Sec8p and Sec15p Are Components of a Plasma Membrane-associated 19.5S Particle That May Function Downstream of Sec4p to Control Exocytosis

Robert Bowser, Heike Müller, Brinda Govindan, and Peter Novick
Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. The SEC8 and SEC15 genes are essential for exocytosis in the yeast Saccharomyces cerevisiae and exhibit strong genetic interactions with SEC4, a gene of the ras superfamily. The SEC8 gene encodes a hydrophilic protein of 122 kD, while the temperature-sensitive sec8-9 allele encodes a protein prematurely truncated at 82 kD by an opal stop codon. The Sec8p sequence contains a 202 amino acid region that is 25% identical to the leucine rich domain of yeast adenylate cyclase that has been implicated in ras responsiveness. Fractionation, stability, and cross-linking studies indicate that Sec8p is a component of a 19.5S particle that also contains Sec15p. This particle is found both in the cytosol and peripherally associated with the plasma membrane, but it is not associated with secretory vesicles. Gel filtration studies suggest that a portion of Sec4p is in association with the Sec8p/Sec15p particle. We propose that this particle may function as a downstream effector of Sec4p, serving to direct the fusion of secretory vesicles with the plasma membrane.

The transfer of proteins from the ER, through the various subcompartments of the Golgi apparatus to the cell surface, is mediated by carrier vesicles which faithfully ferry the transported proteins from one organelle to the next along the secretory pathway. Since there are a number of organelles which can serve as vesicle donors and/or acceptors the fidelity of vesicular transport must be tightly regulated to achieve this orderly flow. Genetic and biochemical approaches have been used to identify components that function to regulate transport at each step of the secretory pathway (Rothman and Orci, 1992). In the yeast Saccharomyces cerevisiae genetic selections have defined a large number of gene products that function within the secretory pathway (Nakajima et al., 1991; Newman and Ferro-Novick, 1987; Newman et al., 1990; Novick et al., 1980). A set of 10 SEC genes have been identified whose products function in transport from the Golgi apparatus to the plasma membrane (Novick et al., 1981).

One of the late-acting SEC gene products, Sec4p, is a GTP-binding protein of the ras superfamily (Salminen and Novick, 1987) that is associated with both the cytoplasmic surface of secretory vesicles and the plasma membrane (Goud et al., 1988). A small soluble pool of Sec4p also is present in yeast. The cycle of GTP binding and hydrolysis by Sec4p is thought to be coupled to a cycle of localization in which Sec4p first associates with the membrane of secretory vesicles, exocytotic fusion then brings Sec4p to the plasma membrane and dissociation of Sec4p from the plasma membrane allows recycling (Bourne, 1988; Walworth et al., 1989a). This cycle may function to regulate the binding and fusion of secretory vesicles with the cell surface. By analogy with other GTP-binding proteins it is thought that the GTP-bound form of Sec4p interacts with an effector, thereby stimulating its activity and leading to exocytotic fusion.

The yeast adenylate cyclase protein is the first identified downstream effector of a GTP-binding protein of the ras superfamily. In yeast, Raslp and Ras2p function through activation of adenylate cyclase to regulate the intracellular level of cAMP, which in turn controls cell growth (Matsumoto et al., 1985; Mitts et al., 1990). Adenylate cyclase behaves as a large particle and has been shown to associate with a 70-kD protein encoded by CAP and the yeast GTPase-activating protein Iralp (Field et al., 1988; Mitts et al. 1991). A leucine-rich repeat domain of adenylate cyclase has been shown through in vitro mutagenesis to be required for Ras responsiveness (Colicelli et al., 1990; Field et al., 1990) and may define a site of direct physical interaction with Ras.

The Sec4p effector is as yet unknown. However, the SEC4 gene has been shown to genetically interact with a subset of the other late-acting SEC genes. Double mutants combining sec4-8 and sec2-41, sec5-24, sec8-9, sec10-2, or sec15-1 are lethal under conditions which are permissive to any of the single mutants (Salminen and Novick, 1987). The same set of mutants are partially suppressed by duplication of SEC4. The products of these genes therefore are candidates for proteins that interact with Sec4p and one or more of these proteins may define the Sec4p effector.

The SEC15 gene has been sequenced and its product characterized as a 115-kD protein that is located both in a
high molecular weight, soluble particle and peripherally associated with the plasma membrane (Bowser and Novick, 1991). Overproduction of Sec15p results in impaired growth, accumulation of aggregated vesicles, and a patch of Sec15p seen by immunofluorescence (Salminen and Novick, 1989). The appearance of this patch of Sec15p upon overexpression is dependent on the function of Sec2p and Sec4p. These data suggested that Sec15p may act downstream of both Sec2p and Sec4p, possibly to dock secretory vesicles to the plasma membrane before fusion. The subcellular distribution of Sec15p is influenced by Sec8p function, as there is an apparent increase in the fraction of Sec15p found on the plasma membrane in sec8-9 mutant extracts (Bowser and Novick, 1991). This evidence suggested that Sec8p and Sec15p may interact.

In this paper we report the sequence of the SEC8 gene, and the characterization of the Sec8 protein. SEC8 encodes a hydrophilic protein of 122 kD that is associated with Sec15p both on the plasma membrane and in a 19.5S soluble complex. The Sec8p sequence contains a region that shares similarity with the domain of adenylate cyclase necessary for Ras responsiveness. By analogy, this suggests that Sec8p may respond to Sec4p. A portion of the soluble pool of Sec8p is found to co-elute with the Sec8p/Sec15p complex upon gel filtration. This complex may function as the downstream effector of Sec4p to regulate the binding and fusion of secretory vesicles with the plasma membrane.

**Materials and Methods**

**Yeast Genetics**

*S. cerevisiae* strains used in this study are listed in Table I. Cells were grown in YP medium containing 1% Bacto-yeast extract, 2% Bacto-peptone (Difco Laboratories, Detroit, MI), and 2% glucose, or in minimal medium YP medium containing 1% Bacto-yeast extract, 2% Bacto-peptone (Difco Laboratories, Detroit, MI) and 2% glucose, supplemented for auxotrophic requirements when necessary.

Yeast transformation was generally performed by alkali cation treatment

| Strain | Genotype |
|--------|----------|
| NY13   | MaTa, ura2-53 |
| NY15   | MaTa, his4-619, ura3-52 |
| NY17   | MaTa, ura3-52, sec-6-4 |
| NY64   | MaTa, ura3-52, sec15-1 |
| NY410  | MaTa, ura3-52, sec-8-9 |
| NY411  | MaTa, his4-619, sec-8-9 |
| NY580  | MaTa, leu2-3,112, ura3-52, pep4::URA3 |
| NY813  | MaTa, leu2-3,112, leu2-3,112, ura3-52, ura3-52, SEC8/sec8::pNB338 (SEC8 disruption) |
| NY862  | MaTa, NY13 + pNB328 (Sec8 overproduced on YEYp24) |
| NY863  | MaTa, NY13 + pNB329 (Sec8 on YCP50) |
| NY864  | MaTa, NY410 + pNB329 (Sec8 on YCP50 in sec8-9 background) |
| NY905  | NY410 + pNB305 (opal tRNA suppressor) |
| NY906  | NY410 + pNB306 (amber tRNA suppressor) |
| NY907  | NY410 + pNB307 (ochre tRNA suppressor) |
| NY910  | NY410 + pNB139 (SEC4 on YCP50) |
| NY911  | NY410 + pNB446 (sec8 BglII truncation) |
| NY912  | NY410 + pNB447 (sec8 PatI truncation) |
| NY913  | NY410 + pNB448 (sec8 NsiI truncation) |

**Nucleic Acid Techniques**

E. coli strain DH1 (F-, recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1) was used for all cloning experiments. For production of fusion proteins to produce Sec8p antibodies, the SEC8 gene was first transformed into BU1255 cells (dam-) and further subcloned in DH5α cells (supE44, d4αU169 [p80lacZAM15], hsdR17, recA1, endA1, gyrA96, thi-1, relA1) as described below.

Plasmids used are listed in Table II. Plasmid pNB328 was isolated from a yeast genomic DNA library in the episomal vector YEYp24 (Carlson and Botstein, 1982). pNB329, a centromere based plasmid containing SEC8, was constructed by ligating the gel purified 4.4-kb Smal–SalI fragment from pNB328 into YCP50 as follows. YCP50 DNA was first digested with BamHI, filled in with Klenow, phosphatase treated, phenol extracted, and precipitated. This linear blunt-end DNA was then further digested with Sall and ligated to a gel purified 4.4-kb Smal–Sall fragment from pNB328. To construct pNB330, Ylp5 DNA was digested with BamHI and Sall as described above for YCP50 and ligated with the 4.4-kb Smal–Sall fragment from pNB328.

For disruption of the SEC8 gene, plasmid pNB338 was constructed by purification of a 1150-bp EcoRI–EcoRV fragment from pNB330 and ligation into SspI–EcoRI-digested Ylp5. DH1 cells were transformed with the ligation mix and transformants selected on LB containing tetracycline plates. Plasmids were isolated and checked for the proper insertion. pNB338 was then digested with XbaI and used to transform the diploid yeast strain NY648 to disrupt one copy of the SEC8 gene. Transformants were selected on SD plates containing leucine at 25°C, single colony purified, and sporulated. The resulting tetrad was dissected and viable spores scored for the presence or absence of the Ura + marker.

**Production of Sec8 Antibody**

A fusion between trpE and SEC8 was constructed using the pATH11 vector. A 0.74-kb EcoRI–XbaI fragment from pNB330 was ligated into EcoRI–XbaI-digested pATH11. To do this BU1255 cells were transformed with pNB330 to avoid methylation of the XbaI site within SEC8. Plasmid isolated from this transformant was then further digested with EcoRI–XbaI, gel purified, and ligated into gel purified EcoRI–XbaI-digested pATH11 to produce pNB356. This ligation was transformed into DH5α cells. pNB356 encodes a TrpE–Sec8 fusion protein of 63 kD. Fusion protein induction and isolation was performed as previously described (Salminen and Novick, 1989), and rabbit polyclonal antibodies produced from gel purified fusion protein (Pocono Rabbit Farm, Canadensis, PA). Antiserum was collected after multiple secondary injections. Affinity purification of the antibodies was performed as previously described (Goud et al., 1988).

**DNA Sequencing and Analysis**

Nucleotide sequencing was performed by the standard dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical, Cleveland, OH). The samples were electrophoresed on 8% acrylamide gels containing 8 M urea. Single stranded template DNA was obtained by subcloning the BamH1–SalI fragment of pNB350 into BlueScript (Stratagene, La Jolla, CA) and both strands were sequenced.

The predicted protein sequence was compared with the National Biomedical Research Foundation GenBank database, release number 580, June 1992.
1991, with the FASTA program, which performs a Pearson and Lipman homology search (Pearson and Lipman, 1988). The GenBank accession number for the SEC8 sequence is X64693, located in the EMBL data library.

**Cell Fractionation and Sec8p Localization**

NY13 cells were used for characterization and localization of Sec8p in wild-type cells. Cell lysates were prepared and used for Sephacryl S-500 gel filtration chromatography, sucrose velocity gradients, and sucrose density gradient as previously described (Bowser and Novick, 1991). Sec8p localization was determined by immunoblot analysis using 8% polyacrylamide gels. Proteins were transferred onto nitrocellulose (BA 83, 0.22 μm; Schleicher & Schuell, Inc., Keene, NH) and Sec8p labeled with affinity purified antiserum at 1/1,000 dilution for 2 h, washed, and subsequently labeled with 125I-Protein A (0.5 μCi/ml; Amersham Corp., Arlington Heights, IL) secondary antibody for 1.5 h. Individual bands were excised from the nitrocellulose and 125I-Protein A quantitated on a gamma counter. Enzyme assays were performed as previously described (Bowser and Novick, 1991; Walworth et al., 1989b).

**Immunoprecipitation**

NY580 cells ( pep4::URA3) were used for immunoprecipitation studies. 50 A600 U of cells were treated with Zymolyase 100-T for 45 min at 37°C to form spheroplasts. Spheroplasts were then lysed in 300 μl of ice cold lysis buffer (PBS containing 0.1 M sorbitol, 1 mM EDTA, 1 mM MgCl2, 1 mM PMSE and 1/1,000 volume of protease inhibitor cocktail: leupeptin, chymostatin, pepstatin, aprotinin, and antipain, each at 1 mg/ml) by resuspension and repeated pipetting until a homogeneous suspension was achieved. The following steps were performed at 4°C: the lysate was centrifuged at 10,000 g for 10 min and the supernatant removed. For immunoprecipitation from the membrane fraction, the pellet was first washed once in lysis buffer and the final membrane pellet resuspended in lysis buffer at a protein concentration of 20 mg/ml, as determined by Bradford analysis (Bio-Rad Laboratories, Cambridge, MA), and 50 μl was used for immunoprecipitation as follows: to the reaction tube containing 50 μl of supernatant, 0.02 ml (or 0.04 vol) of dithio-bis-(succinimidylpropionate) (DSP) 1, (Pierce Chemical Co., Rockford, IL) was added and the samples incubated at 4°C for 30 min. 25 μl of 0.4 M ammonium acetate was added to the tubes and incubated an additional 10 min to quench the reaction. SDS was added to 1% and the samples incubated at 65°C for 10 min. After cooling on ice, 700 μl of immunoprecipitation buffer (lysis buffer containing 1% TX-100) BSA to 0.1% were added. For immunoprecipitation in the absence of crosslinker the samples were simply diluted with 700 μl of immunoprecipitation buffer. All samples were first preclared by addition of 4 μl of preimmune antiserum and protein A-Sepharose CL4B beads (Sigma Chemical Co., St. Louis, MO), followed by incubation for 30 min and centrifugation in a microcentrifuge for 30 s. The supernatant fraction was transferred to a fresh microcentrifuge tube and boiled with 10 μl of 10% SDS, 50 mM Tris, pH 8.0, 100 mM DTT, 1% BSA, and 1 μg of proteinase K. The reaction was terminated after 15 min at 70°C. The reaction was chilled on ice, mixed with 40 μl of 5X sample buffer, boiled for 5 min and stored at -20°C.

Having localized the smallest complementing region of the SEC8 gene to a 3.34-kb fragment the nucleotide sequence of this region was determined. A single large open reading frame was found that contained without a stop codon to the NsiI site. Therefore DNA sequencing was continued beyond the site and a stop codon 192-bp downstream from the NsiI restriction site was discovered (Fig. 2 a). The 3.34-kb Sau3A-NsiI fragment fully complements the sec8-9 mutation suggests that the extreme carboxy terminus of the Sec8 protein is dispensable. The DNA sequence identifies an open reading frame of 3,197 bp (Fig. 2 a). Upstream of the proposed initiation codon, at position -99, is a TATA sequence element. The open reading frame is terminated by a TGA stop codon, which is followed by six additional termination codons within the next 20 potential codons. This open reading frame predicts a protein of 1,065 amino acids with an estimated molecular mass of 122 kD. The predicted amino acid sequence is hydrophilic and does not contain either an NH2-terminal signal sequence or any potential membrane spanning domains.

1. Abbreviation used in this paper: DSP, dithio-bis-(succinimidylpropionate).
of the truncations account for read through into vector sequence. The molecular weights measured by immunoblot analysis are also listed. The plasmid pNB366 encodes a fusion of TrpE to the indicated region of Sec8. The plasmid pNB338 consists of an integrating vector carrying an internal fragment of SEC8. The restriction enzymes are as follows: S, Sau3A; E, EcoRI; H, HindIII; X, XbaI; EV, EcoRV; B, BglII; P, PstI; N, NsiI. Not all sites for these enzymes are shown. (B) Integration of pNB338 into the SEC8 locus results in disruption of the gene. A partial duplication is formed in which one copy is truncated at the EcoRV site and the second copy lacks a promoter as well as the start of the SEC8 gene.

A search for sequence motifs using the Wisconsin Genetics Computer Group package Motifs program (GCG Inc., Madison, WI) revealed a leucine-rich domain (10 leucine residues in 40 amino acids) containing a possible leucine zipper motif near the NH2 terminus of the protein (Fig. 2a). However, a proline residue is present within this domain which may disrupt the alpha-helical characteristic of leucine zipper binding domains. Comparison of the predicted protein sequence with those in the GenBank database by the GCG FASTA program revealed a 189 amino acid region of Sec8p that shared 21.2% identity with an 185 amino acid domain of the yeast adenylate cyclase protein, encoded by the CYR1 gene. This region of similarity includes the leucine-rich domain of Sec8p and a leucine-rich domain of adenylate cyclase. Comparison of this 185 amino acid leucine-rich domain of adenylate cyclase to the Sec8p sequence using the GCG BESTFIT program revealed a 202 amino acid region of Sec8p with 25.4% identity and 44% overall homology (Fig. 2b). Mutational analysis of adenylate cyclase has demonstrated that this leucine rich region is required for activation by RAS (Colicelli et al., 1990; Field et al., 1990). By analogy, the leucine rich domain of Sec8p may provide a site for protein-protein interaction with the ras-related protein Sec4p.

To determine the significance of this similarity, we shuffled the Sec8p sequence using the BESTFIT program 300 independent times and compared each with the domain of adenylate cyclase. If the similarity is significant, then upon shuffling the percentage of identity should decrease. The results are recorded as a measurement of quality points for each comparison and the overall results reported as the average quality point value ± the standard deviation. The initial quality point value for the comparison of Sec8p and adenylate cyclase is 77.1. After sequence shuffling the average qual-

Table II. Plasmids Used in Study

| Plasmid | Derivation |
|---------|------------|
| pNB328  | YEp24, SEC8, 4.4-kb genomic library insert |
| pNB329  | YCp50, SEC8, 4.4-kb SmaI–Sall fragment from pNB328 into SmaI–Sall-digested YCp50 |
| pNB330  | Ylp5, SEC8, 4.4-kb SmaI–Sall fragment from pNB328 into SmaI–Sall-digested Ylp5 |
| pNB338  | Ylp5, 1.15-kb EcoRI–EcoRV fragment from pNB330 into SspI–EcoRI-digested Ylp5 |
| pNB366  | pATH11, TrpE–SEC8 fusion; 0.7-kb EcoRI–XbaI fragment from pNB330 into EcoRI–XbaI-digested pATH11 |
| pNB446  | YCp50, 2.32-kb Sau3A–BglII fragment from pNB330 into Sau3A–BglII-digested YCp50 |
| pNB447  | YCp50, 2.59-kb Sau3A–PstI fragment from pNB330 into Sau3A–PstI-digested YCp50 |
| pNB448  | YCp50, 3.34-kb Sau3A–NsiI fragment from pNB328 into Sau3A–NsiI-digested YCp50 |
**Table III. Cloned Sequence Integrates at SEC8 Locus**

NY15/PNB330 (MATa his4-619, ura3-52, SEC8::SEC8, URA43) x NY410 (MATa ura3-52, sec8-9)

| Parental diplo | Tetratype | NY410/PNB330 |
|---------------|-----------|--------------|
| 2 Ura"Ts", 1 Ura"Ts", 1 Ura"Ts" | 2 Ura"Ts", 2 Ura"Ts" | 0 Nonparental diplo: 2 Ura"Ts", 2 Ura"Ts" |

The z value of this comparison is 3.6. A z value between 3 and 6 indicates that the similarity is possible (Lipman and Pearson, 1985). Therefore the significance of this similarity must be considered speculative. Since both sequences are rich in leucine residues, even after shuffling the sequences will still contain a disproportionate number of leucine residues that may artificially elevate the randomized quality score. Another criteria to consider is the number of times, among the shuffled comparisons, that the quality score is equal to or greater than the initial quality score (Pearson and Lipman, 1988). In comparisons between 300 shuffled Sec8p sequences and adenylate cyclase, no random quality scores were equal to or greater than the initial quality score.

**SEC8 Encodes an Essential Gene Product**

A null allele of SEC8 was constructed to determine if Sec8p function is required for cell viability. Plasmid pNB338 contains an internal EcoRI–EcoRV fragment from pNB330 in YIp5. This plasmid was cleaved within the SEC8 sequence by digestion with XbaI and used for transformation. Integration of this plasmid into NY648 cells, a diploid homozygous for the ura3-52 mutation, should result in the disruption of one chromosomal copy of SEC8 by generating a duplication in which both copies of SEC8 are truncated (Fig. 1b). Transformants were selected at 25°C on minimal plates containing leucine, and single colonies were sporulated and dissected. All 24 tetrads analyzed yielded two viable spores that were Ura" and two inviable spores. This result implies that this putative null allele of sec8 is a recessive lethal mutation and that the SEC8 gene is an essential locus.

**Double Mutants of sec8-9 and Late-acting sec Mutants Are Lethal**

In a previous report we demonstrated that sec4-8 is lethal in combination with a subset of late-acting sec mutants, including sec8-9 (Salminen and Novick, 1987). To extend this genetic analysis, sec8-9 was crossed with representative alleles of all other sec complementation groups. The diploids were sporulated, tetrads dissected, and germinated at 25°C. In crosses with all of the late-acting sec mutants except sec1-l, lethality was observed in approximately one-fourth of the meiotic products (Table IV). The pattern of inviability indicates that it is the combination of sec mutations that is lethal. Complementation analysis was performed on the viable spores and verified that the inviable spores were double mutants. In the cross with sec1-l, all meiotic products were viable at 25°C, but double mutants were inviable at 30°C, a temperature at which either sec1-l or sec8-9 alone are viable. As in the case of sec4-8, sec8-9 is also lethal in combination with sec9-1 and bet2-1 (Table IV). While the bet2-1 mutant displays an ER-blocked phenotype, the BET2 gene product has been shown to function in the membrane attachment of both Yptlp and Sec4p (Rossi et al., 1991). The lethality seen in double mutants of sec8-9 and bet2-1 may be a consequence of a defect in Sec4p function (Rossi et al., 1991). SEC9 may function at several points in the secretory pathway as the sec9-1 mutant accumulates intermediates indicative of blocks at the ER to Golgi, Golgi, and post-Golgi stages of the pathway (Novick et al., 1981).

Since overexpression of Sec4p suppresses the growth defect of a subset of late-acting sec mutants, including sec8-9, the ability of Sec8p overexpression to suppress the growth defects of late-acting sec mutants was tested. Late-acting sec mutants were transformed with pNB328, the multicopy plasmid containing SEC8, and tested for the ability to grow at 37°C. It was found that overexpression of Sec8p failed to suppress the growth defects of any other late-acting sec mutants.

**Generation of Antiseras Against Sec8p and Identification of the Protein**

To identify and characterize the Sec8p protein, polyclonal antiserum was generated against a TrpE–Sec8 fusion protein. A 0.7-kb EcoRI–XbaI fragment of SEC8 encoding a peptide sequence predicted to be hydrophilic in nature was fused in frame to TrpE in the pATH1 vector. This construct encodes a 63-KD fusion protein that was gel purified and used to immunize rabbits. By immunoblot analysis of yeast cell lysates, this polyclonal antibody (αSec8p) recognizes a protein of ~122 kD (Fig. 3a). To confirm that this protein represents the SEC8 gene product, we transformed NY13 cells with the original high copy number SEC8 clone (pNB328) to overproduce the protein. Immunoblots of lysates from cells containing this plasmid (NY862) showed an amplification of the 122-kD band and the appearance of multiple bands of lower molecular weight, which may reflect degradation products of the overexpressed protein. This demonstrates that the antibody recognizes Sec8p. Analysis of a sec8-9 strain, NY410, revealed that the mutant protein was shifted to a higher mobility and was present at a somewhat reduced level relative to the wild type protein (Fig. 3a, lane 6 vs. lane 2). To determine if the sec8-9 mutation causes a premature termination of the protein, sec8-9 cells were transformed with plasmids carrying various tRNA suppressor genes. Cells containing an opal tRNA suppressor expressed a significant quantity of native molecular weight Sec8p, 122 kD, at 25°C (Fig. 3a, lane 3). The presence of the opal tRNA suppressor also allowed growth of sec8-9 cells and expression of full-length Sec8p at 37°C (Fig. 3a, lane 8). Cells containing the amber and ochre tRNA suppressors failed to synthesize full length protein (Fig. 3a, lanes 4 and 5) and also failed to rescue growth at 37°C. Therefore the sec8-9 mutation results in a premature opal stop codon.

To determine the approximate location of the stop codon in the sec8-9 gene COOH-terminal truncations of SEC8 were subcloned into YCP50 and introduced into sec8-9 cells. Immunoblots were performed on lysates to determine the apparent molecular mass of each truncated protein. The con-
Table IV. Summary of Genetic Interactions between sec8 and Other sec Genes

| sec8-9 × Genotype | Viability of double mutant |
|-------------------|---------------------------|
| NY3 sec1-1        | (+) at 25°C, (−) at 30°C   |
| NY130 sec2-41     | −                         |
| NY412 sec3-2      | −                         |
| NY405 sec4-8      | −                         |
| NY402 sec5-24     | −                         |
| NY17 sec6-4       | −                         |
| NY57 sec9-4       | −                         |
| NY61 sec10-2      | −                         |
| NY64 sec15-1      | −                         |
| NY756 sec7-1      | +                         |
| NY728 sec12-4     | +                         |
| NY414 sec13-1     | +                         |
| NY415 sec16-2     | +                         |
| NY418 sec17-1     | +                         |
| NY431 sec18-1     | +                         |
| NY420 sec19-1     | (+) at 25°C, (−) at 30°C   |
| NY422 sec20-1     | −                         |
| NY423 sec21-2     | +                         |
| NY426 sec22-2     | +                         |
| NY806 sec23-1     | +                         |
| NY904 bet1-1      | +                         |
| NY120 bet2-1      | −                         |

Structs used and the size of the resulting polypeptides are depicted in Fig. 1a. Truncation at the NsiI site results in a loss of 60 amino acids from the protein and sec8-9 cells transformed with a plasmid containing this truncation, pNB448, are fully complemented at 37°C and express a Sec8 protein with an apparent molecular mass that is ~2 kD less than that of the wild type protein (Fig. 1a). A truncation at the PstI site, pNB447, allows complementation to 34°C, a temperature that is otherwise lethal to sec8-9 cells, but will not restore growth at 37°C. Analysis of this truncated protein by immunoblot revealed a band of 84 kD (Fig. 1a). Further truncation at the BglII site (Fig. 1a) also fails to complement the sec8-9 mutation at 37°C but does suppress at 34°C. This truncated protein has a higher mobility on SDS–polyacrylamide gels than the mutant Sec8-9 protein, and corresponds to a molecular weight of 78 kD (Fig. 1a). These COOH-terminal truncations have roughly mapped the sec8-9 mutation to the region between the BglII and PstII sites, assuming a linear relationship between the degree of truncation and the mobility of the protein on SDS–polyacrylamide gels. A further truncation to the EcoRV site within SEC8 results in a loss of complementing activity (Fig. 1a).

Sec8p Is Located on Both the Plasma Membrane and in a Soluble Particle

To determine the subcellular localization of Sec8p differen-
Subcerebral fractionation of NY13 cells and localization of seC8-9

Table V. Percent of Sec8 Localized in Subcellular Fractions of NY13 Cells

| Subcellular Fraction | Total Lysate | S2 | P2 | S3 | P3 |
|----------------------|-------------|----|----|----|----|
| 100%                 | 86          | 21 | 39 | 33 |

Subcellular fractionation of NY13 cells and localization of Sec8p by quantitative immunoblot analysis using anti-Sec8 antibody. NY13 cells were osmotically lysed and centrifuged at 450 g for 3 min. The supernatant was then centrifuged at 10,000 g for 10 min to form S2 and P2. The S2 was further centrifuged at 100,000 g for 60 min to form S3 and P3. Each fraction was quantitated for Sec8p and the results expressed as the percentage of the total cell lysate. The results shown are the average of five independent experiments.

The Sec8-9 mutant protein identified in a sec8-9 lysate has an apparent molecular weight of 82 kDa (lane 6) indicated by the arrow on the right. In lanes 3-5 we transformed sec8-9 cells with plasmids containing opal, amber, and ochre tRNA suppressor genes. Cells containing the opal tRNA suppressor gene (lane 3) produce full-length Sec8p. In lanes 7-9 WT, sec8-9 cells containing the opal tRNA suppressor gene, and sec8-9 cells were incubated at 37°C and analyzed by immunoblot analysis with anti-Sec8 antibody.
Figure 4. Localization of Sec8p and organelle enzyme marker activities in sucrose gradient fractions of a 10,000 g membrane pellet from NY13 cells. A 10,000 g pellet was resuspended in 2 ml of 55% sucrose, 10 mM MES pH 6.5 and placed at the bottom of a 30–55% sucrose gradient. After centrifugation to equilibrium, the gradient was fractionated and aliquots of each fraction analyzed for Sec8p, plasma membrane ATPase, Secl5p, cytochrome c reductase, GDPase, and sucrose density. (A) Sec8p and plasma membrane ATPase co-fractionate within the gradient. Sec8p was quantitated from immunoblots by determination of 125I-Protein A secondary antibody on cut out strips of nitrocellulose and expressed as the number of 125I counts per 50 μl of each fraction (■). The recovery of Sec8p from the gradient was 87% of the loaded pellet fraction. Plasma membrane ATPase activity was determined by measuring the release of inorganic phosphate for 10 min at 37°C and the result expressed as the nmoles of liberated phosphate per fraction per min (●). (B) The gradient profiles of Sec15p (●), quantitated in each fraction by immunoblot analysis as above, and cytochrome c reductase activity (○). The cytochrome c reductase activity is expressed as the rate of increase in the A550nm of the enzyme reaction using 20 μl of each fraction. (C) Localization of GDPase (○) within the gradient, expressed as the nmoles of liberated phosphate per fraction per min using 30 μl of each fraction. (△) Percent sucrose of each fraction. Fraction 1 is the gradient pellet and fraction 19 is the top of the gradient.

Figure 5. Sec8p is not associated with isolated secretory vesicles from a 100,000 g pellet from NY17 cells. The pellet was resuspended in 1 ml of lysis buffer and analyzed by Sephacryl S-1000 gel filtration. (A) Sec8p (●) was localized in the column fractions by quantitative immunoblot analysis as described in Fig. 4. The recovery of Sec8p from the column was 94%. The protein concentration of each fraction was determined by Bradford analysis (○). (B) Markers for secretory vesicles (●), expressed as the μmol of glucose released per fraction per min, and Sec15p (○). (C) Elution profiles of GDPase (○) and Kex2 (●). The Kex2 activity is expressed as the units of latent Kex2 activity per 50 μl of each fraction.

associated with secretory vesicles (Fig. 5 b) or other identified organelles (Fig. 5 c). This elution profile is identical to that observed with Sec15p (Fig. 5 b; and Bowser and Novick, 1991). These results suggest that the pool of Sec8p that is not associated with the plasma membrane is a component of a soluble protein aggregate or complex.

Soluble Sec8p Is Found in a High Molecular Weight Particle with Properties Identical to That of Soluble Sec15p

To further characterize the soluble pool of Sec8p, Sephacryl
The stability of the soluble complex containing Sec8p and Sec15p is affected by mutations in the SEC8 or SEC15 genes. 10,000 g supernatants from wild type, secS-9, and sec15-1 cells were analyzed by Sephacryl S-500 gel filtration. The column fractions were analyzed for the presence of Sec8p and Sec15p by quantitative immunoblot analysis and expressed as the number of 125I secondary antibody counts (cpm) per 50 µl of each fraction. The protein concentration of each fraction was determined by Bradford analysis. (A) Gel filtration of NY13 supernatant. 1 ml of a 20 mg/ml 10,000 g supernatant was loaded on the column and analyzed for the elution profile of Sec8p (■) and Sec15p (○). The recovery of Sec8p from the column was 65% and the recovery of Sec15p was 60%. Vertical arrows, from left to right, mark the position of thyroglobulin (669 kD), β-amylase (200 kD), and BSA (66 kD). (B) Gel filtration of a supernatant from secS-9 cells. The elution profile of the secS-9 mutant protein shifts to fractions 53-54 (■) and reduced levels of Sec15p elute from the column (○). The recovery of the mutant Sec8-9 protein from the column was 95%. (C) Gel filtration of a supernatant from sec15-1 cells. Sec8p fails to elute from the column in fractions 47-48 as in wild type cells. The recovery of Sec8p from the column was 29%.

Figure 6. The stability of the soluble complex containing Sec8p and Sec15p is affected by mutations in the SEC8 or SEC15 genes. 10,000 g supernatants from wild type, secS-9, and sec15-1 cells were analyzed by Sephacryl S-500 gel filtration. The column fractions were analyzed for the presence of Sec8p and Sec15p by quantitative immunoblot analysis and expressed as the number of 125I secondary antibody counts (cpm) per 50 µl of each fraction. The protein concentration of each fraction was determined by Bradford analysis. (A) Gel filtration of NY13 supernatant. 1 ml of a 20 mg/ml 10,000 g supernatant was loaded on the column and analyzed for the elution profile of Sec8p (■) and Sec15p (○). The recovery of Sec8p from the column was 65% and the recovery of Sec15p was 60%. Vertical arrows, from left to right, mark the position of thyroglobulin (669 kD), β-amylase (200 kD), and BSA (66 kD). (B) Gel filtration of a supernatant from secS-9 cells. The elution profile of the secS-9 mutant protein shifts to fractions 53-54 (■) and reduced levels of Sec15p elute from the column (○). The recovery of the mutant Sec8-9 protein from the column was 95%. (C) Gel filtration of a supernatant from sec15-1 cells. Sec8p fails to elute from the column in fractions 47-48 as in wild type cells. The recovery of Sec8p from the column was 29%.

Figure 7. Soluble Sec8p is associated with a 19.5S particle identified by sucrose velocity gradient centrifugation of a 10,000 g supernatant of NY11 cells. Sec8p (■) and Sec15p (○) were localized in each fraction by immunoblot analysis and the sedimentation coefficient of both Sec8p and Sec15p was found to correspond to a value of 19.5S. The recovery of Sec8p and Sec15p in the gradient fractions was 93 and 91%, respectively. Vertical arrows, from left to right, mark the position of thyroglobulin (19.5S), catalase (11.3S), and BSA (4.5S).

S-500 gel filtration of a 10,000 g supernatant from NY13 cells was performed. This supernatant contains all Sec8p not associated with the plasma membrane. The resulting column fractions were analyzed by immunoblot analysis for Sec8p and Sec15p as described. We observe that Sec8p co-elutes with Sec15p in a single peak with an apparent molecular mass of 1,000-2,000 kD (Fig. 6 a). No monomer form of Sec8p is apparent. This high molecular mass particle containing Sec8p is stable to treatment with 500 mM NaCl during the column fractionation (data not shown), as in the case of Sec15p (Bowser and Novick, 1991).

Previous results demonstrated that the soluble particle containing Sec15p has a sedimentation coefficient of 19.5S (Bowser and Novick, 1991). To determine the sedimentation coefficient of soluble Sec8p a 10,000 g supernatant from NY13 cells was analyzed by sucrose velocity gradient centrifugation. As shown in Fig. 7, the soluble particle of Sec8 also has a sedimentation coefficient of 19.5S. Therefore the soluble pool of Sec8p has properties identical to those of soluble Sec15p.

Sec8p and Sec15p Associate in a Protein Complex

The results shown above suggest that Sec8p and Sec15p may each be a component of a soluble high molecular weight complex. To directly determine if Sec8p and Sec15p are associated with each other we attempted to co-precipitate Sec15p with anti-Sec8 antibody. A 10,000 g supernatant was prepared from wild-type, protease deficient cells (NY580) and immunoprecipitations were performed either under native conditions without prior cross-linking or under denaturing conditions after treatment with the thiol-reversible cross-linking agent DSP. If present, the cross-linker was then cleaved by reduction and the proteins evaluated by SDS-PAGE and immunoblot analysis using anti-Sec8 and anti-Sec15 antibodies.

The anti-Sec8 antibody (αSec8p) successfully immunoprecipitates Sec8p under native conditions (Fig. 8). How-
Figure 8. Identification of a soluble complex containing Sec8p and Sec15p by immunoprecipitation with anti-Sec8 antibody. A 10,000 g supernatant from NY580 cells was incubated in the absence (lanes 1 and 6) or presence of the chemical crosslinker DSP for 30 min at 4°C. Lanes 2 and 7 contain 1 mM DSP and lanes 4 and 9 were treated with 2 mM of previously inactivated DSP. Lanes 3 and 8, samples were treated with 2 mM DSP. Cross-linking reactions were quenched and subjected to immunoprecipitation with 8 μg of affinity purified anti-Sec8 IgG. Within a yeast lysate the control IgG recognizes several proteins by immunoblot analysis. Lanes 5 and 10, samples were immunoprecipitated with 8 μg of a control rabbit anti-mouse IgG. (A) Immunoprecipitated Sec8p and Sec15p identified by immunoblot analysis using affinity purified anti-Sec8 (left) and anti-Sec15 (right) antibodies. (B) Quantitative determination of Sec8p and Sec15p from cut out nitrocellulose strips of A. (C) Hexokinase and Sec6p are not precipitated with anti-Sec8 IgG. Samples were treated with 2 mM DSP and immunoprecipitated with anti-Sec8 IgG. The resulting pellet and supernatant were analyzed for hexokinase and Sec6p localization by immunoblot analysis. Hexokinase immunoreactivity remains in the supernatant (lane 1) and fails to precipitate with anti-Sec8 antibody (lane 2). Overexposure of the blot confirms these results (lanes 3 and 4). Lanes 1 and 2 are from a 1-h exposure of the blot, lanes 3 and 4 are from a 3-h exposure. The arrows indicate the locations of the molecular mass standards. They are from top to bottom: phosphorylase B, 97.5 kD; BSA, 66 kD; ovalbumin, 45 kD.
25% of Sec15p are associated with the plasma membrane. More slowly than the Sec8p/Sec15p complex (Potenza et al., 1992). Sec6p also fails to precipitate (data not shown). Immunoprecipitation with anti-Sec8 antibody after crosslinking (Fig. 9). Immunoprecipitated with anti-Sec8 antibody in the absence of DSP. In lanes 4 and 8 the membrane pellet was washed once with lysis buffer and centrifuged. After resuspension in 50 μl of lysis buffer, the sample was crosslinked with DSP. Upon solubilization of the membrane with SDS, we immunoprecipitated with anti-Sec8 antibody. For all samples we performed immunoblot analysis of the precipitate and probed for Sec8p (left) and Sec15p (right).

ever, very little Sec15p was precipitated using these conditions. By quantitative immunoblot analysis 30% of the total Sec8p and 0.2% of the total Sec15p in the 10,000 g supernatant precipitated by native immunoprecipitation. Treatment with DSP (0.02 and 0.04 vol of a 20 mg/ml stock) and precipitation under denaturing conditions resulted in increased amounts of precipitable Sec15p (5–6% of the total), with no change in the level of Sec8p precipitation (Fig. 8). Addition of DSP above 0.04 vol decreased the level of immunoprecipitation of both Sec8p and Sec15p. Prior inactivation of the cross-linker by incubation with DTT resulted in a loss of Sec15p precipitation, to levels precipitated under native conditions, indicating that Sec15p precipitation is dependent upon cross-linking. As a control, immunoprecipitation was performed with a control rabbit IgG, using identical quantities of IgG that were present in the anti-Sec8 immunoprecipitations. The control IgG failed to precipitate either Sec8p or Sec15p (Fig. 8).

As an additional control for nonspecific precipitation in the presence of cross-linker, we performed immunoblot analysis with both anti-hexokinase and anti-Sec6 antibodies after immunoprecipitation with anti-Sec8 antibody. As shown in Fig. 8c, hexokinase fails to precipitate with αSec8p antibody and remains soluble. Overexposure of the labeled blot also failed to detect hexokinase immunoreactivity in the precipitated fraction. Sec6p is a soluble protein implicated in transport from the Golgi apparatus to the plasma membrane that sediments on sucrose velocity gradients more slowly than the Sec8p/Sec15p complex (Potenza et al., 1992). Sec6p also fails to precipitate (data not shown).

As previously discussed, 21% of the cellular Sec8p and 25% of Sec15p are associated with the plasma membrane (Fig. 4). It is possible that Sec8p and Sec15p associate with the plasma membrane in a complex or independently. To examine these possibilities a 10,000 g membrane pellet from NY580 cells was resuspended in lysis buffer, treated with DSP, and solubilized with SDS. Proteins were immunoprecipitated with αSec8p antibody. The results show that Sec15p from the membrane fraction can be co-precipitated with Sec8p after treatment with cross-linker (Fig. 9, lanes 4 and 8). This data suggests that Sec8p and Sec15p are associated with each other in a complex on the plasma membrane, as they are in the soluble fraction.

We have previously demonstrated that Sec15p can be removed from the plasma membrane by treatment with 0.5 M NaCl at high pH (Bowser and Novick, 1991), and that the released Sec15p has an apparent molecular mass of 1,000 kD by gel filtration. These conditions were used to determine if Sec8p also can be released from the plasma membrane. A 10,000 g pellet from NY13 cells was incubated with lysis buffer, pH 8.0, containing 500 mM NaCl for 1 h and the membrane was then repelleted. The supernatant was assayed for the presence of Sec8p by immunoblot and also analyzed by gel filtration. Sec8p was found to be released from the plasma membrane and co-eluted with Sec15p in a soluble particle of 1,000 kD (data not shown).

Since both Sec8p and Sec15p can be extracted from the plasma membrane by high pH treatment and then co-eluted by gel filtration, we attempted to co-precipitate the released Sec15p with αSec8p antibody. A 10,000 g pellet from NY580 cells was incubated in lysis buffer at pH 8.0 for 2.5 h on ice. The sample was then centrifuged at 10,000 g for 10 min to repellet the membrane. The supernatant was removed and used for immunoprecipitation with or without prior crosslinking as described above. The results show that Sec15p released from the plasma membrane is precipitated with αSec8p antibody after crosslinking (Fig. 9). Immunoprecipitation in the presence of a control IgG failed to precipitate.
either Sec8p or Sec15p. These data indicate that a protein complex containing both Sec8p and Sec15p can be disassociated from the plasma membrane.

**The Stability of the Complex Is Affected by Mutations in Either SEC8 or SEC15**

The stability of protein complexes is often adversely affected by defects in one of the components. To further probe the interaction of Sec8p and Sec15p we determined if the size or stability of the Sec8p/Sec15p complex could be affected by mutations in either gene. S-500 gel filtration chromatography was performed on 10,000 g supernatants derived from wild type, sec8-9, and sec15-1 cells. Equal amounts of protein were loaded onto the column and quantitative immunoblot analysis was performed on aliquots of each fraction to determine the location and quantity of Sec8p, Sec15p, and the mutant proteins.

As shown in Fig. 6 a, Sec8p and Sec15p co-elute by S-500 gel filtration of a supernatant derived from NY13 (SEC8, SEC15) cells. However, upon fractionation of a supernatant from sec8-9 cells greatly reduced levels of Sec15p were found to elute from the column, and no distinct peak was seen (Fig. 6 b). Degradation products of Sec15p were present on the nitrocellulose blot. We also found that the truncated Sec8-9 protein did not elute in fractions 47-49 as in the wild type column profile, but instead eluted in fractions 53-54 (Fig. 6 b), corresponding to an apparent molecular mass of 200 kD. These data suggest that the sec8-9 mutation results in a partial disruption of the soluble particle, and that in response to this disruption, Sec15p is destabilized.

Analysis of a supernatant from sec15-1 cells by gel filtration revealed that the level of Sec8p was reduced in the elution profile of this mutant supernatant and distributed throughout the profile, rather than concentrated in fractions 47-49 (Fig. 6 c). Detection of the mutant Sec15-1 protein by immunoblot was problematic due to its low abundance and the presence of a cross-reacting protein of similar mobility. To determine if the instability of Sec8p or Sec15p was specific to mutations in either the SEC15 or SEC8 genes, gel filtration chromatography of a supernatant derived from another late-acting sec mutant, sec6-4, was carried out. In this column profile both Sec8p and Sec15p elute in fractions 47-49 as in the case of a wild-type column profile (data not shown).

These results indicate that the stability of the soluble complex is affected by mutations in either SEC8 or SEC15. The observed instability of the soluble complex in these mutant lysates may reflect an overall decrease in the amount of complex present in vivo, or decreased stability during column fractionation. Since it is possible that dilution of the supernatant may affect the degree of instability of the proteins in mutant lysates, we made dilutions of each supernatant to 0.5 mg/ml to approximate the concentration of protein after starting material and the diluted material were analyzed after incubation for Sec8p and Sec15p by quantitative immunoblots. These values were expressed as the CPM of ATP-Protein A bound per mg of protein.

The supernatants of wild type, sec8-9, sec15-1, and sec6-4 cells contained similar quantities of Sec8p and Sec15p (Fig. 10, solid bars). However upon subsequent dilution and incubation, the level of Sec8p decreased in the supernatant derived from sec15-1 cells (Fig. 10 a, hatched bars), but not in the supernatants derived from wild type cells or sec6-4 cells. Approximately 98-100% of the Sec8p present in the diluted wild type or sec6-4 lysate was present after 3 h at 4°C, whereas only 53% of Sec8p in a diluted sec15-1 lysate remained. Likewise, the stability of Sec15p was decreased in sec8-9 cells (Fig. 10 b). Only 38% of the Sec15p contained in a diluted sec8-9 lysate remained after a 3-h incubation at 4°C. The stability of Sec15p was unaffected in sec6-4 cells, consistent with the gel filtration results. These data indicate that the stability of the complex containing Sec8p and Sec15p is influenced in vitro by mutations in either the SEC8 or SEC15 genes.

**Sec8p May Interact With Sec4p**

As the amino terminus of Sec8p is similar in sequence to the...
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Since the Sec8p/Sec15p complex is also associated with the plasma membrane and found in the cytoplasm, Sec8p could associate with Sec4p in either location.

To determine if Sec4p associates with the soluble complex containing Sec8p, Sephacryl S-500 gel filtration chromatography of a 10,000 g supernatant derived from NY13 cells was performed and fractions quantitated for both Sec4p and Sec8p by immunoblot analysis. Sec8p elutes from the column in a single peak (Fig. 11 a), as was previously demonstrated. Sec4p elutes from the column in two distinct peaks (Fig. 11 a). The first peak co-elutes with Sec8p while the second elutes with an apparent molecular mass of 60-70 kD. Integration of the two peaks indicates that ~20% of the eluted Sec4p co-fractionates with the peak of Sec8p, suggesting that a portion of the total cellular Sec4p may be associated with the complex containing Sec8p.

It is also possible that Sec4p associates with another high molecular weight particle or protein aggregate that coincidentally elutes at the position of the Sec8/Sec15p complex. As shown in Figs. 6 and 10, the complex containing Sec8p and Sec15p becomes unstable in sec8-9 or sec15-1 lysates. If Sec4p is associated with this complex then the initial peak of Sec4p should also be sensitive to mutations in SEC8 and SEC15. Therefore we carried out gel filtration chromatography of cytosol derived from sec8-9 and sec15-1 and localized Sec4p.

As shown in Fig. 11 b, the initial peak of Sec4p is not seen in the elution profile of a sec8-9 lysate. The 60-70-kD pool of Sec4p is somewhat enlarged, possibly due to release of Sec4p from the Sec8p/Sec15p complex and incorporation into the 60-70-kD pool. The Sec8-9 mutant protein that elutes in fractions 51-54 may also retain the ability to interact with Sec4p.

A sec15-1 lysate was next analyzed by S-500 column fractionation. Again the initial peak of Sec4p was absent and the 60-70-kD pool was enlarged relative to that of a wild type lysate (Fig. 11 c). These results suggest that a portion of the soluble pool of Sec4p is in association with the Sec8p/Sec15p complex, and under conditions that render the Sec8p/Sec15p complex unstable, this pool of Sec4p is not detected. This result supports the hypothesis that Sec8p interacts with Sec4p.

**Discussion**

We have shown that the SEC8 gene encodes a 122-kD hydrophobic protein that functions, together with the Sec15 protein, in a 19.5S complex that can peripherally associate with the plasma membrane. Several lines of evidence suggest that this complex may serve as the downstream effector of Sec4p to regulate vesicular transport from the Golgi apparatus to the cell surface in yeast cells.

The first line of evidence is based on the strong genetic interactions seen among mutants defective in the SEC4, SEC8, and SEC15 genes. These are three members of a set of late-acting sec mutants that display synthetic lethality in combination with one another (Salminen and Novick, 1987). The growth defects of these mutants are partially suppressed by a twofold increase in the expression of SEC4. This genetic evidence cannot, by itself, establish that physical interactions occur between the gene products. It does, however, suggest that the encoded proteins are acting in a concerted fash-
ion and that Sec4p may play a key regulatory role on the pathway in which they function.

Biochemical evidence suggests a direct, physical interaction of Sec4p with the Sec8p/Sec15p complex. Analysis of a soluble fraction derived from wild type cells by gel filtration indicated that a portion of Sec4p co-elutes with the Sec8/Sec15p complex (Fig. 11). The presence of this species of Sec4p was found to be sensitive to mutations in either Sec8 or Sec15 (Fig. 11), supporting a model in which Sec4p directly associates with the Sec8p/Sec15p complex.

Sequence analysis of Sec8p may provide a clue to the nature of the proposed interaction of Sec4p with the Sec8p/Sec15p complex. A 202 amino acid region of Sec8p shares 25% sequence identity with the region of yeast adenylate cyclase that is necessary for Ras responsive regulation of cAMP synthesis (Colicelli et al., 1990; Field et al., 1990). This region of cyclase may make direct contact with the Ras protein. The observed sequence similarity suggests that Sec8p could have an analogous relationship to Sec4p as adenylate cyclase has to Ras, i.e., Sec8p may be the downstream effector of Sec4p. Since Sec4p is only 32% identical in sequence to Sec8p (Salminen and Novick, 1987) it is not surprising that Sec8p and adenylate cyclase do not exhibit a high degree of similarity. Nevertheless, since Sec8p and adenylate cyclase share a relatively low level of sequence identity, further biochemical evidence will be necessary to establish this analogy.

Some insight into the function of the Sec8p/Sec15p protein complex can be gained from the subcellular localization data. Approximately 20–25% of the Sec8p/Sec15p complex was associated with the plasma membrane, and the remainder was found to be soluble. The Sec8p/Sec15p complex was released from an isolated plasma membrane fraction by a salt wash (0.5 M NaCl, pH 8.0) indicating peripheral membrane attachment. The association of the complex with the plasma membrane therefore, may be mediated by an ionic interaction with a membrane protein. Sec4p itself, could serve as the attachment site for the Sec8p/Sec15p complex, since the major pool of Sec4p is tightly associated with the cytoplasmic face of the plasma membrane. However, if Sec4p serves as the attachment site, there must be additional levels of regulation as well, because secretory vesicles carry Sec4p but do not carry Sec8p or Sec15p. Furthermore, sec4-8 mutants are not altered in their distribution of Sec8 and Sec15 (R. Bowser, unpublished observation). One interesting possibility is that the complex containing Sec8p and Sec15p normally cycles between the plasma membrane and the cytoplasm in conjunction with vesicle docking or fusion events.

The 19.5S Sec8p/Sec15p complex may contain additional subunits. Candidates for such components are the products of the other genes that interact with SEC4, SEC8, and SEC15. Sec8p and Sec15p may be in direct contact with each other, or they may interact through intervening subunits. Deeper insight into the structure of the complex and a detailed understanding of the proposed interaction with Sec4p will require purification of the native complex. These goals will require the development of more specific reagents.

A combination of genetic and biochemical analysis has uncovered a complex containing Sec8p and Sec15p that functions at the final step of the secretory pathway, possibly in response to Sec4p function. Since there are numerous Sec4p homologs that regulate different stages of the exocytic and endocytic pathways (Chavrier et al., 1990a, b; Gorvel et al., 1991; Johnston et al., 1991), additional complexes containing subunits analogous and possibly homologous to Sec8p and Sec15p may be present in yeast and animal cells serving to respond to distinct members of the SEC4/YPT1/rab family of GTP-binding proteins.

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References

Abeijon, C., P. Orlean, P. W. Robbins, and C. B. Hirschberg. 1989. Characterization of GDP-Manose transport and luminal guanosine diphosphatase activities in Golgi like vesicles. Proc. Natl. Acad. Sci. USA. 86:6935-6939.

Bourne, H. R. 1988. Do GTPases direct cytoskeleton-related growth? Cell. 53:669-671.

Bowser, R., and P. Novick. 1991. Sec15 protein, an essential component of the exocytic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. J. Cell Biol. 112:1117-1131.

Carlson, M., and D. Botstein. 1990. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell. 28:145-154.

Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990a. Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. Cell. 62:317-329.

Chavrier, P., M. Vingron, S. Cander, K. Simons, and M. Zerial. 1990b. Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. Mol. Cell. Biol. 10:6578-6585.

Colicelli, J., J. Field, R. Ballester, N. Chester, D. Young, and M. Wigler. 1990. Mutational mapping of RAS-responsive domains of the Saccharomyces cerevisiae adenyl cyclase. Mol. Cell. Biol. 10:2539-2543.

Field, J., N. Ikawa, D. Brock, B. MacDonald, L. Rodgers, J. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. Mol. Cell. Biol. 8:2159-2165.

Field, J., H.-P. Xu, T. Michaeli, R. Ballester, P. Saa, M. Wigler, and J. Carlsson. 1990. Mutations of the adenyl cyclase gene that block RAS function in Saccharomyces cerevisiae. Science (Wash. DC). 247:464-467.

Gorvel, J., P. Chavrier, M. Zerial, and J. Gruenberg. 1991. ras5 controls early endosome fusion in vitro. Cell. 64:915-925.

Goud, B., A. Salminen, N. C. Walworth, and P. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. J. Cell Biol. 103:763-768.

Ito, H., Y. Fukuda, K. Murata, and A. Kikurura. 1983. Transformation of intact yeast cells with alkali cations. J. Bacteriol. 153:163-168.

Johnston, P. A., B. T. Archer, III, K. Robinson, G. A. Mignery, R. Jahn, and T. C. Sudhof. 1991. rasA5A attachment to the synaptic vesicle membrane mediated by a conserved polyisoprenylated carboxy-terminal sequence. Neuron. 7:101-109.

Kreibich, G., P. Debezy, and D. D. Sabatini. 1973. Selective release of contents from micromesos vesicles without membrane disassembly. I. Permeability changes induced by low detergent concentrations. J. Cell Biol. 58:436-462.

Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity searches. Science (Wash. DC). 227:1435-1441.

Matsumoto, K., I. Uno, and S. Takahashi. 1985. Genetic analysis of the role of CAMP in yeast. Yeast. 1:15-24.

Mitts, M. R., D. B. Grant, and W. Heidenman. 1990. Adenylate cyclase in Saccharomyces cerevisiae is a peripheral membrane protein. Mol. Cell. Biol. 10:3873-3883.

Mitts, M. R., J. Bradshaw-Rouse, and W. Heidenman. 1991. Interactions between adenylate cyclase and the yeast GT Pase-activating protein IRA1. Mol. Cell. Biol. 11:4501-4508.

Nakajima, H., A. Hira, T. Yonehara, K. Yoda, and M. Yamashita. 1991. A cytoskeleton-related gene, USO1, is required for intracellular protein transport in Saccharomyces cerevisiae. J. Cell Biol. 113:245-260.

Newman, A., and S. Ferro-Novick. 1987. Characterization of new mutants in the early part of the yeast secretory pathway isolated by a [H]mannoside suicide selection. J. Cell Biol. 105:1587-1594.

Newman, A. P., J. Shim, and S. Ferro-Novick. 1990. BETI, BOS1, and SEC22 are members of a group of interacting yeast genes required for transport from the endoplasmic reticulum to the Golgi complex. Mol. Cell. Biol. 10:3405-3414.
Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell. 21:205–215.

Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. Cell. 25:461–469.

Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. 85:2444–2448.

Potenza, M., R. Bowser, H. Muller, and P. Novick. 1992. SEC6 encodes an 85 kD soluble protein required for exocytosis in yeast. Yeast. In press.

Rossi, G., Y. Jiang, A. P. Newman, and S. Ferro-Novick. 1991. Dependence of Ypt1 and Sec4 membrane attachment on Bet2. Nature (Lond.). 351:158–161.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. Nature (Lond.). 355:409–415.

Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. Cell. 49:527–538.

Salminen, A., and P. J. Novick. 1989. The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control traffic in yeast. J. Cell Biol. 109:1023–1036.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463–5467.

Vidoli, R., H. Yamazaki, A. Nasim, and I. A. Veliky. 1982. A novel procedure for the recovery of hybrid products from protoplast fusion. Biotech. Lett. 4:781–784.

Walworth, N., and P. Novick. 1987. Purification and characterization of constitutive secretory vesicles from yeast. J. Cell Biol. 105:163–174.

Walworth, N. C., B. Goud, A. K. Kabcenell, and P. J. Novick. 1989a. Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. EMBO (Eur. Mol. Biol. Organ.) J. 8:1685–1693.

Walworth, N. C., B. Goud, H. Ruohola, and P. Novick. 1989b. Fractionation of yeast organelles. Methods Cell Biol. 31:335–354.