluorescence and electron microscopy

Five OD units of cells of an overnight culture that was grown to OD_{599} = 1 were centrifuged, fixed and converted to spheroplasts as described previously (40). Spheroplasts were adhered to poly-L-lysine-coated slides for 15 min before they were washed three times with PBST (PBS buffer, pH 7.4, 0.1% tween-20, 10 mg/ml BSA and 0.1% NaN_3). Lysed spheroplasts were incubated overnight with 30 μl primary antibody in a humid chamber at 4 °C, washed 10 times in PBST and then incubated with 30 μl of secondary antibody for 90 min in a humid chamber at 4 °C. Double immunofluorescence of Sec34p-*myc* and Och1p-HA was achieved by incubating with a mixture of mouse anti-*myc* monoclonal antibody 9E10 (1 : 100 dilution; Babco, CA, USA) and rabbit anti-HA polyclonal antibody (1 : 500 dilution; Babco, CA, USA) and then with a mixture of donkey anti-mouse IgG antibody conjugated to Cy3 (1 : 1000 dilution; Jackson Immunoresearch Laboratories, PA, USA) and donkey anti-rabbit IgG antibody conjugated to FITC (1 : 1000 dilution; Jackson Immunoresearch Laboratories, PA, USA). Double immunofluorescence of Sec34p-*myc* and Sec7p-GFP required primary incubation with mouse anti-*myc* monoclonal antibody 9E10 (1 : 100 dilution) followed by secondary incubation with donkey anti-mouse IgG antibody conjugated to Cy3 (1 : 700 dilution). Following the antibody incubations, the slides were washed 30 times with PBST and three times with PBS (pH 7.4) before the coverslips were applied with Fluoromount G (Southern Biotechnology Associates, AL, USA). Samples were viewed under a fluorescence microscope, and images were obtained using a charge-coupled device camera and manipulated with Openlab software (Improvision, MA, USA). Adobe Photoshop and Illustrator software were used to produce figures.

To quantify the colocalization of Sec34p-*myc* and Och1p-HA, 316 Sec34p-*myc*-positive puncta and 340 Och1p-HA-positive puncta were scored in 73 cells by eye after image capture. A total of 251 puncta stained for both proteins. In order to quantify the colocalization of Sec34p-*myc* and Sec7p-GFP, 251 Sec34p-*myc*-positive puncta and 257 Sec7p-GFP-positive puncta were scored in 70 cells. A total of 70 puncta stained for both proteins.

For electron microscopy, wild-type and SFNY1031 were incubated for 12 h at 25 °C in YP medium containing 2% glucose, while *sec34–2* and *sec35–1* mutants were shifted to 38 °C for 2 h, and processed as described before (34).

Screen for high-copy suppressors of sec35–1

To isolate high-copy suppressors of *sec35–1*, the *sec35–1* mutant (SFNY 816) was transformed with a yeast genomic high-copy library and Ura^+ transformants were selected at 25°C and then screened for growth at 37°C. Of the 77 000 transformants screened, 81 were found to grow at 37°C. Plasmids were retrieved from these 81 transformants, amplified in *E. coli and* reintroduced into *sec35–1* to confirm that the suppression was plasmid dependent. Plasmids containing the *SEC34* and *SEC35* structural genes were identified by restriction analysis. *SEC35* has a unique 717 bp *Psh*AI and *Bst*EII fragment, while *SEC34* contains a unique 2.2 kb *Bst*EII and *Bgl*II fragment. Those plasmids that did not contain *SEC34* or *SEC35* were sequenced. A combination of restriction analysis and DNA sequencing revealed that the 81 suppressors contained five different regions of genomic DNA. Two of the genomic regions of DNA contained the *SEC35* and *SEC34* structural genes. The third was found to contain the *SBP1* and *RPL8A* genes. The insert (8.14 kb) in the fourth genomic region was digested with *Kpn*I and *Spe*I and the resulting 3.75 kb fragment was inserted into the *Kpn*I-*Spe*I sites of pRS426. It contained a hypothetical open reading frame (YPR105C) that conferred suppression. We named this ORF *SGF1* (*s*uppressor *g*ene of *sec35–1*). The insert (8.65 kb) in the fifth genomic region of DNA was digested with *Ase*I and the resulting two fragments were inserted into the same site of YEp24. One (2.5 kb) of these fragments contained YML068 which retained full suppression activity.

Antibody production

Antibody to Sgf1p was prepared against an MBP-Sgf1p recombinant form of the protein. To construct the plasmid encoding MBP-Sgf1p, the *SGF1* ORF was amplified by PCR, placing a *Bam*HI site before the start codon and a *Hin*dIII site after the stop codon (sense primer [5′−3′], CAT CTA GGA TCC ATG GAA GGG CAA AAA TCG AAT G; antisense primer [5′-3′], CAT CTA AAG CTT CTA TTA CTG TGT TCT ATC AAT CTT C). The PCR product was digested with *Bam*HI and *Hin*dIII and then ligated into the same sites of pMAL-c2 (New England Biolabs, MA, USA). The construct was transformed into BL21 cells (Novagen, WI, USA), and the fusion protein was purified as described previously (6).

Two-hybrid analysis

The ORFs of *SGF1, YML068W, SEC34 and SEC35* were amplified by PCR, sequenced, digested with *Nco*I and *Bam*HI, and inserted into the same sites of pACT2 (fish) or pAS1-CYH2 (bait) (16). Two different constructs were introduced into the yeast strain Y190, and Leu^+ and Trp^+ transformants were selected. The transformants were smeared onto filter paper (Whatmans, England) and the filter paper was frozen in liquid nitrogen, thawed, and overlaid onto a filter soaked with Z buffer (0.1 M sodium phosphate buffer, pH 7.0, 0.01 M KCl, 1 mM $MgSO_4.7H_2O$, and 30 mM β-mercaptoethanol) containing X-gal (Boehringer Mannheim, IN, USA). After an overnight incubation at 30°C, ~300 colonies were screened for a change from white to blue color. If the majority of the colonies turned blue, it was designated as a positive interaction.

Partial purification of the Sec34p/Sec35p/Sgf1p complex

A yeast lysate was prepared as described before (6), except the cytosol was centrifuged at 84 000 g for 1 h. A total of 5 mg of protein was loaded onto a 25-ml Superdex-200 gel filtration column, pre-equilibrated with buffer A (25 mM Tris, pH 7.6, 100 mM KCl, 1 mM DTT, 1×Pic), at a flow rate of 0.4 ml/min. Fractions (1 ml) were collected and the peak of Sec34p, Sec35p and Sgf1p, as determined by Western blot analysis, was loaded onto a 1-ml Mono Q column at a flow rate of 1 ml/min. The column was washed with 5 column volumes of buffer A and the protein was eluted with a linear gradient (15 ml) of buffer A to buffer B (same as buffer A with 500 mM KCl). Proteins were detected by Western blot analysis.

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