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

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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 19.5*S* 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., 1989*a*). 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

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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 19.5*S* 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 Sec4p 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 (SD) containing 0.7% yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose, supplemented for auxotrophic requirements when necessary.

Yeast transformation was generally performed by alkali cation treatment (Ito et al., 1983), and transformants selected on SD medium, supplemented for additional auxotrophs as noted, at 25°C.

The *SEC8* gene was cloned by complementation of the temperature-sensitive growth defect of *sec8-9* cells with a yeast genomic DNA library contained in the high copy number vector YEp24 (Carlson and Botstein, 1982). 100 A_{599} U of NY410 cells (*sec8-9, ura3-52*) were transformed with 800 ng of library DNA by the alginic acid method (Vidoli et al., 1982) as follows: yeast spheroplasts were resuspended in 0.5 ml of 1 M sorbitol, 10 mM Tris, pH 7.5, 10 mM $CaCl_2$, and incubated for 5 min with 600 ng of DNA and 5 ml of 40% PEG containing 10 mM $CaCl_2$ and 10 mM Tris, pH 7.5. The cells were then pelleted and resuspended in 450 μl of 1.2 M sorbitol. 45 μl were placed in a sterile tube, and an equal volume of 1.2 M sorbitol containing 4% alginate was added. The suspension was plated on a SD medium containing 1.2 M sorbitol and 50 mM $CaCl_2$. Ura^+ transformants were selected at 25°C and then replica plated to 37°C on YPD plates. Total DNA was isolated from temperature resistant colonies. Approximately 50 ng of DNA was used to transform DH1 cells and transformants selected on LB plates containing ampicillin. The plasmid DNA was then isolated from *Escherichia coli* cells and purified.

Table I. Yeast Strains

Strain	Genotype
NY13	*MATa, ura2-53*
NY15	*MATα, his4-619, ura3-52*
NY17	*MATa, ura3-52, sec6-4*
NY64	*MATa, ura3-52, sec15-1*
NY410	*MATa, ura3-52, sec8-9*
NY411	*MATα, his4-619, sec8-9*
NY580	*MATa, leu2-3,112, ura3-52, pep4::URA3*
NY813	*MATa/α, leu2-3,112/leu2-3,112, ura3-52/ura3-52, SEC8/sec8::*pNB338 (*SEC8* disruption)
NY862	*MATa*, NY13 + pNB328 (Sec8 overproduced on YEp24)
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* PstI truncation)
NY913	NY410 + pNB448 (*sec8* NsiI truncation)

Nucleic Acid Techniques

E. coli strain DH1 (F-, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1) was used for all cloning experiments. For production of fusion proteins to produce Sec8 antibodies, the *SEC8* gene was first transformed into BU1255 cells (*dam*-) and further subcloned in DH5α cells (*supE*44, Δ*lac*U169 [φ80*lacZ*ΔM15], *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) as described below.

Plasmids used are listed in Table II. Plasmid pNB328 was isolated from a yeast genomic DNA library in the episomal vector YEp24 (Carlson and Botstein, 1982). pNB329, a centromere based plasmid containing *SEC8*, was constructed by ligating the gel purified 4.4-kb SmaI-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 SalI and ligated to a gel purified 4.4-kb SmaI-SalI fragment from pNB328. To construct pNB330, YIp5 DNA was digested with BamHI and SalI as described above for YCp50 and ligated with the 4.4-kb SmaI-SalI 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 YIp5. 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 tetrads were 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 0.7-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 digested with EcoRI-XbaI, gel purified, and ligated into gel purified EcoRI-XbaI-digested pATH11 to produce pNB366. This ligation was transformed into DH5α cells. pNB366 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 BamHI-SalI fragment of pNB330 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 68.0, June

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 antisera at 1/1,000 dilution for 2 h, washed, and subsequently labeled with ^{125}I-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 ^{125}I-Protein A quantitated on a gamma counter. Enzyme assays were performed as previously described (Bowser and Novick, 1991; Walworth et al., 1989*b*).

Immunoprecipitation

NY580 cells (*pep4::URA3*) were used for immunoprecipitation studies. 50 A_{600} 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 $MgCl_2$, 1 mM PMSF, 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 vol (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 cross-linker the samples were simply diluted with 700 μl of immunoprecipitation buffer. All samples were first precleared by addition of 4 μl of preimmune antisera 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 tube and either 8 μg of affinity purified α-Sec8 antibody or a control rabbit IgG was added for overnight incubation at 4°C. The control IgG was a rabbit anti-mouse polyclonal serum, which also recognizes a number of cross-reacting proteins in a yeast whole cell lysate. After 1 2-h incubation with protein A-Sepharose CL4B beads at 4°C, the beads were collected by microcentrifugation for 15 s. The beads were washed once with immunoprecipitation buffer containing 0.2% SDS (IPS buffer) and then twice with IPS containing 2 M urea, twice with IPS containing 0.5 M NaCl, and once with IPS. For immunoprecipitation under native conditions the antibody was added to the reaction tube in the absence of detergents and after overnight incubation the immune complexes collected as above. The beads were washed five times in lysis buffer containing 0.5 M NaCl. Antigens were released from beads by heating in SDS-PAGE buffer for 5 min at 100°C and loaded onto 8% SDS-polyacrylamide gels.

Results

Cloning and Characterization of the SEC8 Locus

The *SEC8* gene was cloned by complementation of the temperature sensitive *sec8-9* allele using a yeast genomic DNA library in the high copy number vector YEp24. NY410 cells (*MATa, sec8-9, ura3-52*) were transformed and ~7,500 Ura$^+$ transformants were selected at 25°C, and then replica plated onto YPD plates at 37°C. Plasmid DNA was isolated from temperature resistant colonies, amplified in *E. coli* and re-introduced into *sec8-9* cells. Plasmids that complemented the *sec8-9* growth defect were analyzed by restriction digestion and the smallest plasmid, pNB328, (Table II) was used for further studies.

Analysis of plasmid pNB328 revealed a genomic DNA insert of 3.8 kb (Fig. 1 *a*). The ability of this insert to complement *sec8-9* cells at single copy was tested by ligating a 4.4-kb SmaI-SalI fragment, which contains the complete genomic insert, into the centromere based vector YCp50. Introduction of this plasmid, pNB329, into *sec8-9* cells fully restored growth at 37°C. Various restriction fragments were subcloned into YCp50 and analyzed for complementing activity (Fig. 1 *a*). The smallest fully complementing fragment contains all but ~400 bp of the original genomic isolate and is contained in a Sau3A-NsiI fragment of 3,344 bp.

To determine if the cloned fragment represents the *SEC8* gene, the ability of the sequence to direct integration into the *SEC8* locus was analyzed. The 4.4-kb SmaI-SalI fragment of pNB328 was inserted into the yeast integrating vector, YIp5, which contains the selectable marker *URA3*. This plasmid, pNB330, was cleaved within the complementing region by digestion with BglII and introduced into the wild type haploid yeast strain, NY15 (*MATα, ura3-52*). Integration at the *SEC8* locus results in the duplication of the cloned sequence, with the *URA3* marker between the two copies. This transformant was mated with NY410 (*MATa, sec8-9, ura3-52*) and the resulting diploid was sporulated and analyzed by tetrad dissection. If the cloned sequence represents the *SEC8* gene, then the Ura$^+$ and Ts$^+$ phenotypes should co-segregate. Of 36 tetrads analyzed, 34 segregated 2 Ura$^+$ Ts$^+$: 2 Ura$^-$ Ts$^-$ (Table III). No Ts$^-$, Ura$^+$ spores were found, yet in two tetrads Ura$^-$, Ts$^+$ spores were seen, possibly the result of gene conversion events. The tight linkage observed between the *sec8-9* locus and the site of plasmid integration supports the conclusion that the cloned sequence represents the *SEC8* locus.

SEC8 Sequence

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 continued 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*). That 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 NH_2-terminal signal sequence or any potential membrane spanning domains.

1. *Abbreviation used in this paper*: DSP, dithio-*bis*-(succinimidylpropionate).

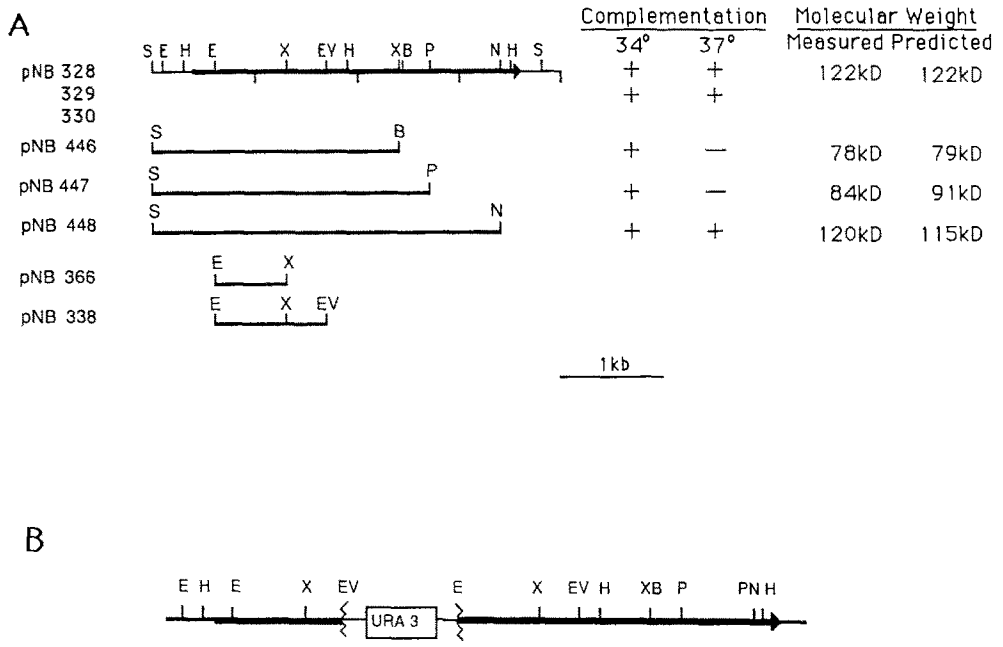

Figure 1. (*A*) A restriction map of the *sec8-9* complementing region. Plasmid pNB328 was isolated from a yeast genomic library in the high copy number vector YEp24. The plasmid pNB329 contains the entire genomic sequence inserted into the centromere based vector YCp50. The plasmid pNB330 contains the entire genomic sequence inserted into the integrating vector YIp5. Various fragments were also subcloned into the centromere based vector and tested for complementation of the growth defect of a *sec8-9* mutant at 34° and 37°C as shown. The Sec8p open reading frame is shown as a bold arrow. The molecular weights of the proteins encoded by the *SEC8* gene and the various truncations are listed. The predicted weights 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*, *Sau*3A; *E*, *Eco*RI; *H*, *Hind*III; *X*, *Xba*I; *EV*, *Eco*RV; *B*, *Bgl*II; *P*, *Pst*I; *N*, *Nsi*I. 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 *Eco*RV 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 NH_2 terminus of the protein (Fig. 2 *a*). 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 a 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. 2 *b*). 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–SalI fragment from pNB328 into SmaI–SalI-digested YCp50
pNB330	YIp5, *SEC8*, 4.4-kb SmaI–SalI fragment from pNB328 into SmaI–SalI-digested YIp5
pNB338	YIp5, 1.15-kb EcoRI–EcoRV fragment from pNB330 into SspI–EcoRI-digested YIp5
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 (*MATα his4-619, ura3-52, SEC8::SEC8, URA3*) × NY410 (*MATa ura3-52, sec8-9*)
2 Tetratype: 2 Ura^+Ts^+, 1 Ura^-Ts^+, 1 Ura^-Ts^-
34 Parental ditype: 2 Ura^+Ts^+, 2 Ura^-Ts^-
0 Nonparental ditype: 2 Ura^+Ts^+, 2 Ura^-Ts^+

ity point value is 65.3 ± 3.3. The significance of these values can be expressed as the z value where

z = (initial quality score − average quality score after randomization)/SD

The z value of this comparison is 3.6. A z value between 3 and 6 indicates the comparison is possibly significant (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. 1 *b*). 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 *sec1-1*, 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 *sec1-1*, all meiotic products were viable at 25°C, but double mutants were inviable at 30°C, a temperature at which either *sec1-1* or *sec8-9* alone are viable. As in the case of *sec4-8*, *sec8-9* is also lethal in combination with *sec19-1* 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). *SEC19* may function at several points in the secretory pathway as the *sec19-1* 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 Antisera Against Sec8p and Identification of the Protein

To identify and characterize the Sec8 protein, polyclonal antisera 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 pATH11 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. 3 *a*). 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. 3 *a*, 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. 3 *a*, 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. 3 *a*, lane *8*). Cells containing the amber and ochre tRNA suppressors failed to synthesize full length protein (Fig. 3 *a*, 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-

A

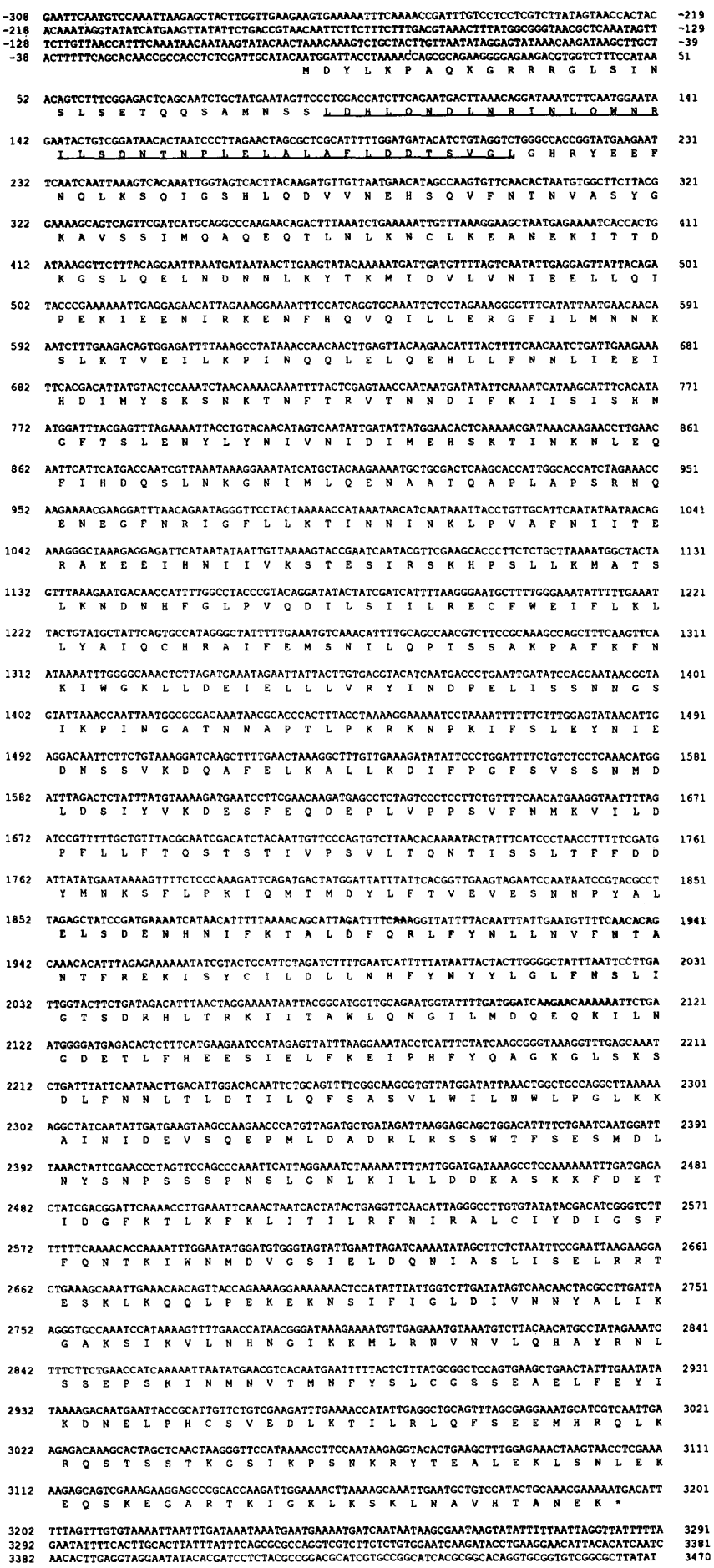

Figure 2. Nucleotide sequence of *SEC8*. (*A*) The DNA and amino acid sequence of the *SEC8* open reading frame are shown. The GenBank database accession number of *SEC8* is X64693. The leucine rich domain is underlined. (*B*) A 202 amino acid region of Sec8p containing the leucine rich domain exhibits 25.4% identity and 44% similarity to a 185 amino acid domain of adenylate cyclase. Identical amino acids are distinguished by a "|" and conserved amino acids with a "*." The homology search between Sec8p and adenylate cyclase was performed using the Wisconsin Genetics Computer Group BESTFIT program. (*C*) The region of adenylate cyclase that exhibits homology with Sec8p is contained within the leucine repeat domain of adenylate cyclase.

B

```
ADEN. 972 IGDLSE.............MTDLRTLNLRYNRISSIKTNASNLQNLFLTDNRI
          |  |||                ||  :|| :||| |  ||: :|   || |  :
SEC8   17 INSLSETQQSAMNSSLDHLQNDLNRINLQWNRILSDNTNPLELALAFLDDTSV

SNFEDTLPKLRALEIQENPITSISFKDFYPKNMTSLTLNKAQLSSIPGELLTKLSFL.......
: ::     :  |  |          :      | :      :|||       | :
G.LGHRYEEFNQLKSQIGSHLQDVVNEHSQVFNTNVASYGKAVSSIMQAQEQTLNLKNCLKEAN

EKLELNQNNLTRLPQEISKLTKLVFLSVARNKLEYIPPELSQLKSLRTLDLHSNNI...RDFVD
||:  :   |  | ::  | ||:: : |  : |  ||  : :   :|  ::|   |   |:|:
EKITTDKGSLQELNDNNLKYTKMIDVLVNIEELLQIPEKIEE..NIRKENFHQVQILLERGFI.

GMENLELTSLNISSNAFGNSSLENSFY  1157
 |:|  |  ::|         |:: :
LMNNKSLKTVEILKPINQQLELQEHLL  220
```

C

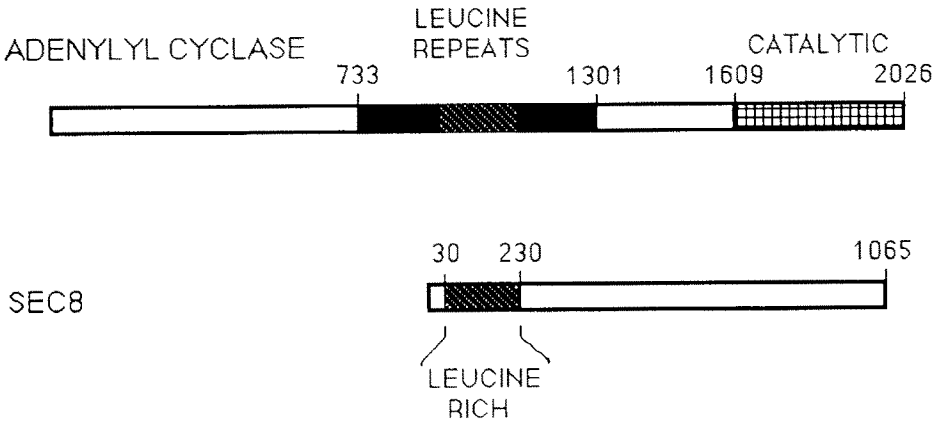

Table IV. Summary of Genetic Interactions between sec8 and Other sec Genes

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*	+
ANY114 *bet1-1*	+
ANY120 *bet2-1*	−
NY806 *sec23-1*	+

structs used and the size of the resulting polypeptides are depicted in Fig. 1 *a*. 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. 1 *a*). 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. 1 *a*). Further truncation at the BglII site (Fig. 1 *a*) 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. 1 *a*). These COOH-terminal truncations have roughly mapped the *sec8-9* mutation to the region between the BglII and PstI 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. 1 *a*).

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

To determine the subcellular localization of Sec8p differen-

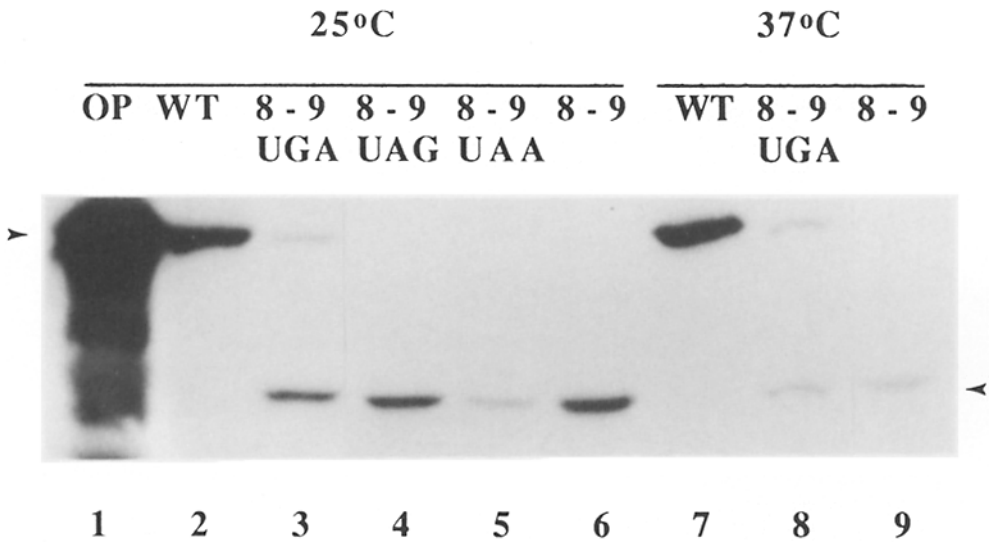

Figure 3. Identification and localization of Sec8p using TrpE-Sec8 fusion protein generated polyclonal antibody. A glass bead lysate of NY13 cells was analyzed by immunoblot analysis using the anti-Sec8 polyclonal antiserum. This antiserum recognizes a protein of ~122 kD (lane *2*) indicated by the arrow on the left. To verify that this protein is Sec8p we introduced pNB-328, a high copy number plasmid containing the *SEC8* gene into NY13 cells to overproduce the Sec8 protein. The 122-kD band is amplified in this lysate (lane *1*) and several bands appear that may correspond to degradation products. The Sec8-9 mutant protein identified in a *sec8-9* lysate has an apparent molecular weight of 82 kD (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.

tial centrifugation was carried out on wild-type yeast cell lysates and immunoblot analysis performed using affinity purified anti-Sec8 antibody. Approximately 21% of the total cellular Sec8p is found in a 10,000 *g* membrane pellet (Table V). Centrifugation of the 10,000 *g* supernatant at 100,000 *g*, pellets an additional 33% of the cellular Sec8p. The remainder is located in the 100,000 *g* supernatant (Table V). To determine the membrane component with which Sec8p is associated in the 10,000 *g* pellet, we performed sucrose gradient fractionation. A 10,000 *g* pellet from NY13 cells was resuspended in 55% sucrose and overlaid with a step gradient made by addition of various sucrose solutions decreasing in concentration to 30%. After centrifugation to equilibrium, the gradient was fractionated and aliquots were subjected to SDS-gel electrophoresis and transferred to nitrocellulose. The Sec8p present in each fraction was quantitated using αSec8p antibody and ^{125}I-protein A, and the results were expressed as the CPM specifically bound per 50 μl of fraction.

The profile of a typical 10,000 *g* sucrose gradient from wild type cells is shown in Fig. 4. Sec8p co-fractionates with both the plasma membrane marker enzyme, vanadate sensitive Mg^{2+}-ATPase activity, and Sec15p. During differential centrifugation we typically observe 50–60% of the total cellular plasma membrane ATPase activity associated with the 10,000 *g* pellet. Approximately 20% of the ATPase activity is associated with the 100,000 *g* membrane pellet while the activity remaining in the soluble fraction is attributed to interfering ATPases. Sec8p does not co-fractionate with NADPH-cytochrome *c* reductase activity, a marker for the ER (Kreibich et al., 1973). Sec8p also fails to co-fractionate with a marker for the yeast Golgi apparatus (Abeijon et al., 1989), GDPase activity (Fig. 4 *c*). We typically observe 40–45% of the total GDPase activity associated with the 10,000 *g* pellet. From this result we conclude that the Sec8p found in a 10,000 *g* pellet from wild type cells is associated with the plasma membrane.

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

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 lysate. The results shown are the average of five independent experiments.

We next determined if the pool of Sec8p that pellets at 100,000 *g* is associated with a membrane. It is possible that Sec8p associates with small membrane bounded organelles, such as secretory vesicles, that are found in a high speed (100,000 *g*) pellet of a yeast lysate. Alternatively, Sec8p may be a component of a proteinaceous particle and not associated with a membrane. To address these possibilities sucrose density gradient fractionation was performed using a 100,000 *g* pellet derived from wild-type yeast cells. Sec8p did not float into the gradient with membrane components but remained at the bottom of the gradient with free protein (data not shown).

Since few secretory vesicles are present in wild type yeast cells, we determined if Sec8p was associated with the enlarged pool of secretory vesicles isolated by Sephacryl S-1,000 gel filtration chromatography from the late-acting *sec* mutant NY17 (*sec6-4*) (Walworth and Novick, 1987). NY17 cells were incubated at the restrictive temperature (37°C) for 1 h and simultaneously shifted into low glucose containing media to derepress synthesis of the secretory protein invertase. Thus invertase can be used as a lumenal enzyme marker for secretory vesicles. Each fraction eluted from the column was assayed for marker enzymes and for the abundance of Sec8p by immunoblot analysis. The results of this fractionation indicate that Sec8p elutes from the column with the leading edge of free protein (Fig. 5 *a*) and is not as-

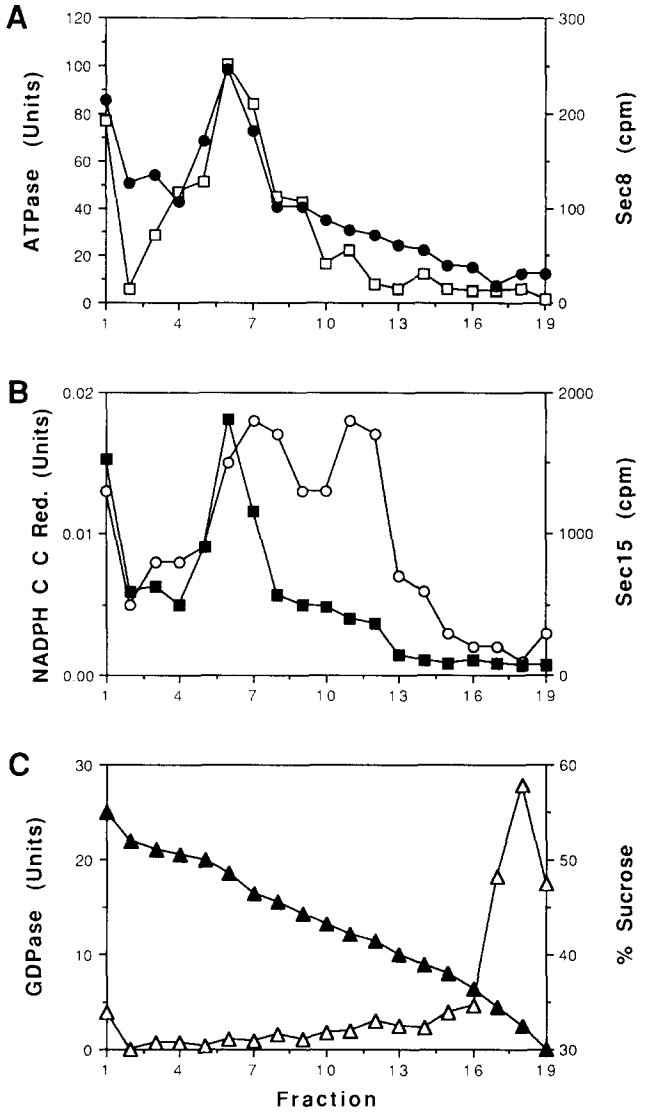

Figure 4. Localization of Sec8p and organelle enzyme marker activities in sucrose gradient fractions of a 10,000 *g* membrane pellet from NY13 cells. A 10,000 *g* pellet was resuspended in 2 ml of 55% sucrose, 10 mM MES pH 6.5 and placed at the bottom of a 30–55% sucrose gradient. After centrifugation to equilibrium, the gradient was fractionated and aliquots of each fraction analyzed for Sec8p, plasma membrane ATPase, Sec15p, 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 ^{125}I-Protein A secondary antibody on cut out strips of nitrocellulose and expressed as the number of ^{125}I 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 A_{550nm} 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.

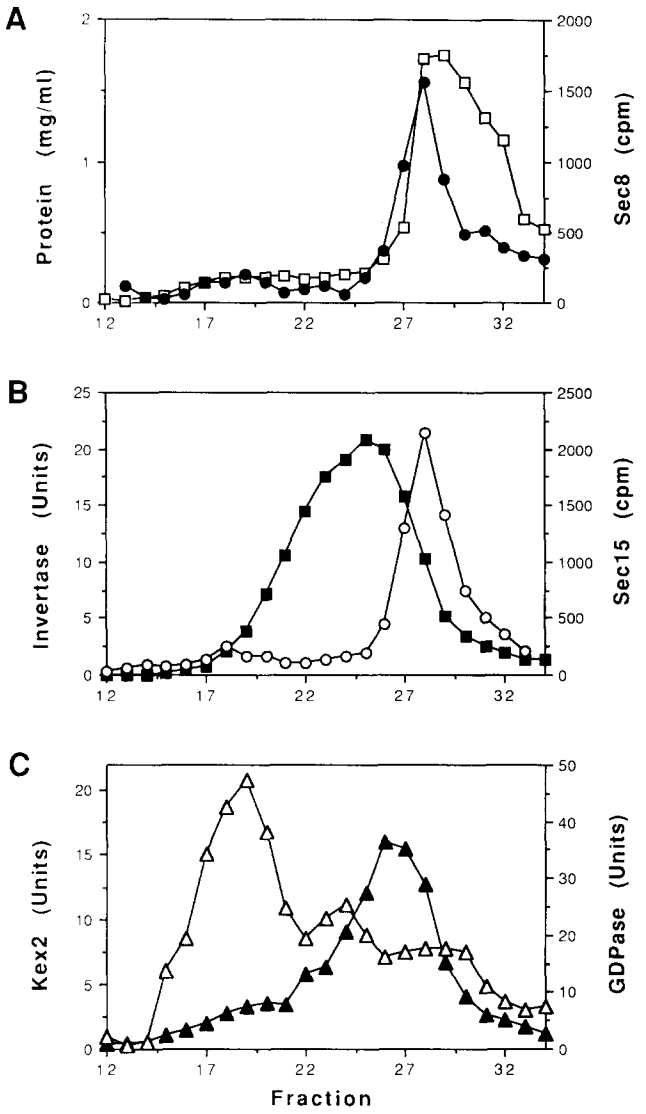

Figure 5. Sec8p is not associated with isolated secretory vesicles from a 100,000 *g* pellet from NY17 cells. The pellet was resuspended in 1 ml of lysis buffer and analyzed by Sephacryl S-1000 gel filtration. (*A*) Sec8p (-●-) was localized in the column fractions by quantitative immunoblot analysis 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.

sociated 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

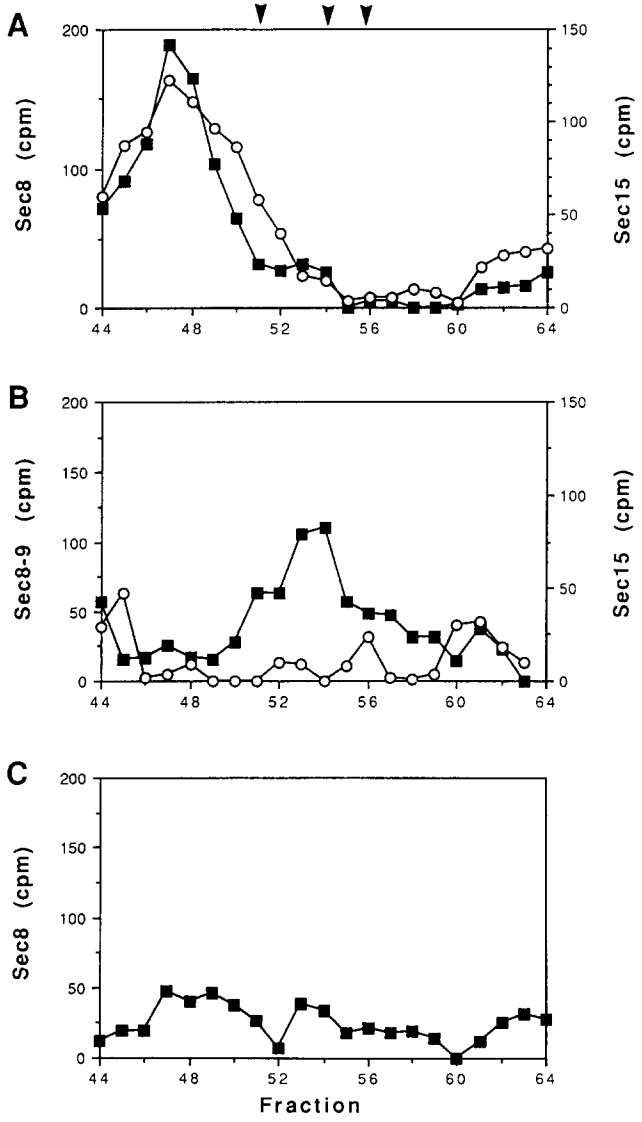

Figure 6. The stability of the soluble complex containing Sec8p and Sec15p is affected by mutations in the *SEC8* or *SEC15* genes. 10,000 *g* supernatants from wild type, *sec8-9*, and *sec15-1* cells were analyzed by Sephacryl S-500 gel filtration. The column fractions were analyzed for the presence of Sec8p and Sec15p by quantitative immunoblot analysis and expressed as the number of ^{125}I 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 *sec8-9* cells. The elution profile of the sec8-9 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%.

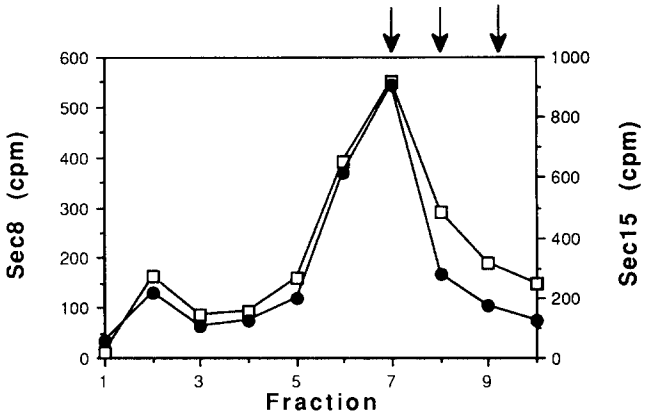

Figure 7. Soluble Sec8p is associated with a 19.5*S* particle identified by sucrose velocity gradient centrifugation of a 10,000 *g* supernatant of NY11 cells. Sec8p (-□-) and Sec15p (-●-) were localized in each fraction by immunoblot analysis and the sedimentation coefficient of both Sec8p and Sec15p was found to correspond to a value of 19.5*S*. 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 (19.5*S*), catalase (11.3*S*), and BSA (4.5*S*).

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 19.5*S* (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 19.5*S*. 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-

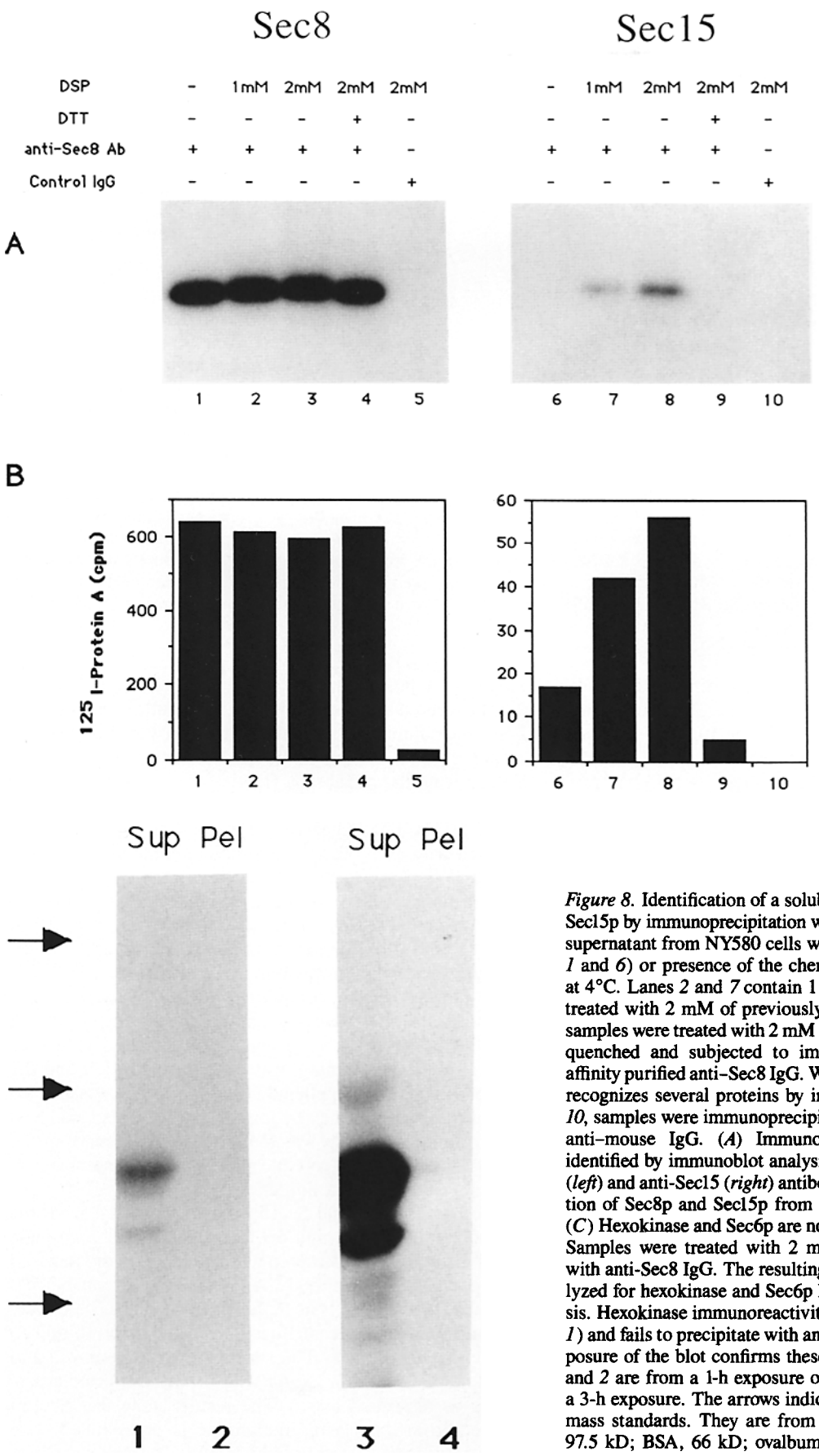

Figure 8. Identification of a soluble complex containing Sec8p and Sec15p by immunoprecipitation with anti-Sec8 antibody. A 10,000 *g* supernatant from NY580 cells was incubated in the absence (lanes *1* and *6*) or presence of the chemical crosslinker DSP for 30 min at 4°C. Lanes *2* and *7* contain 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.

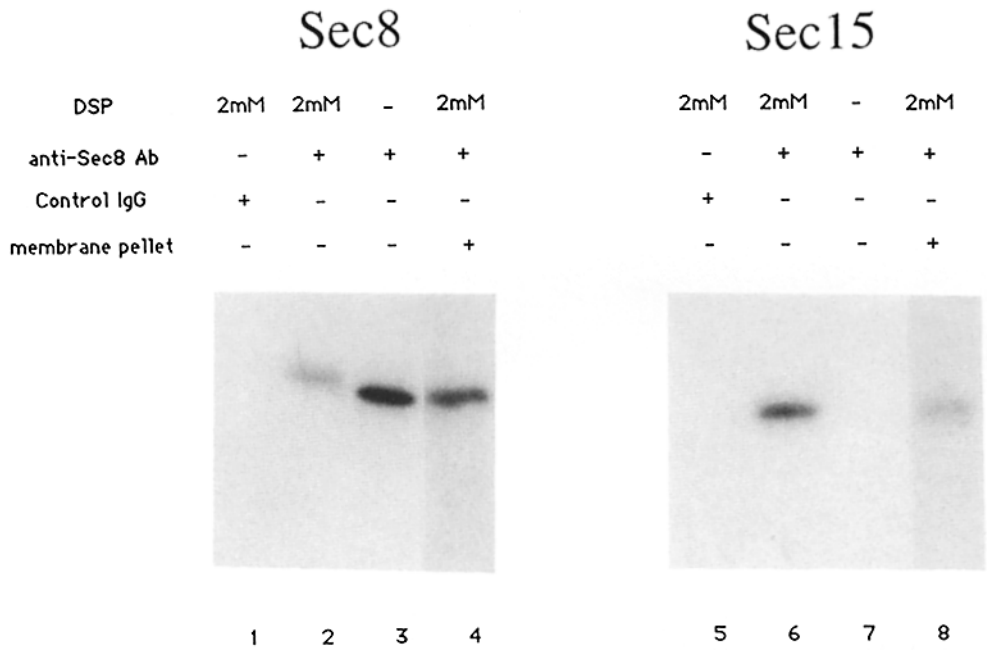

Figure 9. Sec8p and Sec15p are associated in a complex on the plasma membrane and can be released from the membrane in a complex. A plasma membrane containing pellet was isolated from NY13 cells. In lanes *1–3* and *5–7* we incubated the membrane in lysis buffer at pH 8.0 for 2 h. The sample was then centrifuged at 10,000 *g* for 10 min to pellet the membrane. The supernatant was removed and analyzed as follows. In lanes *1* and *5* the supernatant was immunoprecipitated with a rabbit anti-mouse control IgG. Sec8p and Sec15p fail to precipitate in the presence of the control IgG. In lanes *2* and *6* the supernatant was cross-linked with DSP and immunoprecipitated with anti-Sec8 antibody. In lanes *3* and *7* the supernatant was 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. 8 *c*, 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-elute 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-elute 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 cross-linking 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 dissociated 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 column fractionation and incubated them at 4°C for 3 h, the time it takes to perform the column fractionation. Both the 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 ^{125}I-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.

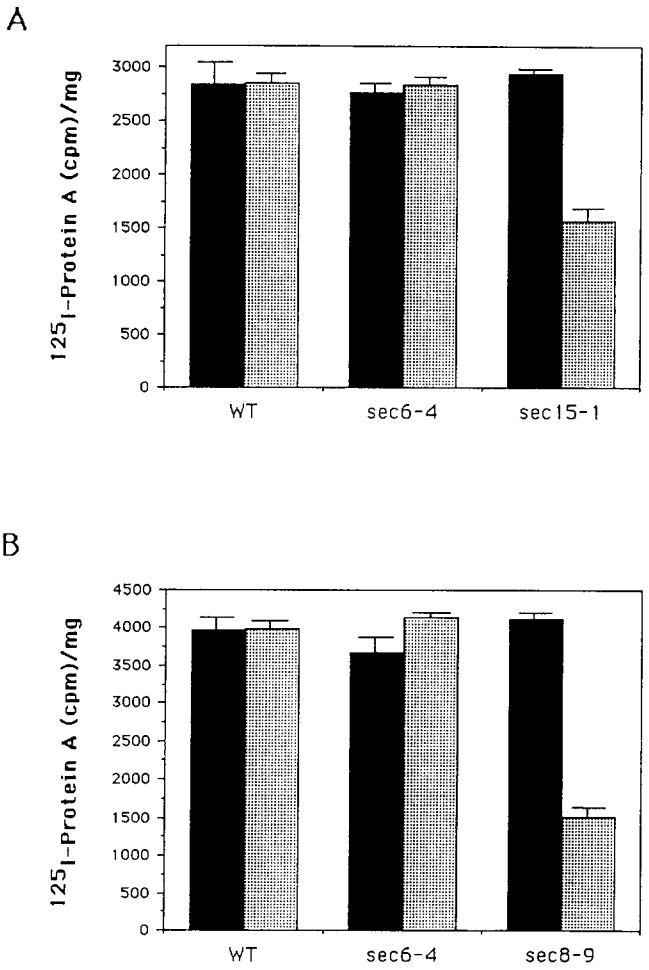

Figure 10. The stability of Sec8p and Sec15p is affected by *sec8-9* and *sec15-1* mutations but not by a *sec6-4* mutation. 10,000 *g* supernatants were prepared from NY13 (*SEC8, SEC15*), NY17 (*sec6-4*), NY410 (*sec8-9*), and NY64 (*sec15-1*) cells. All supernatants had a protein concentration of 20 mg/ml. Equal volumes of each sample were analyzed by immunoblot analysis either immediately (■) or after dilution in lysis buffer to 0.5 mg/ml and incubation at 4°C for 3 h (▨). The samples were probed for Sec8p (*A*) or Sec15p (*B*). The results, normalized for protein concentration, represent the average of two to four independent experiments and standard deviations are shown.

Sec8p May Interact With Sec4p

As the amino terminus of Sec8p is similar in sequence to the

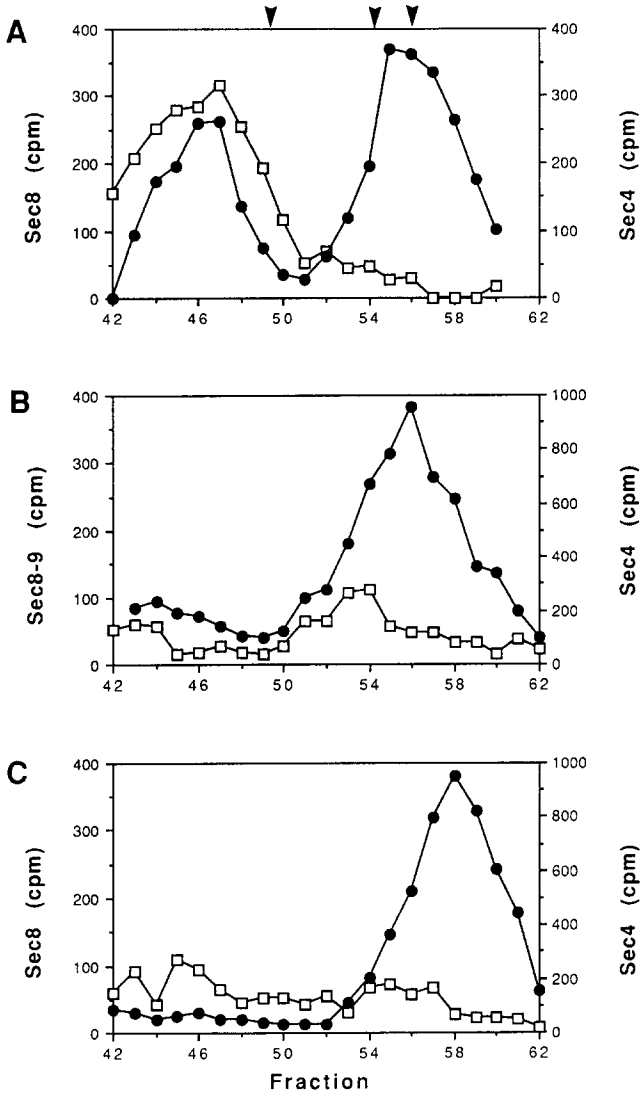

Figure 11. Sec4p co-elutes with the Sec8p/Sec15p complex by gel filtration. 10,000 *g* supernatants from NY13 (*SEC8, SEC15*), NY17 (*sec8-9*), and NY64 (*sec15-1*) cells were analyzed by Sephacryl S-500 gel filtration and the fractions assayed for the presence of Sec8p and Sec4p by quantitative immunoblot analysis. The results are expressed as the amount of ^{125}I-Protein A (*cpm*) specifically bound per sample. (*A*) Column fractionation of 20.5 mg of a NY13 supernatant. Sec8p (-□-) elutes in a single peak as observed in Fig. 9. Sec4p (-●-) elutes in two distinct peaks. The recovery of Sec8p from the column was 97%. Vertical arrows, from left to right, mark the location of thyroglobulin (669 kD), β-amylase (200 kD), and BSA (66 kD). (*B*) S-500 gel filtration of 20.5 mg of a *sec8-9* supernatant. The sec8-9 protein (-□-) and Sec4p (-●-) elution profiles are shown. The recovery of sec8-9 protein from the column was 95% and the recovery of Sec4p was 50%. (*C*) S-500 gel filtration of 20.5 mg of a *sec15-1* supernatant. Sec8p (-□-) and Sec4p (-●-) elution profiles. The recovery of Sec8p from the column was 40% and the recovery of Sec4p from the column was 70%.

region of yeast adenylate cyclase implicated in interaction with *RAS* (Fig. 2 *b*), we determined if Sec8p may associate with Sec4p, the member of the *ras* superfamily that functions in post-Golgi transport. It has been previously shown that Sec4p cycles between the plasma membrane and secretory vesicles through a soluble intermediate (Goud et al., 1988). 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 coincidently 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 Sec8/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 hydrophilic protein that functions, together with the Sec15 protein, in a 19.5*S* 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 *SEC8* 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 Ras (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 19.5*S* 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 exocytotic and endocytic pathways (Chavrier et al., 1990*a*,*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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