Sec34p, a Protein Required for Vesicle Tethering to the Yeast Golgi Apparatus, Is in a Complex with Sec35p

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Abstract. A screen for mutants of Saccharomyces cerevisiae secretory pathway components previously yielded sec34, a mutant that accumulates numerous vesicles and fails to transport proteins from the ER to the Golgi complex at the restrictive temperature (Wuestehube, L. J., R. Duden, A. Eun, S. Hamamoto, P. Korn, R. Ram, and R. Schekman. 1996. Genetics. 142:393–406). We find that SEC34 encodes a novel protein of 93-kD, peripherally associated with membranes. The temperature-sensitive phenotype of sec34-2 is suppressed by the rab GTPase Ypt1p that functions early in the secretory pathway, or by the dominant form of the ER to Golgi complex target-SNARE (soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor)-associated protein Sly1p, Sly1-20p. Weaker suppression is evident upon overexpression of genes encoding the vesicle tethering factor Uso1p or the vesicle-SNAREs Sec22p, Bet1p, or Ykt6p. This genetic suppression profile is similar to that of sec35-1, a mutant allele of a gene encoding an ER to Golgi vesicle tethering factor and, like Sec35p, Sec34p is required in vitro for vesicle tethering, sec34-2 and sec35-1 display a synthetic lethal interaction, a genetic result explained by the finding that Sec34p and Sec35p can interact by two-hybrid analysis. Fractionation of yeast cytosol indicates that Sec34p and Sec35p exist in an ~750-kD protein complex. Finally, we describe RUD3, a novel gene identified through a genetic screen for multicopy suppressors of a mutation in USO1, which suppresses the sec34-2 mutation as well.

Key words: Sec34p • Sec35p • Rud3p • vesicle tethering • secretory pathway

The flow of material through the secretory pathway is mediated, at least in part, by membrane-bound vesicles or larger membrane-delimited structures (Palade, 1975). These transport intermediates, which are generated from numerous intracellular compartments, dock and fuse specifically with the next membrane compartment on their itinerary. Therefore, there must be a mechanism that allows the vesicle and target membranes to specifically recognize one another.

For some time, members of the rab GTPase family were suspected to be the principle determinants of this targeting specificity because distinct family members display unique organellar localizations that correlate with their site of action (for review see Simons and Zerial, 1993). However, it has been demonstrated that a single chimeric rab protein can function at two transport steps (Brennwald and Novick, 1993; Dunn et al., 1993), indicating that rabs cannot be the sole targeting determinants. In a similar fashion, SNARE1 (soluble N-ethylmaleimide sensitive fusion protein protein attachment protein receptor) proteins, which are integral membrane proteins found predominantly on vesicles (v-SNAREs) or target membranes (t-SNAREs), were proposed to encode specificity (Söllner et al., 1993). Once more, evidence suggests that SNAREs cannot be the only targeting components: some SNAREs can function at several transport steps in vivo (Fischer von Mollard et al., 1997; Fischer von Mollard and Stevens, 1999), SNAREs that faithfully function at different transport

1. Abbreviations used in this paper: CEN, centromere; Gal4p-A-D, Gal4p transcriptional activation domain; Gal4p-BD, Gal4p DNA-binding domain; gp-α-factor, glycosylated pro-α-factor; GST, glutathione S-transferase, ORF, open reading frame; PGK, phosphoglycerate kinase; SNARE, soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor; t-SNAREs, SNAREs found predominantly on target membranes; v-SNAREs, SNAREs found predominantly on vesicles.
steps in vitro can interact promiscuously in vitro (Fas-shauer et al., 1999; Yang et al., 1999), and some SNAREs have been found in multiple SNARE complexes by co-immunoprecipitation (Lupashin et al., 1997; Holthuis et al., 1998).

Given that neither rabs nor SNAREs are the sole governors of targeting specificity, other components are likely to play important roles. A dditional candidate players include the so-called tethering factors (Lowe et al., 1998), which are proteins that bind membranes together before SNARE interactions (Cao et al., 1998). Tethering factors are a diverse group of proteins whose only common feature is their tendency to form elongated, coiled-coil structures and/or to assemble into large, multimeric complexes (for reviews see Pfeffer, 1999; Waters and Pfeffer, 1999). However, because they are peripheral membrane proteins, and therefore must interact with integral membrane components with distinct localizations, once again, they cannot be the sole determinants of targeting specificity. Taken together, these results suggest that specificity in vesicle traffic might be generated by the interaction of several factors in such a way that no individual component plays the dominant role.

ER to Golgi complex traffic in the yeast Saccharomyces cerevisiae is one of the most intensively studied steps in membrane trafficking. The components involved in the consumption of ER-derived vesicles at the Golgi complex are related to those of many other steps. These include: the v-SNARE Bet1p, Bos1p, Sec22p, and perhaps Ykt6p (Novick et al., 1980; Newman and Ferro-Novick, 1987; Dacher et al., 1991; Shim et al., 1991; McNamara et al., 1997); the t-SNARE Sec5p (Hawrick and Pelham, 1992); the t-SNARE-associated protein Sly1p (Dacher et al., 1991); and the rab GTPase Ypt1p (Schmitt et al., 1992); the t-SNARE–associated protein Sly1p (Dascher et al., 1991), a homodimeric molecule with two heads and a long neck that may function in the tethering of ER-derived vesicles with the Golgi complex. This Uso1p- and Sec35p-dependent tethering, or stable interaction, or tethering of ER-derived vesicles may function in this process. Indeed, vesicle tethering was defective in an in vitro system generated from a sec34 mutant. Interestingly, we find that Sec34p is present in a large protein complex that contains Sec35p. These findings indicate that the Sec34p/Sec35p complex is a novel component required for tethering ER-derived vesicles to the yeast Golgi complex and, as such, may help to impart targeting specificity to this transport step. Lastly, we describe a novel gene, RUD3, which was originally identified as a high copy suppressor of a sos1 tethering mutant (Sapperstein, 1997), and which we now find acts as a multicopy suppressor of sec34 as well. This genetic result implicates RUD3 as functioning in, or downstream of, ER to Golgi vesicle tethering.

### Materials and Methods

#### Media and Microbial Techniques

Bacterial media was prepared by standard protocols (Miller, 1972). Yeast strains were maintained on rich media (YEP) containing 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose, or on synthetic complete media (SC) containing 0.67% yeast nitrogen base without amino acids, 2% glucose, and the appropriate supplements (Rose et al., 1990). SC media lacking histidine, leucine, and tryptophan used in the two-hybrid assay contained 2.5 mM 3-amino-triazole. Diploid strains were sporulated at room temperature in liquid media consisting of 1% potassium acetate and 0.02% glucose. Escherichia coli transformations were performed by the method of Hanahan (1983) and yeast transformations were performed by the method of Elledge (1992), except for the yeast genomic library transformation, which was by the method of Schiestl and Gietz (1989).

#### Plasmid and Strain Construction

Plasmids used in this work are described in Table I. Plasmid construction was as follows. To generate pSV22, the genomic library plasmid pBS was digested with PvuII and HindIII, and the resulting 2.7-kb fragment containing Tey YER157w/SEC34 was ligated into pRS416 that had been digested with SmaI and HindIII. To create pSV24 and pSV25, SEC34 was liberated from the polylinker of pSV22 with either HindIII and BamHI or XhoI and SpeI, respectively. The insert for bacterial expression plasmids encoding glutathione S-transferase (GST)-Sec34p and His15-Sec34p fusion proteins was generated by PCR including a BamHI site adjacent to the codon for the first amino acid of Sec34p and a Smal site downstream of the stop codon (5′ primer, 5′ gcc-gga-tcc-att-gag-aga-agt-aga-aag-3′; 3′ primer, 5′ tcc-ccc-ggg-gtt-tat-ttc-gtg-att-gta-tc-3′). The PCR product was digested with BamHI and Smal, and

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ligated into similarly digested pGEX 4T-1 (Pharmacia Biotech, Inc.) and pQE 30 (QIA GEN, Inc.), generating pSV 28 and pSV 30, respectively. To create the constructs expressing the Gal4p DNA binding domain (Gal4p-BD) or transcriptional activation domain (Gal4p-AD) fused to Sec34p, the pSC 34 open reading frame (ORF) was amplified by PCR, placing a BamHI site upstream of the codon for the second amino acid residue of the protein and a PstI site downstream of the stop codon (5′ primer, 5′ cgc-gga-tcc-ttg-cga-gaa-gta-gaa-ag 3′; 3′ primer, 5′ cgc-gct-gca-gtg-tct-gtt-agt-gta-tc 3′). The resulting product was cleaved with BamHI and PstI, and ligated into a similarly digested pA S2 or pGA D 424 (Clonetech, Inc.), yielding COOH-terminal fusions to Gal4p-BD (pSV 37) and Gal4p-AD (pSV 35), respectively. The constructs expressing the Gal4p-BD or Gal4p-AD fused to Sec34p were constructed by transforming GWY 30 with PstI-digested pSV 27. The presence of the SEC34 deletion in the Leu+ transformants was confirmed by PCR amplification of the novel junctions at the deletion locus. The diploid strain heterozygous for both the sec34-2 and sec35-1 alleles was created by mating G Wy 93 to G Wy 95 for 6 h on YPD. The diploid was identified by the distinct morphology of the zygote, and was isolated by micromanipulation.

### Cloning of SEC34

To clone SEC34, the sec34-2 strain G Wy 95 (Wuestehube et al., 1996; V an R heenen et al., 1998) was transformed with a U RA 3 Y Cp 50-based library (R ese et al., 1987), and temperature-resistant colonies were selected at 38.5°C. Over 100,000 transformants were screened and ~90 temperature-resistant colonies were isolated. Library plasmids from nine colonies were isolated, amplified in bacteria, and tested for the ability to confer temperature-resistance when retransformed into the sec34-2 strain. Four plasmids restored growth at 38.5°C to wild-type levels, three yielded partial suppression, and the remaining two did not display plasmid-linked suppression. The ends of the inserts of the seven constructs showing plasmid-linked suppression were sequenced with primers YEp24-F and YEp24-R (Sapperstein et al., 1996) and the resulting sequences were used to search the Saccharomyces Genome Database. To mark the Y ER 157w locus for purposes of integrative mapping, the sec34-2 strain G Wy 95 was transformed with pSV 24 that had been linearized with BglII, an enzyme that cleaves internal to the ORF, such that integration is directed to the Y ER 157w locus.

### Antibody Production and Immunoblotting

GST-Sec34p and His6-Sec34p were expressed in strain XL1-Blue (Stratagene) from plasmids pSV 28 and pSV 30, respectively, and fusion proteins were purified according to the manufacturer’s instructions (Pharmacia Biotech, Inc.; QIA GEN, Inc.). GST-Sec34p was used to immunize rabbits (H. Pelham (MRC, Cambridge, UK), S. Ferro-Novick (Yale University, New Haven, CT). Crude yeast lysates used to characterize the af-

### Table 1. Plasmids Used in this Work

| Plasmid | Description | Source |
|---------|-------------|--------|
| pJG103  | 2μm SEC2 2 URA3 | S. Ferro-Novick (Yale University, New Haven, CT) |
| pSFN20  | 2μm BET1 URA3 | S. Ferro-Novick |
| pNB167  | 2μm YPT1 URA3 | S. Ferro-Novick |
| pGR3    | 2μm BET3 URA3 | S. Ferro-Novick |
| pSDD5   | 2μm SED5 URA3 | H. Pelham (MRC, Cambridge, UK) |
| pNB142  | 2μm SEC4 URA3 | P. Novick (Yale University, New Haven, CT) |
| pSK47   | 2μm USO1 URA3 | This laboratory |
| pSK60   | 2μm YK6 URA3 | This laboratory |
| pSK101  | 2μm BOS1 URA3 | This laboratory |
| pSV11   | CEN SLY1-20 URA3 | This laboratory |
| pSV17   | 2μm SEC3 5 URA3 | This laboratory |
| pVA3    | 2μm Gal4p-BD-p53 TRP1 | Clonetech, Inc. |
| pTD1    | 2μm Gal4p-AD-T-antigen LEU2 | Clonetech, Inc. |
| pB4     | CEN SEC34 URA3 (library plasmid) | This study |
| pA10    | CEN SEC34 URA3 (library plasmid) | This study |
| pSV22   | CEN SEC34 URA3 | This study |
| pSV24   | Ylp SEC34 LEU2 | This study |
| pSV25   | 2μm SEC3 4 URA3 | This study |
| pSV27   | Ylp sec34-construct LEU2 | This study |
| pSV28   | GST-Sec34p | This study |
| pSV30   | His6-Sec34p | This study |
| pSV34   | 2μm Gal4p-BD-Sec35p TRP1 | This study |
| pSV35   | 2μm Gal4p-AD-Sec34p LEU2 | This study |
| pSV36   | 2μm Gal4p-AD-Sec35p LEU2 | This study |
| pSV37   | 2μm Gal4p-BD-Sec34p TRP1 | This study |
| pSOU7   | 2μm RUD3 URA3 (library plasmid) | This study |
| pSK81   | 2μm RUD3 URA3 | This study |
Table II. Strains Used in this Work

| Strain     | Genotype                        | Source                                      |
|------------|---------------------------------|---------------------------------------------|
| RSY255     | MAT a ura3-52 leu2-3,112        | R. Schekman (University of California at Berkeley, Berkeley, CA) |
| RSY942     | MAT sec23-3 ura3-52 lys2-801    | R. Schekman                                 |
| RSY944     | MAT bet1-1 ura3-52 lys2-801     | R. Schekman                                 |
| RSY976     | MAT ypt1-3 ura3-52             | R. Schekman                                 |
| RSY1074    | MAT sly14 ura3-52 trp1 ade2-1   | R. Schekman                                 |
| RSY1157    | MAT pepp4::HIS3 prb::HisG prc::HisG ura3-52 ade2-1 trp1-1 ade2-1 can1-100 | D. Schekman                                 |
| SFNY314    | MAT bet3-1 ura3-52 leu2-3,112  | D. Schekman                                 |
| PW9-4A     | MAT a ura3-52 leu2-3,112        | P. James (Yale University, New Haven, CT)   |
| GSY901     | MAT a ura3-52 trp1 ade2-1 can1-100 | D. Schekman                                 |
| GSY927     | MAT sly14 ura3-52 ade2-1        | D. Schekman                                 |
| GSY944     | MAT bet1-1 ura3-52 lys2-801     | D. Schekman                                 |
| GSY976     | MAT ypt1-3 ura3-52             | D. Schekman                                 |
| GSY1074    | MAT sly14 ura3-52 trp1 ade2-1   | D. Schekman                                 |
| GSY1157    | MAT pepp4::HIS3 prb::HisG prc::HisG ura3-52 ade2-1 trp1-1 ade2-1 can1-100 | D. Schekman                                 |
| GSY1158    | MAT bet3-1 ura3-52 leu2-3,112  | D. Schekman                                 |
| GSY127     | MAT a ura3-52 ura3-52 leu2-3,112 | D. Schekman                                 |
| GSY132     | MAT sec54-1 ura3-52 leu2-3,112  | D. Schekman                                 |
| GSY138     | MAT sec54-1 (bosl+) ura3-52 his4-612 lys2-801 | D. Schekman                                 |

**Extraction and Subcellular Fractionation**

A 250-ml culture of wild-type yeast (RSY255) was grown in YPD at 30°C to midlogarithmic phase (OD600 = 1.4), washed in sterile water, and resuspended at 75 OD600/ml in Buffer 88 (25 mM Hepes, pH 7.0, 150 mM KCl, 5 mM MgCl2, 1 mM DTT) containing protease inhibitors (VanRheenen et al., 1998). 1/2 sample vol of acid-washed glass beads (425-600 μm; Sigma Chemical Co.) was added, and the material was vortexed eight times for 30 s, with 30 s on ice between each burst. The crude yeast lysate was centrifuged at 1,500 g at 30°C (Baker et al., 1988). Before as-

**Partial Purification of the Sec34p/Sec35p Complex**

The protease deficient RSY1157 strain was grown to late log phase (OD600 = 3.4) in 36 liters of YPD at 30°C, after which all manipulations were performed at 0–4°C. The cells were harvested by centrifugation and washed twice with water. The 334 g cell pellet was resuspended in 1 liter of 25 mM Tris-Ci, pH 8.0, 1 M KCi, 2 mM EGTA (lys buffer) with protease inhibitors (0.5 mM 1:10 phenanthroline, 2 μM pepstatin A, 2 μM aprotinin, 0.5 μg/ml leupeptin, 1 μM PMFS, 200 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride (A EBSF)) and 1 mM DTT, and lysed in an Ems- Flex-C5 (Avestin Inc.) at 18,000-20,000 psi. The lysate was centrifuged at 5,000 g (Sorvall SLA 3000 rotor, 6,000 rpm, 10 min), and the supernatant (1.04 liter) was collected and centrifuged at 20,000 g (Sorvall SA 600 rotor, 12,000 rpm, 20 min). The supernatant (960 ml) was removed, avoiding the loose pellet, and centrifuged at 175,000 g (Beckman 45Ti rotor, 44,000 rpm, 120 min). The supernatant (1,715; 750 ml at 7.3 mg protein/ml) was removed, avoiding the pellets. The 715 was made 1 mM in EDTA, and (NH4)2SO4 was added to 35% saturation, dissolved, and the solution was stirred for 60 min. The (NH4)2SO4 precipitate was collected by centrifugation at 17,000 g (SLA 3000 rotor, 10,000 rpm, 10 min), resuspended in enough 25 mM Tris-Ci, pH 8.0, 1 mM DTT to yield a conductivity equiva-

**In Vitro ER to Golgi Complex Transport Assay**

Yeast semi-intact cells from either the wild-type (RSY255) or the sec34-2 strain (GWY95) were prepared from logarithmic phase cultures of strains grown at 23°C and were stored at −70°C (Baker et al., 1988). Before as-

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brane. The concentrated sample was loaded onto a 700 ml Sephacryl S-300 (Pharmacia Biotech, Inc.) column (2.5 cm i.d.) that had been equilibrated in 5 mM Tris pH 7.6, 100 mM KCl, 1 mM DTT (T7.6/100K/D) and chromatographed at 1.5 ml/min in T7.6/100K/D, collecting 10 ml fractions. The fractions containing both Sec34p and Sec35p were isolated from the genomic insert (as shown in Fig. 1 d) and chromatographed at 0.3 ml/min, collecting 2 ml fractions. The fractions containing both Sec34p and Sec35p, which coeluted at ~255 mM KCl, were pooled (16 mg protein in 18 ml, 0.2% mg/ml), dialyzed against 40 mM potassium phosphate, pH 6.8, 0.02 mM KCl, 1 mM DTT (K7.6/0Pi/D) and stored growth at the restrictive temperature to wild-type levels. The remaining three yielded partial suppression. The ends of the inserts of the seven library plasmids were isolated from these colonies and re-tested for their ability to confer growth at 38.5 °C.

Results

Cloning of SEC34

To clone SEC34, the sec34-2 strain was transformed with a low-copy (centromere, CEN) yeast genomic library and temperature-resistant colonies were selected at 38.5 °C. Library plasmids were isolated from these colonies and re-tested for their ability to confer growth at 38.5 °C. Four restored growth at the restrictive temperature to wild-type levels, and the remaining three yielded partial suppression. The ends of the inserts of the seven library plasmids were sequenced and found to contain overlapping regions of the right arm of chromosome V, a portion of which is shown in Fig. 1 a. The only complete ORF contained on each of the plasmids that conferred strong suppression of the temperature-sensitive phenotype (two of which are shown in Fig. 1, b and c) was YER157w. Interestingly, the three plasmids that partially suppress the sec34-2 mutation contained identical inserts in which only the 5' end of YER157w is present; the inability of these plasmids to fully suppress may be due to the absence of the COOH-terminal portion of the protein. The ORF YER157w was isolated from the genomic insert (as shown in Fig. 1 d) and transferred to a low-copy plasmid. This construct was demonstrated to suppress the temperature sensitivity of the sec34-2 strain, confirming that YER157w is responsible for the suppression conferred by each of the library plasmids. To address the possibility that YER157w was a suppressor of the sec34-2 mutation rather than the gene itself, integrative mapping was performed. A sec34-2 strain in which the YER157w locus was marked with LEU2 was constructed and subsequently mated to a wild-type strain. The resulting diploid strain was subjected to tetrad analysis and, of 38 tetrads examined, the temperature-sensitive phenotype did not segregate away from the marked locus. Thus, integrative mapping strongly suggests that YER157w is SEC34.

SEC34 is predicted to encode an 801-amino acid protein (Fig. 1 f) with a molecular weight of 92.5 kDa and a pl of 5.2. SEC34 lacks a signal sequence, as well as transmembrane domains or other motifs that could facilitate membrane anchorage. Therefore, the protein is predicted to encode an 801-amino acid protein

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Figure 1. The SEC34 genomic locus and the sequence of Sec34p. a, Diagrammatic representation of a 10-kb region of the right arm of chromosome V containing BEM2, YER156c, YER157w, and YER158c. Relevant restriction enzyme sites (see Materials and Methods) are indicated. b and c, Genomic fragments contained in library plasmids p84 and pA10, respectively. d, The 2.6-kb PvuI/HindIII fragment that contains YER157w and restores temperature resistance to the sec34-2 strain. e, Representation of the SEC34 (YER157w) locus in the sec34-2 strain, in which the region between PvuI and HindIII is replaced by sequences in the LEU2 integrating vector. f, Predicted amino acid sequence of Sec34p. The nucleotide accession number for SEC34 is U18917 and the protein accession number is S50660.

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fission yeast Schizosaccharomyces pombe contains a 735-

amino acid protein (GenBank/EMBL/DDJB accession number CA B51337, PID g5579050) that is 26% identical and 40% similar to Sec34p. Finally, several overlapping human expressed sequence tags (ESTs; GenBank/EMBL/DDJB accession numbers A A 280321, A A 603511, A A 429818, and z21241) have been isolated that show high similarity to Sec34p. Sequencing of clones containing the first two ESTs (provided by Genome Systems, Inc., St. Louis, MO) allowed us to analyze additional sequences previously unavailable in GenBank (data not shown). By combining our newly sequenced regions with the overlapping ESTs in the database, we obtained a 263-amino acid portion of the putative human protein. Comparison of this partial protein to Sec34p using the BLAST algorithm (Altschul et al., 1990) revealed a 175-amino acid region of homology (encompassing amino acids 531 to 706 of Sec34p) between the human sequence and Sec34p. Within this region the two proteins are 25% identical and 50% similar. Since the 86-

amino acid region 5 to this homologous region does not display a high degree of similarity to Sec34p, the protein encoded by these ESTs may not be a true Sec34p homolog, but instead may contain a Sec34p-like domain.

The SEC34 Deletion Strain Displays a Severe Growth Defect

To evaluate the phenotype of a strain lacking SEC34, we constructed a diploid strain in which one allele of SEC34 had been deleted (as diagrammed in Fig. 1 e) and replaced with the gene LEU2. This strain (sec34::LEU2/SEC34) was sporulated and dissected, and the resulting tetrads were incubated on rich media at 30°C. As shown in Fig. 2 (left), a clear 2:2 segregation pattern was observed in which each tetrad contained two large and two very small colonies. The large colonies were without exception Leu+ and thus contained the wild-type copy of the gene, while the small colonies were Leu−, indicating the presence of the sec34Δ locus. Therefore, although SEC34 is not an essential gene, haploid strains lacking SEC34 are at an extreme growth disadvantage. The growth defect of the sec34Δ strain is complemented by a plasmid bearing SEC34 since the presence of the construct in the diploid sec34Δ/SEC34 strain resulted in the restoration of a 4:0 segregation pattern (Fig. 2, right). In each tetrad, two segregants were Leu+ and Ura+, indicating the presence of both the deletion and the plasmid bearing SEC34, respectively.

Genetic Analysis of SEC34

Previous analysis of the sec34 mutant strain indicated a role for Sec34p in the docking or fusion of ER-derived vesicles with the cis-Golgi complex (Wuestehube et al., 1996). By testing whether SEC34 displayed genetic interactions with other factors required at this stage, as well as at other stages of transport, we sought to confirm this assignment and to further explore the function of Sec34p. We thus tested whether the temperature-sensitive growth defect of the sec34-2 strain could be suppressed by overexpression of other genes required for transport. Two of the best suppressors were found to encode the rab protein Ypt1p and the dominant allele of the t-SNARE-associated protein, SLY1, termed SLY1-20 (Fig. 3). Although neither gene could restore growth to the levels observed when the strain was complemented by SEC34 on a plasmid, both conferred a significant growth advantage to the mutant strain. A second gene, USO1, which encodes a tethering factor, also suppressed the temperature sensitivity, but to a lesser extent than either YPT1 or SLY1-20. Weak suppression was also observed upon overexpression of the v-SNAREs Sec22p, Bet1p, and Ykt6p, each of which has been implicated in the ER to Golgi complex stage of transport; no suppression was seen for another v-SNARE involved at this step, Bos1p (Fig. 3).

Several other genes were tested for their ability to suppress the sec34-2 mutation when overexpressed, but were found to have no effect. These genes encode the tethering factor Sec35p, the TRAPP complex component Bet3p, the cis-Golgi complex t-SNARE Sed5p, and the Golgi complex to plasma membrane rab protein Sec4p. The lack of suppression by the Golgi complex to plasma membrane rab indicates that suppression by the ER to Golgi complex rab is specific.

We also tested whether overexpression of SEC34 could suppress temperature-sensitive mutant alleles of several ER to Golgi complex docking factors. Interestingly, overexpression of SEC34 was capable of weakly suppressing the temperature-sensitive growth defect of the sec35-1 strain (data not shown). However, multicopy SEC34 was unable to suppress mutant alleles of all other secretory factors tested, including: the v-SNAREs Sec22p, Bos1p, and Bet1p; the t-SNARE Sed5p; the tethering factor Uso1p; the rab Ypt1p; and the TRAPP complex component Bet3p (data not shown). Therefore, overexpression of either SEC34 or SEC35 (Van Rheenen et al., 1998) is unable to suppress the temperature-sensitive growth defects of the majority of tethering and docking factors, yet multicopy SEC34 can improve the growth defect of a compromised allele of SEC35, and thus the two genes show a genetic interaction.

Since YPT1 and SLY1-20 were efficient suppressors of the temperature-sensitive growth defect of the sec34-2
strains, we explored whether they could also improve the severe growth defect of the sec34Δ strain. The sec34Δ/SEC34 diploid strain was therefore transformed with plasmids expressing either multicopy YPT1 or low-copy SLY1-20, and the resulting diploids were sporulated and subjected to tetrad analysis. Indeed, the presence of either plasmid significantly improved the growth of the sec34Δ strain (Fig. 2, right). This result implies that Ypt1p and Sly1p most likely function downstream of, or in a parallel pathway with, Sec34p.

Isolation of RUD3 and its Genetic Interactions with SEC34 and USO1

Suppression of the sec34-2 temperature-sensitive growth defect was also observed upon overexpression of a gene designated RUD3 (Fig. 3). RUD3 was isolated previously in our lab through a screen for genes that, when overexpressed, are able to suppress the temperature-sensitive growth defect of the uso1-1 strain (Sapperstein et al., 1996; Sapperstein, 1997). From a panel of multicopy suppressors, four library plasmids were isolated that contained overlapping regions of chromosome XV that include two hypothetical ORFs and one known gene. The suppressing activity was localized to the hypothetical ORF YOR216c, and this gene was named RUD3 because it relieves the uso1-1 transport defect. RUD3 is an efficient suppressor of the uso1-1 deficiency at 37.5°C, suppressing the growth defect (Fig. 4a), as well as the ER to Golgi transport block, as monitored through movement of the marker protein, carboxypeptidase Y (data not shown).

RUD3 is predicted to encode a 484-amino acid protein with a molecular mass of 56.1 kD and a pI of 4.67 (Fig. 4b). Like Sec34p, this protein lacks any motifs to indicate a localization other than cytosolic. Both the PAIRCOIL and COILS programs (Lupas et al., 1991; Berger et al., 1995) indicate a high probability that the protein assumes a coiled-coil secondary structure in a central, ~215-amino acid stretch (Fig. 4, b and c). There is a region near the center of this coiled-coil domain, however, that has a decreased coiled-coil probability; this region could potentially be a hinge in the protein (Lupas, 1996). BLAST searches revealed a single putative homolog of Rud3p in the genome of S. pombe. This 401-amino acid protein (GenBank/EMBL/DDBJ accession number A022117, P1D g2959373) shares 28% identity and 41% similarity with Rud3p, and thus encodes a Rud3p ortholog that we designate spRud3p. Interestingly, spRud3p also contains a region predicted to form a coiled-coil secondary structure, although the homology between the proteins is not restricted to this motif. Indeed, the COOH termini of the proteins (ranging from amino acid 365 to 484 for Rud3p, and 280 to 401 for spRud3p) are most homologous, displaying 49% identity and 62% similarity.

To test whether Rud3p is encoded by an essential gene, a diploid strain was created in which one of the alleles of RUD3 was deleted and marked with LEU2. Sporulation and tetrad dissection of this strain yielded tetrads with four viable spores, two of which were Leu'. Sporulation at 25, 30, and 37°C, the haploid rud3Δ strain did not display a significant growth defect as compared with a wild-type strain (data not shown), and thus RUD3 is not an essential gene.

Sec34p Is a Peripheral Membrane Protein

To analyze the Sec34 protein, we generated affinity-purified anti-Sec34p antibody. The antibody recognizes two proteins in crude yeast extracts, the larger of which corresponds to the predicted molecular weight of Sec34p (Fig. 5 a). This protein is absent in a sec34Δ strain (which expresses SLY1-20 to enhance its propagation) and is overexpressed in a strain containing SEC34 on a multicopy plasmid, and thus represents Sec34p. The smaller protein, which is recognized despite the affinity purification, is not related to the Sec34p locus since it is present in the sec34Δ strain and its expression level is unaffected by overexpression of SEC34.

With this antibody, we investigated whether Sec34p was capable of associating with membranes, as might be expected for a protein involved in secretion. A crude yeast extract (designated S1) was centrifuged at 175,000 g to separate the organelles of the secretory pathway, which are found in the pellet fraction, from cytosolic proteins,
which are contained in the supernatant fraction. As shown in Fig. 5 b (left), the majority of Sec34p is found in the pellet fraction, along with the integral membrane protein Sed5p, while a small amount of Sec34p is contained in the supernatant, as is the cytosolic marker, PGK. This result is consistent with a peripheral membrane association for Sec34p. To explore the basis for the sedimentation of Sec34p, we attempted to extract the protein from an enriched membrane fraction using buffers containing Triton X-100, NaCl, or Na$_2$CO$_3$, pH 11.5. As expected for an integral membrane protein, Sed5p was released into the supernatant fraction after incubation with buffer containing Triton X-100, but not after treatment with salt or high pH, whereas the peripheral membrane protein Sec35p was released, at least partially, upon incubation with all three buffers. Sec34p was partially shifted into the supernatant fraction upon treatment with Triton X-100, salt, or high pH, and thus behaves as a peripheral membrane protein (Fig. 5 c).

To further analyze the membrane association of Sec34p, differential centrifugation was employed. The S1 fraction was centrifuged at 10,000 g, separated into supernatant (S10) and pellet (P10) fractions, and the S10 fraction was
Sec34p Is Required for Tethering of ER-derived Vesicles to the cis-Golgi Complex

Because the mutant phenotype (Wuestehube et al., 1996) and suppression profile of sec34-2 is similar to that of other factors required for the tethering stage of ER to Golgi complex transport, we investigated whether Sec34p was required for tethering as well. We thus employed two in vitro assays that, together, are able to distinguish the stages of budding, tethering, and fusion in ER to Golgi complex transport (Barlowe, 1997; Cao et al., 1998).

In the first assay (Fig. 6 a), overall ER to Golgi complex transport is measured in semi-intact cells incubated with a mixture of purified protein components that drive all the stages of transport: vesicle formation from the ER is supported by the addition of COPII proteins, efficient vesicle tethering requires added Uso1p, and vesicle fusion requires a protein complex termed LMA1. Productive transport is monitored by following the addition of 1,6-mannose residues to gp-α-factor, an event that occurs in the cis-Golgi complex. Generation of this transport system from conditional mutants has shown that the system also requires the activities of several peripheral and integral membrane proteins including Ypt1p, Sec35p, and the SNAREs (Cao et al., 1998; VaRheenen et al., 1998). Although there is no requirement for exogenous Sec34p in the system, Sec34p may be supplied to the assay by peripheral association with membranes of the semi-intact cells. Therefore, to test for a requirement for Sec34p, we generated the in vitro system from the sec34-2 strain. In wild-type or sec34-2 mutant cells at 23°C, movement of gp-α-factor from the ER to the Golgi complex in this in vitro system proceeds with a similar efficiency (Fig. 6 a). In contrast, at 29°C in sec34-2 semi-intact cells, this process is very inefficient relative to wild-type, indicating that sec34-2 is defective for overall ER to Golgi transport in vitro.

The second assay we employed examined the functionality of the vesicle budding and vesicle tethering steps in the sec34-2 mutant (Fig. 6 b). In this assay, release of vesicles from semi-intact cells is detected by the appearance of protease-protected gp-α-factor in a low-speed supernatant at the end of the reaction. Vesicles were efficiently generated upon addition of COPII components to wild-type or sec34-2 semi-intact cells at 23 or 29°C (Fig. 6 b). These data indicate that Sec34p is not required for ER-derived vesicle budding. Because addition of Uso1p significantly reduced vesicle release, vesicle tethering was also functional in the semi-intact wild-type cells at 23 or 29°C, as well as in sec34-2 semi-intact cells at 23°C. In contrast, when Uso1p was added to the sec34-2-derived system at the restrictive temperature of 29°C, vesicle release was only slightly diminished (Fig. 6 b). This result indicates that the sec34-2 mutant cannot efficiently tether ER-derived vesicles to the yeast Golgi complex.

sec34 and sec35 Display a Synthetic Lethal Interaction

The similar genetic interactions of SEC34 and SEC35 with genes involved in the docking stage of vesicular transport, taken together with their genetic interaction with one another, and with the finding that both proteins function in
vesicle tethering, lead us to examine whether mutations in the two genes would display a synthetic lethal interaction. To do this, we generated a diploid strain heterozygous for both the sec34-2 and sec35-1 alleles and subjected it to tetrad analysis. Although both the sec34-2 and sec35-1 haploid strains are permissive for growth at both 21 and 30°C, tetrads from the diploid sec34-2/SEC34 SEC35/sec35-1 strain yielded numerous inviable colonies at either temperature (Fig. 7 a). After incubation of the segregants for long periods of time, a small proportion of those previously characterized as inviable would form visible microcolonies. Since this phenotype was variable, we hypothesize that the microcolonies result from either the appearance of spontaneous suppressors of the inviability or from background mutations in the strain. Examination of the viable segregants in each tetrad for temperature sensitivity revealed a pattern in which the inviable segregants are predicted to be those containing both the sec34-2 and the sec35-1 alleles. To confirm this prediction, the diploid strain was transformed with low-copy plasmids bearing either SEC34 or SEC35 before tetrad dissection. The presence of either plasmid lead to a greater proportion of viable segregants than was observed for the untransformed strain, concurrent with the appearance of segregants that were sensitive to the drug 5-fluoro-orotic acid, which is toxic to cells that must maintain the plasmid to survive (data not shown). Therefore, the sec34-2 and sec35-1 alleles display a synthetic lethal phenotype, which can be complemented by the presence of either gene on a plasmid.

Sec34p and Sec35p Interact in the Two-hybrid Assay

In many cases, synthetic lethality between alleles of two genes involved in secretion indicates that their gene products are involved in the same stage of secretion (Aisen and Schekman, 1990). Furthermore, such genetic interactions can also be indicative of a physical interaction of the proteins encoded by those genes, as is the case for mutations in the α and β subunits of tubulin (Huffaker et al., 1987). Therefore, we investigated whether Sec34p and Sec35p physically interact using the two-hybrid system (Fields and Song, 1989). A strain in which transcription of both the ADE2 and HIS3 genes is under the control of the Gal4p transcriptional activator (James et al., 1996) was transformed with plasmids expressing either the Gal4p-BD or Gal4p-AD, either alone or fused to Sec34p or Sec35p. A cistron of transcription of the ADE2 and HIS3 genes was assessed by the ability of the strain to grow on media lacking adenine or histidine, respectively. A usual example of a positive two-hybrid interaction, coexpression of p53 fused to the Gal4p-BD and the large T-antigen fused to the Gal4p-AD was demonstrated to activate both reporter genes (Fig. 7 b), as has been described previously (Iwabuchi et al., 1993). Strains expressing either the Sec34p-Gal4p-BD or the Sec35p-Gal4p-AD fusion protein were unable to grow on either media. However, when Sec34p-Gal4p-BD was expressed along with the Sec35p-Gal4p-A D, the strain grew well on media lacking either adenine or histidine (Fig. 7 b), indicating that the interaction of Sec34p with Sec35p was able to localize the Gal4p-A D to the promoters of these genes, activating transcription. When the converse experiment was completed, in which Sec35p was fused to the Gal4p-BD and Sec34p was fused to the Gal4p-AD, expression of the ADE2 and HIS3 genes was also observed only when both fusion proteins were expressed. Thus, Sec34p and Sec35p interact.

Sec34p and Sec35p Are Components of a Large Protein Complex

To characterize the interaction of Sec34p and Sec35p further, we employed immunoblotting to monitor the behavior of these proteins during fractionation of yeast cytosol. Sec34p and Sec35p coprecipitated in 35% saturated ammonium sulfate and cofractionated precisely by DEAE anion exchange chromatography (data not shown). A aliquot of the Sec34p/Sec35p anion exchange pool was then subjected to size exclusion chromatography on Superose 6 (Fig. 8 a), and once again, Sec34p and Sec35p precisely cofractionate. Interestingly, they elute from the column slightly before thyroglobulin, a 669-kD globular protein.
fractions 4–7 from the MonoS chromatographic step described, Sec34p and Sec35p cofractionate on ceramic hydroxyapatite. The second gradient elution fraction were analyzed as described in a.

contained in the load (L), flow-through fractions (FT), and every second gradient elution fraction were analyzed as described in a. c, Sec34p and Sec35p cofractionate by MonoS anion exchange chromatography. Fractions 8–11 from the MonoQ step described in b were loaded onto a MonoS anion exchange column and eluted with a linear KCl gradient. Proteins contained in the load (L), flow-through fractions (FT), and every second gradient elution fraction were analyzed as described in a. c, Sec34p and Sec35p cofractionate through sequential chromatographic steps. b, Sec34p and Sec35p cofractionate by MonoQ anion exchange column containing Sec34p and Sec35p were fractionated by size-exclusion chromatography. Proteins contained in the load (L), as well as the column, fractions were separated by SDS-11% PAGE and immunoblotted with antibodies against Sec34p or Sec35p, as indicated. The immunoreactive protein marked with an asterisk (*) is unrelated to Sec34p (see Fig. 5 a). The elution position of 25, 67, 232, and 669 kD protein standards, as well as the location of the void volume, is indicated at the top. b–d, Sec34p and Sec35p cofractionate by size-exclusion chromatography. Proteins contained in the load (L), flow-through fractions (FT), and every second gradient elution fraction were analyzed as described in a. The apparent difference in Sec34p and Sec35p abundance in fractions 10 and 13 is likely an artifact of chemiluminescent detection because reanalysis of these results is consistent with Sec34p and Sec35p existing in a large protein complex with a mass of up to ~750 kD. A small amount of monomeric Sec35p is also evident upon gel filtration, suggesting either that some Sec35p has dissociated from the complex or that a cytosolic pool of monomeric Sec35p exists. No such monomeric Sec35p has been detected in cytosolic fractions. To further purify the Sec34p/Sec35p complex, the remainder of the DEAE anion exchange pool was subjected to several sequential chromatographic steps (see Materials and Methods), including Sephacryl S-300 gel filtration (data not shown), MonoQ anion exchange (Fig. 8 b), MonoS cation exchange (Fig. 8 c), Superose 6 gel filtration (data not shown), and ceramic hydroxyapatite (Fig. 8 d). Once again, Sec34p and Sec35p precisely comigrate through each step, strongly indicating that Sec34p and Sec35p are present in a large protein complex.

**Discussion**

Much effort has been extended towards gaining an understanding of the mechanism of transport vesicle docking in the secretory pathway. Several families of proteins are involved in this event, including the rab family of small GTP-binding proteins and the SNARE family of integral membrane proteins. Recently, another class of proteins has been described, the tethering factors. Although these proteins do not display homology with one another and thus do not define a family, they share a similar function in docking, that of connecting the vesicle to the target compartment before the interaction of v- and t-SNAREs (for reviews see Pfeffer, 1999; Waters and Pfeffer, 1999). The docking event can therefore be separated into two distinct substages, tethering and SNARE-dependent docking.

A recent genetic screen identified temperature-sensitive alleles of two genes, SEC34 and SEC35, that, when incubated at the restrictive temperature, are defective in ER to Golgi complex transport and accumulate large numbers of vesicles (Wuestehube et al., 1996). Mutant alleles of these genes are also able to be suppressed by the dominant allele of SLY1, SLY1-20, a trait shared with all previously characterized ER to Golgi complex tethering factors (Sapp-sterstein et al., 1996; Cao et al., 1998; VanRheenen et al., 1998). Based on these data, we hypothesized that SEC34 and SEC35 might be involved in tethering, and this was demonstrated to be the case for SEC34 by the discovery that this gene both displayed a genetic interaction with genes involved in tethering and is required in this process as revealed by an in vitro assay (VanRheenen et al., 1998). We therefore investigated whether Sec34p functions in tethering as well.

in c were subjected to Superose 6 gel filtration chromatography and the Sec34p- and Sec35p-containing fractions were pooled, loaded onto a ceramic hydroxyapatite column, and eluted with a linear potassium phosphate ($\text{PO}_4^{2-}$) gradient. Proteins in the load (L), flow-through (FT), and every third gradient elution fraction were analyzed as described in a. The apparent difference in Sec34p and Sec35p abundance in fractions 10 and 13 is likely an artifact of chemiluminescent detection because reanalysis of every fraction in this region yields precise comigration and relative abundance (data not shown).
To begin our study of SEC34, we cloned the gene by complementation of the temperature-sensitive phenotype of a strain bearing the sec34-2 mutation. SEC34 was discovered to be a novel gene encoding a protein with a predicted molecular weight of 93 kD. Deletion of SEC34 in a haploid strain resulted in a severe growth defect, and thus SEC34 is essential for wild-type growth rates, although not for viability.

To investigate the genetic interactions of SEC34 we employed multicy copy suppressor analysis. The best suppression of the sec34-2 temperature-sensitive growth defect was conferred by overexpression of YPT1, the rab required in ER to Golgi complex transport, or by expression of SLY1-20p, the dominant form of the t-SNARE-associated factor, SLY1p. Suppression of the SEC34 deletion strain allowed us to order the action of SEC34 with respect to YPT1 and SLY1p. Since either YPT1 or SLY1-20p can suppress both mutations in, and a deletion of, SEC34, yet overexpression of SEC34 cannot suppress mutations in either YPT1 or SLY1, we hypothesize that YPT1 and SLY1 function downstream of SEC34.

Weaker suppression of the sec34-2 mutation was observed upon overexpression of the tethering factor USO1p, or the v-SNAREs SEC22p, BET1p, or YKT6p. The suppression of sec34-2 by the v-SNAREs may be through mass action, in which vesicles containing supernumerary v-SNAREs are able to compensate for a deficiency in tethering, albeit with a very low efficiency. This phenomenon has been observed previously for mutations in the tethering factors USO1p (Sapperstein et al., 1996) and SEC35p (VanRheenen et al., 1998), as well as components of the putative tethering complex TRAPP (Jiang et al., 1998). Interestingly, no suppression of the sec34-2 mutation was observed upon overexpression of either the v-SNAREs BOS1p or the cis-Golgi complex t-SNARE SED5p. Overexpression of BOS1p was also unable to suppress a temperature-sensitive growth defect of the sec35-1 strain (VanRheenen et al., 1998) or the inviability of theuso1Δ strain (Sapperstein et al., 1996). This lack of suppression could result from inefficient expression of the gene or may indicate a functional difference between BOS1p and the other v-SNAREs that mediate the ER to Golgi complex transport step. The lack of suppression of the sec34-2 mutant strain by high-copy expression of SED5p was unexpected, because it has been demonstrated that overexpression of this t-SNARE is toxic to cells (Hardwick and Pelham, 1992).

Biochemical analysis of the SEC34 protein reveals that it is a peripheral membrane protein. Although a small amount of the protein is soluble, the remaining portions between the P10 and P175 fractions, similar to the Golgi protein SED5p; it is possible, therefore, that SEC34p is associated with the Golgi complex. Due to the association of SEC34p with membranes, we used semi-intact cells made from the sec34-2 strain to test the requirement for SEC34p in tethering through an assay that reconstitutes ER to Golgi complex transport. These semi-intact cells were demonstrated to be able to bud vesicles from the ER, but these vesicles failed to efficiently tether to the Golgi complex at the restrictive temperature, indicating that SEC34p is required for the tethering of ER-derived vesicles to the cis-Golgi complex. Since cytosolic proteins are removed from the sec34-2 semi-intact cells, the membrane-associated pool of SEC34p is most likely the source of the tethering defect. In addition, since the membranes involved in tethering are restricted to those of the vesicle and the cis-Golgi complex, SEC34p is most likely associating with one, or both, of these membranes.

SEC34 was found to display two interesting genetic interactions with the tethering factor gene SEC35. First, multicy copy SEC34 weakly suppresses a temperature-sensitive allele of SEC35. Since overexpression of SEC34 cannot suppress the cold-sensitive lethality of the sec35Δ strain, SEC34p is able to assist a handicapped allele of SEC35, but cannot replace its function. Second, the sec34-2 and sec35-1 alleles display a synthetic lethal interaction. A lethal strain bearing either allele alone are permissive for growth at 23 and 30°C, a haploid strain containing both the sec34-2 and sec35-1 alleles is inviable at either temperature. This synthetic phenotype is more severe than the conditional synthetic lethality of the sec35-1 allele in combination with a mutant allele of either YPT1 or USO1, in which the double mutants are viable at 23°C, but not at 30°C (VanRheenen et al., 1998). This finding suggests a close functional interaction of the SEC34 and SEC35 proteins.

Based on these results, we investigated whether SEC34p and SEC35p could physically interact through the two-hybrid assay. Indeed, SEC34p and SEC35p were found to interact. The interaction between the two proteins may explain the ability of multicy copy SEC34 to suppress the sec35-1 allele, but not the sec35Δ allele: increased levels of SEC34p could stabilize a defective form of SEC35p but would be ineffectual in the absence of SEC35p, especially if the interaction of the two proteins is essential to their function in tethering. To further explore the interaction of SEC34p and SEC35p we examined the behavior of the soluble pool of these proteins through several chromatographic steps. The proteins cofractionated through ammonium sulfate precipitation and anion exchange, cation exchange, ceramic hydroxyapatite, and size exclusion chromatographic steps, providing strong evidence that the two proteins are in a complex with one another. Intriguingly, the SEC34p/SEC35p complex appears quite large, with an estimated molecular weight (if globular) of ~750 kD. This size, which is larger than the combined molecular weights of the two proteins (124 kD), suggests several possibilities for the structure of the complex. First, the complex could be homodimeric, containing one molecule of each protein, but highly elongated such that it migrates rapidly through a size exclusion column. We consider this unlikely because the sequences of SEC34p and SEC35p lack motifs (such as coiled-coil domains) that would be indicative of an elongated structure. Second, the complex could contain two or more molecules of at least one protein, resulting in a more massive structure. Finally, the complex could be multimeric, containing heretofore unidentified component(s) in addition to SEC34p and SEC35p. We are currently purifying the SEC34p/SEC35p complex to address this issue and identify any additional components. It appears, however, that USO1p is unlikely to be a component of the SEC34p/SEC35p complex since immunoblotting fractions from the purification with an antibody against this protein revealed that USO1p did not comigrate with SEC34p and SEC35p (data not shown).
The 750-kD complex containing Sec34p and Sec35p is reminiscent of the TRAPP complex, which migrates at 800 kD by size exclusion chromatography (Sacher et al., 1998). However, two pieces of data indicate that the TRAPP complex is distinct from the Sec34p/Sec35p complex. First, the identities of the low molecular weight members of the TRAPP complex have been elucidated, and none corresponds to Sec35p, whose mobility on SDS-PAGE was within the range of the proteins that have been sequenced (Sacher et al., 1998). In addition, the known members of the TRAPP complex display genetic interactions with one another (Jiang et al., 1998; Sacher et al., 1998), yet no interaction was discerned between the gene encoding the TRAPP component Bet3p and either sec34 or sec35 (this work and VanRheenen et al., 1998).

Since many secretory factors are evolutionarily conserved, we explored whether the components of the Sec34p/Sec35p complex were conserved in higher eukaryotes. The genome of the nematode C. elegans was discovered to contain a protein designated Y71F9A 290.A that is very similar to Sec34p. However, the C. elegans protein is 50% the size of Sec34p and therefore may not be a true ortholog. We also discovered a C. elegans protein with moderate homology to Sec35p (22% identical and 33% similar), designated C35A5.6. While the similarity is not high, the proteins are similar in size (C35A5.6 is comprised of 273 amino acid residues, whereas Sec35p is comprised of 275 amino acid residues), and thus, this C. elegans protein is a putative ortholog of Sec35p. Searches of GenBank for additional homologs of these proteins did not reveal additional Sec35p homologs, but several human ESTs were discovered with a high degree of similarity to Sec34p. Interestingly, the sequences contained on these ESTs were homologous to Sec34p over only a portion of the analyzed region of the putative human protein, and thus the protein may contain a Sec34p-like domain and may not be a true Sec34p ortholog. These data indicate that there may be orthologs of the Sec34p/Sec35p complex in higher organisms, but functional experiments will be required to unambiguously address this point. Finally, a putative ortholog of Sec34p was discovered in the genome of S. pombe. No paralogs of either Sec34p or Sec35p exist in S. cerevisiae, and thus these proteins do not define a family of related proteins.

Finally, we describe the identification and characterization of a gene designated RUD3 that displays a genetic interaction with SE C34. RUD3, which encodes a novel non-essential protein with a predicted molecular weight of 56 kD, was originally identified in a screen for multicopy suppressors of a temperature-sensitive allele of the tethering factor, USO1 (Sapp et al., 1996; Sappet et al., 1997), and is also able to suppress the temperature-sensitive growth defect of the sec34-2 strain. Interestingly, RUD3 is unable to suppress mutations in other ER to Golgi complex defects such as Sec35p, Ypt1p, Sec22p, Bet1p, and Bos1p, and thus the suppression is specific to mutant alleles of SE C34 and USO1. Overexpression of RUD3 can weakly suppress the viability of the uso1Δ strain (data not shown). Taken together, these data suggest that RUD3 either acts at, or downstream of, the tethering stage of ER to Golgi complex transport. RUD3 does not appear to be a component of the Sec34p/Sec35p complex from the majority of the protein fractionates away from the complex during its purification (data not shown).

In summary, we characterize the formation of a novel secretory factor, Sec34p, and its role in tethering of ER-derived vesicles to the cis-Golgi complex. Unlike the SNAREs and rabs, the tethering factors described thus far at different intracellular transport steps are not members of a protein family. Nevertheless, they do share structural similarity, since they are either elongated or present in a large multimeric complex (Pfeffer, 1999; Waters and Pfeffer, 1999). The large size may be related to the requirement for the tethering factors to span the distance between the vesicle and the target compartment, before trans-SNARE complex formation. Interestingly, three factors meet this criteria in the yeast ER to Golgi complex transport step: the extended homodimer Uso1p, the TRAPP complex, and the Sec34p/Sec35p complex. It will be very exciting to discover in the future how these large protein complexes function to secure a vesicle to its target membrane, and whether their function is more complex than simply connecting vesicle and target membranes.

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