Sec6, Sec8, and Sec15 Are Components of a Multisubunit Complex Which Localizes to Small Bud Tips in *Saccharomyces cerevisiae*

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Abstract. In the yeast *Saccharomyces cerevisiae*, the products of at least 14 genes are involved specifically in vesicular transport from the Golgi apparatus to the plasma membrane. Two of these genes, *SEC8* and *SEC15*, encode components of a 1–2-million D multisubunit complex that is found in the cytoplasm and associated with the plasma membrane. In this study, oligonucleotide-directed mutagenesis is used to alter the COOH-terminal portion of Sec8 with a 6-histidine tag, a 9E10 c-*myc* epitope, or both, to allow the isolation of the Sec8/15 complex from yeast lysates either by immobilized metal affinity chromatography or by immunoprecipitation. Sec6 cofractionates with Sec8/15 by immobilized metal affinity chromatography, gel filtration chromatography, and by sucrose velocity centrifugation. Sec6 and Sec15 coimmunoprecipitate from lysates with c-*myc*–tagged Sec8. These data indicate that the Sec8/15 complex contains Sec6 as a stable component. Additional proteins associated with Sec6/8/15 were identified by immunoprecipitations from radiolabeled lysates. The entire Sec6/8/15 complex contains at least eight polypeptides which range in molecular mass from 70 to 144 kD. Yeast strains containing temperature sensitive mutations in the *SEC* genes were also transformed with the *SEC8-c-myc-6-histidine* construct and analyzed by immunoprecipitation. The composition of the Sec6/8/15 complex is disrupted specifically in the *sec3-2*, *sec5-24*, and *sec10-2* strain backgrounds. The c-*myc*-Sec8 protein is localized by immunofluorescence to small bud tips indicating that the Sec6/8/15 complex may function at sites of exocytosis.

In the yeast *Saccharomyces cerevisiae*, genetic selections have identified ten *SEC* (Novick et al., 1981), two *SNC* (Gerst et al., 1992), and two *SSO* (Aalto et al., 1993) genes that are uniquely required for protein transport from the Golgi apparatus to the plasma membrane. One of these genes, *SEC4*, encodes a small GTP-binding protein of the rab branch of the ras superfamily (Salminen and Novick, 1987). The cycle of GTP binding and hydrolysis by Sec4 is thought to be coupled to a cycle of localization in which GTP-bound Sec4 associates first with secretory vesicles, which are then targeted to and fuse with the plasma membrane, followed by hydrolysis of GTP to GDP by Sec4 (Bourne, 1988; Goud et al., 1988; Walworth et al., 1989). The GDP-bound Sec4 is then extracted from the plasma membrane by Gdi1 (GDP dissociation inhibitor) into the cytosol where it is available for another cycle (Garrett et al., 1993, 1994). Genetic evidence indicates that Sec4 is a key regulator of vesicular transport (Salminen and Novick, 1987), but cannot represent the sole source of vesicle targeting information (Brennwald and Novick, 1993).

A recent proposal regarding the molecular basis of targeting is that each class of carrier vesicles has a unique integral membrane protein, termed a vesicle SNARE (v-SNARE)[1], which interacts with a specific protein on the target organelle, a target SNARE (t-SNARE), and through the interaction of these proteins, vesicles fuse with the appropriate membrane compartment (Söllner et al., 1993; Rothman and Warren, 1994). The hypothesis is based on the observation that synaptobrevin, a protein localized to the surface of synaptic vesicles (also called VAMP) (Trimble et al., 1988; Baumert et al., 1989), associates with syntaxin and SNAP-25, proteins localized to the presynaptic membrane (Oyler et al., 1989; Bennett et al., 1992). A stable association of these proteins is detected when detergent-solubilized membranes are incubated in the presence

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1. *Abbreviations used in this paper*: IDA, iminodiacetate; NSF, *N*-ethylmaleimide–sensitive factor; PBT, PBS containing 5 mg/ml BSA, 5 mg/ml bovine gelatin, 5 mg/ml fish skin collagen, and 0.5% Tween 20; PIC, protease inhibitor cocktail containing 1 μg/ml of leupeptin, pepstatin, chymostatin, aprotinin, and antipain; S10, the supernatant of a 10,000 *g* spin for 10 min; S30, the supernatant a 30,000 *g* spin for 30 min; SD, synthetic minimal medium plus 2% dextrose; SNAP, soluble NSF attachment protein; t-SNARE, target SNARE; v-SNARE, vesicle SNARE; YPD, medium containing 1% bacto-yeast extract, 2% bacto-peptone, and 2% dextrose.

of recombinant NSF (*N*-ethylmaleimide–sensitive factor), α- and γ-SNAPs (soluble NSF attachment proteins), and ATPγS (Söllner et al., 1993). Binding of the cytoplasmic domains of synaptobrevin and syntaxin can be seen even in the absence of NSF and SNAP (Calakos et al., 1994). These mammalian proteins each have yeast counterparts: Sncl and 2 are homologous to synaptobrevin and are localized on the surface of secretory vesicles (Gerst et al., 1992; Protopopov et al., 1993); Ssol and 2 are homologous to syntaxin and Sec9 is homologous to SNAP-25 and they are localized to the plasma membrane (Aalto et al., 1993; Brennwald et al., 1994); Sec18 is homologous to NSF (Wilson et al., 1989), and Sec17 is homologous to α-SNAP (Griff et al., 1992). In addition, Snc and Sso both coimmunoprecipitate with Sec9 from detergent solublized yeast lysates indicating that these components interact in yeast (Brennwald et al., 1994).

There are several complicating issues yet to be resolved with the SNARE hypothesis as it applies to post–Golgi secretion in yeast. First, Sso and Snc are integral membrane proteins which must traffic through the entire secretory pathway to get to the sites of their biological activity. For the cell to prevent mistargeting of transport vesicles containing these SNAREs, there must be a way to regulate their activity. Recently, the homologue of Sec4 involved in ER to Golgi transport in yeast, Ypt1, was shown to be required for the interaction of the v-SNAREs, Sec22, and Bos1 (Lian et al., 1994) and subsequent binding of these v-SNAREs to the t-SNARE, Sed5 (Søgaard et al., 1994). Ypt1 did not appear to directly activate the v-SNAREs for binding, but seemed to require other protein factors (Lian et al., 1994). Second, Sso and Sec9, the presumed t-SNARES, are distributed over the entire inner surface of the plasma membrane and are not concentrated at sites of exocytosis. Thus, they cannot solely determine the appropriate targeting of a secretory vesicle. An analysis of the genetic interactions between the ten late-acting *SEC* genes and those genes encoding the SNAREs and the SNARE regulatory proteins may yield a clue as to which encoded proteins may be important for v-SNARE activation or vesicle targeting. Overexpression of *SSO1* or *SSO2* partially suppresses the temperature-sensitive growth defects of *sec1-1*, *sec3-2*, *sec5-24*, *sec9-4*, and *sec15-1* (Aalto et al., 1993), while overexpression of *SEC9* suppresses the growth defects of *sec1-1*, *sec3-2*, *sec8-9*, and *sec15-1* (Brennwald et al., 1994). Duplication of *SEC4* suppresses the growth defects of *sec2-41*, *sec8-9*, and *sec15-1* very well, and those of *sec1-1*, *sec5-24*, *sec10-2*, and *sec19-1* somewhat (Salminen and Novick, 1987). Thus, one or more of these gene products may be involved in regulating the activation of the SNAREs or providing the necessary information for correct vesicle targeting.

Prior studies have shown that Sec8 and Sec15 are part of a multiprotein complex. Sec8 and Sec15 comigrate by gel filtration chromatography and by sucrose velocity gradient centrifugation with an apparent size of 1 to 2 million D (Bowser and Novick, 1991; Bowser et al., 1992). The subcellular distributions of Sec8 and Sec15 are virtually identical with ~20–25% of each associated with the plasma membrane (Bowser and Novick, 1991; Bowser et al., 1992). The complex is disrupted in lysates from either *sec8-9* or *sec15-1* strains (Bowser et al., 1992). In addition, Sec15 can be detected in native Sec8 immunoprecipitates (Bowser et al., 1992). Taken together, these data indicate that Sec8 and Sec15 are components of a large complex, but further analysis has been hindered by the lack of appropriate immunological tools necessary to efficiently recover the complex (Bowser et al., 1992).

In this report, we have tagged the dispensable, COOH-terminal portion of Sec8 with a 6-histidine sequence, a c-*myc* epitope, or both. These tags allow the isolation of the Sec8/15 complex from yeast lysates by immobilized metal affinity chromatography or by immunoprecipitation. In addition to Sec8 and Sec15, the isolated complex contains Sec6 and five unidentified polypeptides which range in molecular mass from 144 to 70 kD. The composition of the Sec6/8/15 complex immunoisolated from radiolabeled strains containing the *sec3-2*, *sec5-24*, and *sec10-2* backgrounds is disrupted; in each strain there is a distinct and specific deletion of a subset of the eight polypeptides from the Sec6/8/15 complex. Thus, at least one biochemical function of the Sec3, Sec5, and Sec10 proteins is to promote the assembly of, or maintain the integrity of, the Sec6/8/15 complex. The presence of the c-*myc* tag on Sec8 has allowed us to localize the Sec6/8/15 complex by immunofluorescence to small bud tips. Based on the genetic interactions and the bud tip localization of the Sec6/8/15 complex, we suggest that it may function as an additional discriminator which determines whether a secretory vesicle is targeted to the plasma membrane.

Materials and Methods

Reagents

Oligonucleotides for mutagenesis and primers for sequencing and PCR were prepared by DNA Laboratory, Group No. 241, Yale University (New Haven, CT). Restriction enzymes, T4 DNA ligase, and polymerase were from New England Biolabs Inc. (Beverly, MA). Taq polymerase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bacto peptone, bacto tryptone, bacto agar, yeast nitrogen base without amino acids, and bacto yeast extract were from Difco Laboratories Inc. (Detroit, MI). Zymolyase 100T was from Seikagaku Corp. (Tokyo, Japan). Secondary, tertiary, and quaternary antibodies used for immunofluorescence were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The 9E10 ascites was prepared by the Pocono Rabbit Farm & Laboratory, Inc. (Canadensis, PA). The Nutridoma-grown 9E10 antibody was prepared at the Department of Cell Biology Core Facility, Yale University. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), J. T. Baker Chemical Co. (Phillipsburg, NJ) or from American Bioanalytical (Natick, MA) except as noted in the text.

Antibodies

A Sec1-glutathione S-transferase fusion protein was constructed for the preparation of polyclonal antibodies (Smith and Johnson, 1988). PCR was performed using pNB498, a YcP50-based plasmid containing *SEC1*, as template. The PCR primers were constructed to place a BamHI site at the 3′ end and an EcoRI site at the 5′ end of the 515-bp segment of *SEC*1 (bp 1660–2175 which correspond to amino acids 554–724). The PCR yielded a single 0.5-kb fragment of DNA which was gel purified and ligated into a BamHI-EcoRI–digested pGex-2T (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to yield pNB648. This ligation was transformed into XL1Blue cells and encodes a GST-Sec1 fusion protein of 45 kD which is expressed in response to induction with isopropyl-β-D-thiogalactopyranoside. Induced fusion protein was isolated on glutathione–agarose beads, released from the beads by boiling in sample buffer, and further purified by SDS-PAGE (Laemmli, 1970) and electroelution. Rabbit antiserum against the purified, denatured fusion protein was generated by Cocalico Biologicals (Reamstown, PA). The polyclonal serum recognized an 85-kD

protein which was overexpressed in lysates from yeast transformed with a 2-μm vector containing *SEC1* and decreased in lysates from *sec1-1* yeast (data not shown). Taken together, these results indicate that the polyclonal serum recognized yeast Sec1.

Oligonucleotide-directed Mutagenesis

SEC8 was subcloned to facilitate the preparation of ssDNA for oligonucleotide-directed mutagenesis. pBluescript II KS(+) (Stratagene Inc., La Jolla, CA) was digested with SmaI-SalI and gel purified. pNB328 (contains *SEC8* as a 4.4-kb SmaI-SalI fragment in YEp24 vector) (Carlson and Botstein, 1982; Bowser et al., 1992) was digested with SmaI-SalI and gel purified. The 4.4-kb SmaI-SalI, *SEC8*-containing fragment and the SmaI-SalI digested pBluescript II KS(+) were ligated and transformed into TG1 cells. The plasmid (pNB504) was recovered and the construction confirmed by digestion with SmaI, SalI, EcoRI, HindIII, and XhoI. pNB504 was transformed into CJ236 (*dut–, ung–*) and ssDNA was isolated. Next, mutagenesis was carried out as previously described (Kunkel et al., 1987) to introduce a c-*myc* epitope (9E10, [Evan et al., 1985] or a 6-histidine epitope into the DNA sequence corresponding to the dispensable COOH-terminal portion of Sec8 [Bowser et al., 1992]). The products of the mutagenesis reaction were transformed into TG1 cells, colony purified, and both ssDNA and dsDNA was isolated from each colony. The plasmids containing *SEC8-c-myc* and *SEC8-6-his* in pBluescript II KS(+) were designated pNB650 and pNB651, respectively. ssDNA from pNB651 was mutagenized to place a c-*myc* epitope adjacent to the 6-histidine epitope of *SEC8-6-his*. The product of the mutagenesis reaction was transformed into TG1 cells, colony purified, and both ssDNA and dsDNA were isolated. The plasmid containing *SEC8-c-myc-6-his* in pBluescript II KS(+) was designated pNB555. All constructs were verified by direct DNA sequencing of the ssDNA (Sanger et al., 1977).

Construction of Yeast Strains

The epitope-tagged *SEC8* constructs were subcloned into a yeast shuttle vector suitable for integration at *LEU2*. pNB399 (pRS305 in Sikorski and Heiter, 1989) was digested with SacI-XbaI and subsequently treated with dNTPs and T4 polymerase to form blunt-ended DNA. The product was gel purified, ligated, and transformed into TG1 cells to yield pNB499 (which lacks the BstxI site in the polylinker region). pNB499 was digested with SmaI-SalI and gel purified. The SmaI-SalI fragments containing *SEC8-c-myc*, *SEC8-6-his*, and *SEC8-c-myc-6-his* from pNB650, pNB651, and pNB555, respectively, were gel purified. The SmaI-SalI digested pNB499 and was mixed respectively with the SmaI-SalI fragments containing *SEC8-c-myc*, *SEC8-6-his*, and *SEC8-c-myc-6-his*, ligated, and transformed into TG1 cells. The constructions of the recovered plasmids (pNB557, pNB558, and pNB583, respectively) were verified by restriction digest. For integration into the yeast genome at *LEU2*, a double-strand break was introduced at *LEU2* by digestion with BstxI.

Competent NY813 cells (*MATa/α, leu2–3, 112/leu2–3, 112, ura3–52/ura3–52, SEC8/sec8::pNB338URA3*) were prepared by the alkali cation method (Ito et al., 1983) and transformed with BstxI-digested pNB557, pNB558, and pNB583, respectively. Transformants were plated on selective medium at 25°C. Tetrads from positive transformants were dissected and the haploid progeny were analyzed for the presence of the *LEU* and *URA* markers. The presence of the respective epitope-tagged *SEC8* alleles was indicated by the presence of the *LEU2* gene and the lack of a wild-type *SEC8* was indicated by the presence of the *URA3* gene. All strains transformed with the epitope-tagged *SEC8* as the sole copy of *SEC8* grew like wild-type *SEC8* strains at temperatures ranging from 14 to 37°C. The strains containing *SEC8-c-myc*, *SEC8-6-his*, and *SEC8-c-myc-6-his* were designated NY1008, NY1012, and NY1115, respectively.

Additional strains were constructed which contain both *SEC8-c-myc-6-his* and *SEC8* in various other *sec* backgrounds by transformation with BstxI cut pNB583. The genotypes of the untransformed and transformed yeast strains and of the other strains used in this study are found in Table I.

S-500 Column Fractionation and Ni^{2+} Resin Column Fractionation of Lysates

Overnight cultures (300 ml) of NY13 (wild-type Sec8) or NY1012 (6-histidine–tagged Sec8) were grown to an A_{599} of 0.9–1.2 in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) at 25°C. The yeast were pelleted and resuspended in 20 ml of spheroplasting wash buffer (10 mM NaN_3, 20 mM triethanolamine, pH 7.5, 1.4 M sorbitol). The yeast were pelleted again and resuspended in 20 ml of spheroplasting medium (spheroplasting wash buffer containing 40 mM 2-mercaptoethanol and 0.125–0.2 mg/ml of Zymolyase-100T) and incubated at 37°C for 45 min to form spheroplasts. The spheroplasts were washed, pelleted again, and resuspended in 4 ml of ice-cold lysis buffer (0.8 M sorbitol, 1.0 mM EDTA, 20 mM triethanolamine, pH 7.2, 1 mM DTT, 1 mM PMSF, and 1× PIC [Protease inhibitor cocktail; 1× concentrations are 1 μg/ml of leupeptin, pepstatin, chymostatin, aprotinin, and antipain]). The resuspended spheroplasts were Dounce homogenized and 10,000 × *g* supernatants (S10 fraction) prepared as previously described (Bowser et al., 1992). The protein concentration of the supernatants were usually between 15 and 20 mg/ml when measured against bovine IgG (Bradford, 1976).

Typically, 1.5–3.5 ml of S10 lysate was applied to the top of a 90-ml gel filtration column (Sephacryl S-500; Pharmacia LKB Biotechnology Inc.) equilibrated with 40 mM sodium phosphate, pH 7.5, 50 mM NaCl, and 1 mM DTT. 20 drop fractions were collected and the fractions corresponding to the peak containing Sec8, Sec6, and Sec15 were pooled and adjusted to 20 mM imidazole. The pooled fractions were applied to a 2-ml Ni^{2+}-iminodiacetate (IDA) column (Invitrogen, San Diego, CA), and equilibrated with 40 mM sodium phosphate, pH 7.5, 50 mM NaCl, and 20 mM imidazole. The flow-through was collected and reapplied for four more cycles. The column was then washed four times with 8 ml of buffer containing 40 mM sodium phosphate, pH 7.5, 50 mM NaCl, 0.3 mM DTT, and 20 mM imidazole and then eluted four times with the same buffer containing 250 mM imidazole. All of the fractions obtained from the Ni^{2+}-column fractionation were analyzed by SDS-PAGE (Laemmli, 1970) and Western blot as previously described except the incubation times were 1.5 h for the antibodies and the ^{125}I-protein A (Salminen and Novick, 1989). The primary antibodies used to detect the transferred proteins included rabbit anti-$Sec15^{1-241}$ (1:1,000) (Salminen and Novick, 1989), rabbit anti-$Sec8^{78-316}$ (1:1,000) (Bowser and Novick, 1991), rabbit anti-$Sec6^{247-573}$ (1:1,000) (Potenza et al., 1992), rabbit anti-$Sec2^{61-338}$ (1:1,000) (Nair et al., 1990), rabbit anti-$Sec1^{554-724}$ (1:2,000) (see above), rabbit anti-$Sec4^{1-215}$ (1:1,000) (Goud et al., 1988), rabbit anti-$Gdi1^{191-451}$ (1:3,000) (Garrett et al., 1994), and rabbit anti-$Sec9^{582-651}$ (1:250) (Brennwald et al., 1994). In some experiments, fractions from the Ni^{2+}-column fractionation were blotted for GTP-binding proteins using [α-^{32}P]GTP (Amersham Corp. Arlington Heights, IL) according to Lapetina and Reep (1987).

c-myc Immunoprecipitation from Nonradioactive Lysates

Lysates for immunoprecipitations were prepared as above except strains NY13 and NY1115 (c-*myc*– and 6-histidine–tagged Sec8) were typically used. Protein (S10 fraction) was diluted to a final concentration of 4 mg/ml with dilution buffer (PBS, pH 7.2, containing 0.1% Tween 20, 1 mM PMSF, and 2× PIC). The lysates (1 ml each) were precleared by the addition of 5 mg of hydrated protein A for 1.5 h at 4°C followed by spinning for 15 min in an Eppendorf microfuge. The supernatants were transferred to fresh tubes and 4 μl of c-*myc* (9E10) ascites was added to each tube (except minus antibody controls). The tubes were rocked overnight at 4°C on a platform shaker and then 5 mg of hydrated protein A was added to each tube for 1.5 h. The protein A–bound immune complexes were cleared from solution by spinning 10 s in a 4°C microfuge. The supernatants were removed and 1 ml of the dilution buffer lacking PIC and PMSF was added to each tube. The beads were pelleted 10 s in a 4°C microfuge and the supernatants removed. The wash/spin cycle was repeated two more times. 75 μl of sample buffer was added to the beads and the tubes were boiled for 3 min to release the immunoprecipitates. The released proteins were then analyzed by SDS-PAGE and Western blotting as above. Immunoprecipitates were screened with the same antibodies as the Ni^{2+} column fractions and in addition antibodies generated in rabbits against $Myo2^{833-1574}$ (affinity purified, 1:50 dilution) and $Smy1^{410-647}$ (serum, 1:67 dilution) (Lillie and Brown, 1994).

c-myc Immunoprecipitation from [^{35}S]Methionine/Cysteine–labeled Yeast

50-ml cultures of NY13 and NY1115 were grown overnight to an A_{599} of 0.6–0.9 in SD (synthetic minimal medium plus 2% dextrose) supplemented for auxotrophic requirements at 25°C. Aliquots of the cultures were spun down at room temperature and the pellets resuspended in 1 ml of fresh, supplemented SD for each multiple of 0.75 A_{599} U. 10 μl of label mix (label mix contains 14.3 μCi/μl of total labeled [^{35}S]methionine/cysteine, Amersham Corp., Arlington Heights, IL) was added for each 0.75

Table I. Yeast Strains

Strain	Genotype
NY13	*MATa, ura3-52*
NY177	*MATα, leu2–3, 112*
NY179	*MATa, leu2-3, 112, ura3-52*
NY180	*MATα, leu2-3, 112, ura3-52*
NY769	*MATa, leu2–3, 112, sec1-1*
NY771	*MATa, leu2–3, 112, sec2–41*
NY774	*MATα, leu2-3, 112, ura3-52, sec4-8*
NY776	*MATα, leu2–3, 112, ura3-52, sec5-24*
NY779	*MATα, leu2–3, 112, sec6–4*
NY781	*MATα, leu2–3, 112, sec8-9*
NY782	*MATα, leu2–3, 112, ura3-52, sec9-4*
NY784	*MATa, leu2–3, 112, ura3-52, sec10-2*
NY787	*MATα, leu2–3, 112, sec15–1*
NY813	*MATa/α, leu2–3, 112/leu2–3, 112, ura3–52/ura3–52, SEC8/sec8::pNB338Ura+* (*SEC8* disruption)
NY1008	*MATa, ura3–52, sec8Δ::URA3, leu2–3, 112::(LEU2,SEC8-c-myc)* (Sole functional copy of *SEC8* is *c-myc*-tagged)
NY1012	*MATa, ura3–52, sec8Δ::URA3, leu2–3, 112::(LEU2, SEC8-c-myc)* (Sole functional copy of *SEC8* is *6-histidine*-tagged)
NY1115	*MATα, ura3–52, sec8Δ::URA3, leu2–3, 112::(LEU2, SEC8-c-myc-6-his)* (Sole functional copy of *SEC8* is *c-myc*– and *6-histidine*–tagged)
NY1182	*MATα, leu2–3, 112::(LEU2, SEC8-c-myc-6-his)*
NY1183	*MATa, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec2–41*
NY1184	*MATα, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec6–4*
NY1185	*MATα, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec15–1*
NY1192	*MATa, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec1-1*
NY1193	*MATα, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec4-8*
NY1217	*MATa, leu2–3, 112, ura3-52, sec18-1*
NY1219	*MATa, leu2–3, 112, ura3-52, sec17-1*
NY1224	*MATα, leu2–3, 112, ura3-52, sec3-2*
NY1244	*MATα, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec3-2*
NY1245	*MATα, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec5-24*
NY1246	*MATα, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec8-9*
NY1247	*MATa, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec9-4*
NY1248	*MATα, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec10-2*
NY1249	*MATa, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec17-1*
NY1250	*MATa, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his), sec18-1*
NY1266	*MATa, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his)*
NY1267	*MATα, ura3-52, leu2–3, 112::(LEU2, SEC8-c-myc-6-his)*

A_{599} U of yeast and the cultures were incubated for 2 h at 25 or 30°C in a slowly shaking water bath. Spheroplasts were formed as described above except that the volumes were scaled down and the incubation temperature was 25°C for temperature-sensitive strains and their controls. The spheroplasts were lysed with 1 ml of buffer A (40 mM Pipes, pH 6.8, 100 mM NaCl, 0.5% Tween-20, 2× PIC, 1 mM PMSF) for every multiple of 0.75 A_{599} U of starting yeast. The concentration of protein (BCA protein assay; Pierce Chemical Co., Rockford, IL) and the TCA-precipitable counts were determined. After the protein assay, DTT was added to a final concentration of 1 mM. In some experiments, parallel cultures were grown without addition of [^{35}S]methionine/cysteine for protein determinations. To each microfuge tube, 1 ml of the radioactive lysate (250–350 μg total protein/tube), 3 mg of hydrated protein A, and 4 mg of nonradiolabeled protein (P30 fraction made from NY13) were added. The immunoprecipitations were then performed the same as for nonradiolabeled immunoprecipitations (described above) except that Nutridoma-grown 9E10 antibody (20 μl/tube) was used instead of ascites and buffer A lacking PIC and PMSF was used instead of PBS. After the final washes, 25–75 μl of sample buffer was added to each tube. The beads were then boiled for 3 min and the released proteins separated by SDS-PAGE. The gels were stained with Coomassie blue, destained, incubated with water for 15 min, and then Autofluor (National Diagnostics, Inc., Atlanta, GA) for 1 h. The gels were then dried to filter paper and exposed to X-OMAT film (Eastman Kodak Co., Rochester, NY) at –80°C for one or more days. The radioactively labeled bands were cut from the gels using the autoradiogram as a template, depolymerized in 30% H_2O_2 at 80°C overnight, and counted in scintillation fluid.

In some experiments, the radiolabeled proteins were transferred to nylon membranes and the membranes exposed to Kodak X-OMAT film at –80°C for several days to generate an image of the labeled proteins. The membranes were then Western blotted for Sec8, Sec6, or Sec15 using the Rad-Free chemiluminescent detection system (Schleicher & Schuell, Inc., Keene, NH) exactly as detailed in the product literature.

Sucrose Velocity Gradients

Yeast strains containing or lacking c-*myc*-6-histidine–tagged Sec8, were grown overnight at 25°C in 400 ml of YPD to a final A_{599} between 0.8 and 1.0 U. The yeast were spheroplasted and lysates prepared as described above except the concentration of sorbitol in the lysis buffer was 0.2 M. The lysates were then spun 30,000 *g* for 30 min at 4°C. The supernatant (S30 fraction) was carefully removed and the protein measured (Bradford, 1976) using bovine IgG as a standard. The protein concentration was then adjusted to ~10 mg/ml with lysis buffer and Triton X-100 was added to 0.1%.

The lysates or a mixture of molecular mass marker proteins made up in lysis buffer were carefully layered (0.5 ml) on top of 10–30% continuous sucrose gradients (4.5 ml) and spun for 2 h at 49,000 rpm in a rotor (SW50.1; Beckman Instruments, Fullerton, CA) as previously described except that the gradients contained 1 mM DTT (Bowser and Novick, 1991). 15 0.33-ml fractions were obtained by pipetting from the top and the amount of protein in each gradient fraction was determined using bovine IgG as a standard (Bradford, 1976). The fractions collected from the molecular mass standards gradient were run on a 5–15% SDS–polyacrylamide gel and identified by Coomassie blue staining. The fractions from the cell lysate containing gradients were run on 8% SDS–polyacrylamide gels and transferred to nitrocellulose for Western blotting. The amount of detectable Sec8, Sec15, and Sec6 in each fraction was determined by cut-

ting and counting the amount of ^{125}I-protein A bound to the nitrocellulose with a gamma counter (Minigamma 1275; LKB-Wallac, Gaithersburg, MD).

Immunofluorescence

Yeast strains containing or lacking c-*myc*–tagged Sec8, were grown overnight at 25°C in YPD to a final A_{599} between 0.4 and 0.8 U. For asynchronous cultures, 10 ml of the overnight culture was removed to a tube containing 1.6 ml of 1.0 M potassium phosphate buffer, pH 6.5, and 1.6 ml of 37% formaldehyde. The culture was gently rocked for 20 min and then pelleted. The pellet was resuspended in 10 ml of freshly prepared 4% formaldehyde (Roberts et al., 1991) and rocked for 90 min. The yeast were then pelleted, washed with 10 ml of 0.1 M potassium phosphate (pH 7.5), pelleted again, and resuspended with 10 ml of buffer containing 1.2 M sorbitol and 0.1 M potassium phosphate (pH 7.5). The yeast were pelleted a final time and resuspended in 1 ml of the sorbitol/KPi buffer and stored overnight at 4°C. Cultures were sometimes synchronized by α–mating factor arrest before fixation. For synchronization experiments, overnight cultures were pelleted and resuspended in YPD (adjusted to pH 4.0 with HCl) at 0.2 A_{599} containing 2.0–2.5 μM yeast α–mating pheromone. The cultures were then shaken at 25°C for 2 h, pelleted, and then resuspended in fresh YPD (not pH adjusted) lacking α–mating factor. 10-ml aliquots were removed at various times during the α-factor incubation or after the culture was resuspended in fresh YPD. The 10-ml aliquots were fixed in formaldehyde as above.

After storage overnight at 4°C, the cell wall was removed and the yeast were permeabilized and attached to 8-well microslides (Carlson Scientific, Peotone, IL) according to Redding et al. (1991). The cells, after attachment, were washed three times with 20 μl of PBS containing 5 mg/ml BSA, 5 mg/ml bovine gelatin, 5 mg/ml fish skin collagen and 0.5% Tween 20 (PBT buffer). After incubating in PBT buffer for 15 min, the wells were successively incubated with 20 μl of nutridoma grown, 9E10, anti-c-*myc* antibody (1° antibody), 20 μl of goat anti–mouse antibody (2° antibody), 20 μl of mouse anti–goat antibody (3° antibody), and finally with 20 μl of Texas red–conjugated donkey anti–mouse antibody (4° antibody). All antibody incubations were for 1 h and the dilutions are given in the figure legends. The wells were washed nine times with PBT after each antibody incubation. Mounting medium (Roberts et al., 1991) was then carefully dropped in between the wells and covered with a large coverslip. The edges were sealed with nail polish and the slides stored at -20°C. The 2° and 3° antibodies were precleared against fixed, permeabilized yeast cells lacking any c-*myc*–tagged proteins before use as described in Roberts et al. (1991).

The indirect immunofluorescence was visualized with a fluorescence microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) using the ×100 objective. 10–20-s exposures of the fluorescent images were taken with Tri-pan 400 ASA (Eastman Kodak Co.) (see Fig. 8) or T-Max 400 ASA (Eastman Kodak Co.) (see Fig. 7) film which was push-processed to 1600 ASA. All fluorescent images shown in a given figure were matched exactly in terms of exposure time, film developing, printing time, and enlargement.

Results

Sec8-6-his, Sec15, and Sec6 Cofractionate on an Ni^{2+} IDA Column

To facilitate further characterization and eventual purification of the Sec8/15 complex, we introduced several tags into Sec8. Previous work has shown that truncated Sec8, missing the COOH-terminal 60 amino acids, is fully functional for cell growth (Bowser et al., 1992). Various sequences were introduced into this region of Sec8 (Table II). The resulting strains (Table I) NY1008 (*SEC8-c-myc*), NY1012 (*SEC8-6-his*), and NY1115 (*SEC8-c-myc-6-his*) have the tagged *SEC8* allele as their sole copy of *SEC8*, yet grow at the same rate as wild-type with no discernible phenotype at all temperatures tested (14, 25, 30, 34, and 37°C). In this study, we used the tagged Sec8 proteins to facilitate isolation of the Sec8/15 complex by immunoprecipitation and Ni^{2+} resin column fractionation.

The initial approach was to fractionate lysates made from yeast strains containing or lacking 6-histidine–tagged Sec8 on an Ni^{2+} IDA column and analyze the fractions by Western blot for other proteins known to function in secretion between the Golgi apparatus and the plasma membrane. Cell lysates (S10 fraction) prepared from overnight cultures of NY13 (*SEC8*) and NY1012 (*SEC8-6-his*) were fractionated on parallel Sephacryl S-500 columns. Fractions were analyzed for Sec8 and Sec15 immunoactivity by Western blot. For both strains, Sec8 and Sec15 were found in the same fractions with a peak of immunoactivity around fraction 48 as was previously reported (Bowser et al., 1992). S-500 fractions 43–53 were pooled independently for NY13 and NY1012, adjusted to 20 mM imidazole, and applied to separate 2-ml Ni^{2+} IDA columns and the flow-through (*FT*) collected. The columns were washed (*W*) four times with buffer containing 20 mM imidazole and then eluted (*E*) four times with buffer containing 250 mM imidazole. The resulting fractions (*LOAD*, *FT*, *W1*, *W2*, *W3*, *W4*, *E1*, *E2*, *E3*, and *E4*) were run on 8, 12, or 15% polyacrylamide gels, transferred to nitrocellulose, and blotted for Sec8, Sec15, Sec6, Sec4, Sec2, and Gdi1 (Fig. 1). Very small amounts of Sec8 and Sec15 were eluted into the E2 fractions in a 6-histidine–tagged Sec8-independent manner in this experiment. However, both Sec8 and Sec15 were retained on the column and eluted in the E2 fractions in much greater amounts from NY1012 lysates which contain 6-histidine–tagged Sec8. Sec6 specifically eluted in the E2 fraction in a 6-histidine–tagged Sec8-dependent manner. Neither Sec4, Sec2, Sec1 (Fig. 1) nor Sec9 (data not shown) cofractionated with 6-histidine–tagged Sec8 indicating that these proteins are not stable members of the Sec8/15 complex. The transferred Ni^{2+} IDA column fractions were also analyzed for proteins which bind [α-^{32}P] GTP. Seven proteins which bind [α-^{32}P]GTP could be detected in the pooled S-500 column fractions

Table II. Location of c-myc and 6-histidine Tags on Sec8

Strain	Protein sequence
NY13: (wild-type Sec8)	1023-NKRYTEALEKLSNLEKEQSKEGARTKIGKLKSKLNAVHTANEK.-1065
NY1008: (c-*myc*–tagged Sec8; the codon for lysine-1053 was deleted in the mutagenesis reaction)	1023-NKRYTEALEKLSNLEKEQSKEGARTKIGKLEQKLISEEDLHTANEK.-1068
NY1012: (6-histidine–tagged Sec8)	1023-NKRYTEALEKLSNLEKEQSKEGARTKIGKLKAHHHHHHHTANEK.-1065
NY1115: (c-*myc*–and 6-histidine–tagged Sec8)	1023-NKRYTEAEQKLISEEDLQSKEGARTKIGKLKAHHHHHHTANEK.-1065

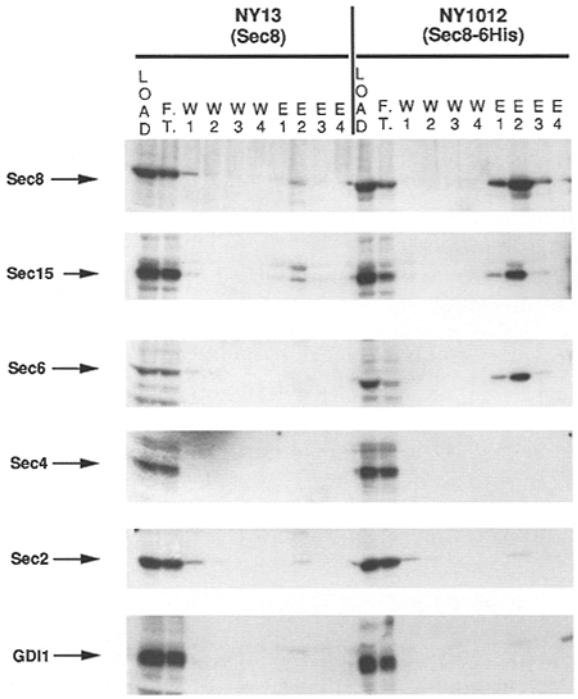


Figure 1. Analysis of proteins associated with 6 histidine–tagged Sec8 by immobilized metal affinity chromatography and Western blotting. S10 fractions from NY13 (wild-type Sec8) and NY1012 (6-histidine–tagged Sec8) were fractionated on an S-500 gel filtration column according to Methods. Peak fractions containing Sec8 immunoactivity were pooled, adjusted to 20 mM imidazole (*LOAD*), and applied to a 2-ml Ni^{2+} IDA column. The flow-through was passed over the Ni^{2+} IDA column four more times and saved (*FT*). The column was then washed four times (*W1*, *W2*, *W3*, and *W4*) with 8-ml aliquots of wash buffer (containing 40 mM $NaPO_4$, pH 7.5, 50 mM NaCl, 20 mM imidazole, and 0.3 mM DTT) and eluted (*E1*, *E2*, *E3*, and *E4*) with four 2-ml aliquots of elution buffer (wash buffer containing 250 mM imidazole). The samples were then analyzed by SDS-PAGE and Western blot for Sec8, Sec15, Sec6, Sec2, Gdi1, or Sec4. The results are representative of two or more experiments for each protein analyzed.

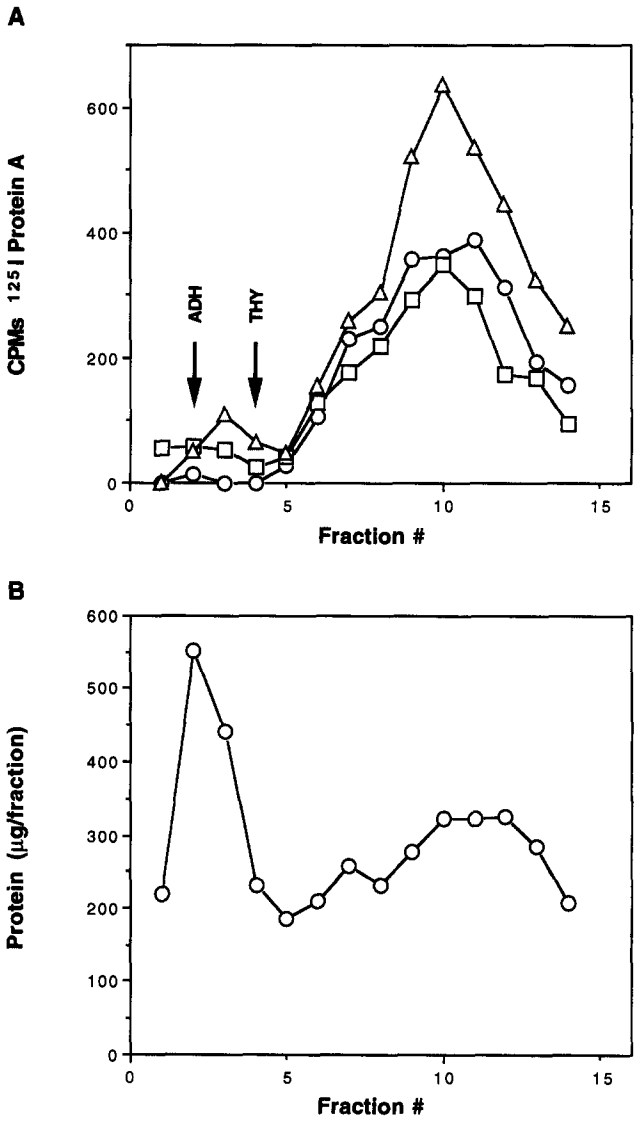


Figure 2. Sec8, Sec6, and Sec15 all cosediment on sucrose velocity gradients. An S30 fraction from NY13 (wild-type Sec8, 9.4 mg/ml protein) or molecular mass markers prepared in the same buffer were separated on continuous sucrose gradients (10–30% sucrose). (*A*) The distribution of Sec8 (○), Sec6 (□), and Sec15 (△) immunoactivity was determined by SDS-PAGE and Western blot. Equal volumes of each gradient fraction were analyzed for Sec8 and Sec15 and twice as much was analyzed for Sec6. The top of the gradient is on the left (*fraction #1*) and the position of yeast alcohol dehydrogenase (*ADH*, 7.4S) and bovine thyroglobulin (*THY*, 19.3S) are indicated by the arrows. (*B*) The distribution of protein across the gradient fractions. The amount of protein recovered was 85% of that loaded on top of the gradient. The results presented here are representative of six separate experiments spun at various times (1.67–8 h).

and the Ni^{2+} column flow-through for both NY13 and NY1012 lysates, but none of the GTP-binding proteins cofractionated with 6-histidine–tagged Sec8 (data not shown).

Analysis of the S-500 column fractions indicated that Sec6 cofractionates with Sec8 and Sec15 (data not shown). However, prior studies had yielded a value for the sedimentation coefficient of Sec6 which was slightly smaller than the value for Sec8 and Sec15 (Bowser et al., 1992; Potenza et al., 1992). We therefore reexamined the sedimentation properties of Sec6, Sec8, and Sec15. Lysate from NY13 (S30 fraction) was subjected to centrifugation through a 10–30% sucrose gradient, fractionated, and Western blotted for Sec8, Sec6, and Sec15. All three proteins cosedimented with their peak of immunoactivity in fraction 10 (Fig. 2). Lysates from NY1115 (*SEC8-c-myc-6-his*) were also analyzed, and yielded identical results indicating that the presence of the epitope tag on Sec8 did not disrupt the complex (data not shown). Using either tagged or untagged Sec8-containing strains, we observed no appreciable monomeric forms of Sec6, Sec8, or Sec15 (data not shown). These data indicate that Sec6 is tightly associated with Sec8 and Sec15 and the complex will hereafter be referred to as the Sec6/8/15 complex.

The S-500 column and Ni^{2+}IDA column steps together gave ~50-fold purification of the Sec6/8/15 complex that was dependent on the presence of the 6-histidine–tagged Sec8 protein (based on quantitative Western blot, data not shown). The fractions from the Ni^{2+} IDA columns were

also examined for the presence of specific protein bands by silver stain. At least 36 protein bands were eluted by 250 mM imidazole in the E2 fractions of both NY13 and NY1012 (data not shown) and there was no discernible difference in the pattern of silver-stained protein bands in the E2 fractions of the two strains. Therefore, this purification protocol was insufficient to allow visualization of Sec8, Sec15, Sec6, or any other specific component of the complex by silver staining.

Sec15 and Sec6 Coimmunoprecipitate with Sec8-c-myc

Independent experiments were performed with the c-*myc*–tagged *SEC8* strain to confirm the Ni^{2+} IDA column results and to attain a greater fold purification. NY13 (*SEC8*) and NY1115 (*SEC8-c-myc-6-his*) strains were grown overnight and cell lysates prepared (S10 fraction). The lysates were incubated overnight with or without 9E10 antibody directed against the c-*myc* epitope. The 9E10 antibody should immunoprecipitate the Sec6/8/15 complex in a c-*myc*–tagged Sec8-dependent manner. The immunoprecipitated proteins were analyzed by Western blot for Sec8, Sec15, Sec6, Sec2, and Sec1 (Fig. 3). Sec8 was only immunoprecipitated by the 9E10 antibody when it was tagged with the c-*myc* epitope. The 9E10 antibody coimmunoprecipitated Sec6 and Sec15 with the c-*myc*–tagged Sec8. In control lysates (lacking c-*myc*–tagged Sec8), neither Sec8, Sec6, nor Sec15 were immunoprecipitated. Sec2 and Sec1 were not immunoprecipitated from either yeast strain. Additional immunoprecipitation experiments have indicated that Sec9, Gdi1, Myo2, and Smy1 are not stable components of the Sec6/8/15 complex (data not shown). The immunoprecipitation experiment results confirm the Ni^{2+} column fractionation results: Sec6, Sec8, and Sec15 are all members of the same multiprotein complex.

Since we have reported previously that a portion of Sec4 cofractionates with Sec8 and Sec15 by gel filtration (Bowser et al., 1992), we examined the immunoisolated Sec6/8/15 complex for the presence of stably associated Sec4. Spheroplasts (strains NY13 and NY1008) were lysed with buffer containing 20 mM Pipes, pH 6.8, 100 mM NaCl, 0.5% Tween 20, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 2 × PIC in the presence or absence of 1 mM GDP/5 mM $MgCl_2$ or in the presence or absence of 1 mM GTP/5 mM $MgCl_2$. S30 fractions were prepared from the lysates and 40 mg of protein from each fraction was precleared, incubated overnight with 20 μl of 9E10 ascites, and then removed from solution with 40 mg of protein A beads. The beads were washed three times with 10 ml of the appropriate lysis buffer (lacking protease inhibitors), boiled in sample buffer, electrophoresed through 5–15% gradient gels, and transferred to nitrocellulose overnight. The resulting blots were probed for GTP-binding proteins with [α-^{32}P] GTP (Lapetina and Reep, 1987) and for Sec4 with anti-$Sec4^{1-215}$ (Goud et al., 1988). There was no detectable Sec4 coimmunoprecipitated with the c-*myc*–tagged Sec8 under any of the conditions examined (data not shown). We estimate that if 0.07% of the total Sec4 present in the starting lysates was associated with the immunoisolated Sec6/8/15 complex, it could be easily detected in this experiment (data not shown). Thus, only a fraction of Sec4 much smaller than this could be stably associated with the Sec6/8/15 complex.

Sec6/8/15 Complex Is Composed of Eight Polypeptides

To identify additional members of the Sec6/8/15 complex, ^{35}S-labeled lysates from NY13 (*SEC8*) and NY1115 (*SEC8-c-myc-6-his*) were incubated with the 9E10 antibody and the immunoprecipitates were subjected to SDS-PAGE on a 5–15% gradient gel (Fig. 4). The antibody specifically and reproducibly immunoprecipitated eight polypeptides in a c-*myc*–tagged Sec8-dependent manner. These range in molecular mass from 144 to 70 kD and the eight bands were assigned the letters *a* through *h* from highest to lowest molecular mass (Fig. 4). If all of the proteins are present in single copy, then the molecular mass of the complex would be 834 kD. This is significantly less than the 1–2-million D estimated by Sephacryl S-500 gel filtration (Bowser and Novick, 1991; Bowser et al., 1992). This suggests that the apparent molecular mass of the Sec6/8/15 complex could be overestimated by gel filtration, that it may be associated with other cellular components, or have a stoichiometry other than one for some or all of the polypeptides. To begin to address the stoichiometry of the components of the complex, radiolabeled bands were cut and counted from c-*myc*–tagged Sec8 immunoprecipitates from several experiments. All of the members of the complex were approximately equally labeled (data not shown) except the 144-kD band (band *a*) which consistently contained one third to one half the radioactivity of the other members. To account for its reduced labeling, band *a* may

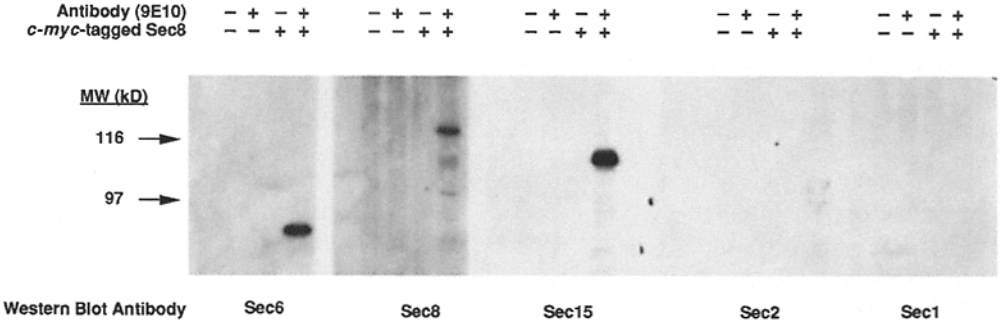
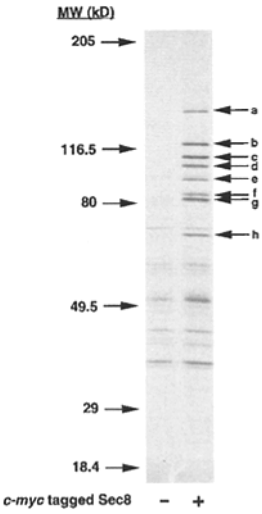


Figure 3. Analysis of proteins associated with c-*myc*–tagged Sec8 by immunoprecipitation and Western blotting. S10 fractions from NY13 (wild-type Sec8) and NY1115 (double-tagged Sec8 with 6-histidines and the c-*myc* epitope) were diluted to 4 mg/ml with PBS containing 0.1% Tween 20 and divided into 1 ml aliquots. The immunoprecipitations were performed according to Methods. After the final wash, the beads were then boiled with sample buffer, pelleted, and the supernatant analyzed by SDS-PAGE and Western blot for Sec6, Sec8, Sec15, Sec2, and Sec1. The results are representative of two or more experiments for each protein blotted.



Band	MW (kD)	Identity
a	144	
b	121	Sec8
c	113	Sec15
d	107	
e	100	
f	91	
g	88	Sec6
h	70	

Figure 4. Eight proteins are specifically immunoprecipitated by the 9E10 c-*myc* mAb from yeast lysates containing c-*myc*–tagged Sec8. Cultures of NY13 (wild-type Sec8) and NY1115 (double-tagged Sec8 with 6-histidines and the *c-myc*-epitope) were grown overnight in SD supplemented with uracil. For each gel lane, 0.75 A_{599} U of each yeast strain were pelleted and resuspended in 1 ml of fresh SD plus uracil. To each tube, 10 μl of 14.3 μCi/μl of [^{35}S]methionine/cysteine label mix was added and the cultures incubated for 2 h at 30°C. The yeast were then spheroplasted, lysed, and immunoprecipitated according to Methods except the protein A beads were washed one time quickly with 1 ml of buffer (containing 40 mM Pipes, pH 6.8, 100 mM NaCl, 0.5% Tween 20, and 1 mM DTT), washed again in the same buffer containing 300 mM NaCl for 3 h with gentle rocking at 4°C, and finally one more time quickly. The protein A beads were then boiled in sample buffer, pelleted, and the supernatant analyzed by SDS-PAGE on a 5–15% gradient gel and autoradiography. The estimated molecular masses are the average of four experiments.

have a low cysteine and methionine content, it may be present in only ~one-third the Sec6/8/15 complexes or all of the other bands may be present in two or three copies. Washing the immune complexes with high salt (300 mM NaCl for 3 h) significantly reduced the nonspecific proteins which adhere to the protein A beads (especially those with molecular masses of 89 and 71 kD which migrate adjacent to bands *g* and *h*, respectively), but only reduced the yield of the specifically immunoprecipitated bands by about one half (data not shown). This indicates that the proteins are tightly and stably associated with Sec8. Note that the strain (NY1115, *SEC8-c-myc-6-his*) used in this experiment contains a 6-histidine tag adjacent to the c-*myc* tag. Experiments with lysates from NY1008, which contains c-*myc*–tagged Sec8 as its only copy of Sec8, gave the same pattern and number of bands as lysates containing the c-*myc*- and 6-histidine–tagged Sec8 (data not shown). Thus, none of the proteins in the immunoprecipitate were due to nonspecific interaction with the 6-histidine tag. Since 20–25% of Sec8 and Sec15 are found on the plasma membrane, we determined if the membrane-associated pool of the complex had a different subunit structure. Immunoprecipitations were performed on radiolabeled S30 and solublized P30 fractions but there was no difference in the pattern or number of bands coimmunoprecipitated (data not shown). Since the solubilized membrane-associated complex and the complex found in the supernatant are identical in composition, there does not appear to be a detergent soluble "receptor" stably bound to the membrane-associated form of the Sec6/8/15 complex.

We were also able to calculate the percentage of cellular protein which is composed of the Sec6/8/15 complex and the fold purification of the complex achieved by immunoprecipitation from radiolabeled lysates. For the calculations, we assumed that the specific activity of the total cellular protein is the same as that for all members of the Sec6/8/15 complex and the yield of the complex from the immunoprecipitations is 10% (a reasonable estimate based on Western blotting of nonradiolabeled immunoprecipitations for Sec6, Sec8, and Sec15). The percentage of total cellular protein composed of the Sec6/8/15 complex ranged from 0.013 to 0.027% (n = 3 experiments) and the fold purification achieved by the immunoprecipitations ranged from 1,150- to 3,962-fold (n = 3 experiments). The radiopurity of the isolated complex was increased significantly without any detectable change in subunit composition by the addition of nonradiolabeled yeast lysate lacking any c-*myc*–tagged proteins (data not shown). Since only 50-fold purification of the Sec6/8/15 complex was achieved by the combination of Sephacryl S-500 gel filtration and Ni^{2+} column fractionation, <1% of the protein in the E2 fraction was the Sec6/8/15 complex. This explains why we were unable to see any proteins specific to the Sec6/8/15 complex in the Ni^{2+} column fractions by SDS-PAGE and silver stain (see above).

Assignment of the Sec6, Sec8, and Sec15 Proteins in the Immunoisolated Complex

The molecular masses of the eight specific proteins were sufficiently similar that additional experiments were necessary to unambiguously assign the identity of Sec8, Sec6, and Sec15 to a given band. To facilitate assignment, we separated by SDS-PAGE immunoprecipitates from ^{35}S-labeled lysates lacking (NY13) or containing (NY1115) c-*myc*–tagged Sec8 and transferred them to nylon membranes. The blots were then exposed to film to give an image of the radiolabeled, immunoprecipitated protein bands. Seven of the specific bands (*b–h*) of the immunoprecipitates transferred sufficiently well to the nylon membrane to be imaged (Fig. 5 *B*, *Radiolabeled IPs*). Band *a* (144 kD) did not transfer in a sufficient amount to be imaged in this experiment. The identical nylon membranes were then Western blotted and chemiluminescent detection was used to detect Sec8, Sec6, and Sec15 (Fig. 5 *B*, *Western Blot*). Chemiluminescent detection of the proteins allowed such short exposure times that the radiolabeled proteins did not contribute to the exposure of the film. The two autoradiograms were then precisely superimposed to unambiguously assign the identity of Sec8 (band *b*), Sec15 (band *c*), and Sec6 (band *g*). Note that even though there is a band of similar molecular mass to Sec6 in the radiolabeled control immunoprecipitation, this band is not recognized by the Sec6 antibody and is clearly not Sec6.

We were able to confirm the identification of Sec6 and Sec15, as well as obtain information regarding the structural stability of the complex by immunoprecipitating the Sec6/8/15 complex isolated from various *sec* mutant strains. We constructed yeast strains expressing *SEC8-c-myc-6-his* with wild-type (NY1182), *sec2-41* (NY1183), *sec6-4* (NY1184), and *sec15-1* (NY1185) backgrounds by transforming haploid yeast with the same plasmid (pNB583 digested with BstxI) used to generate NY1115. To illustrate the ef-

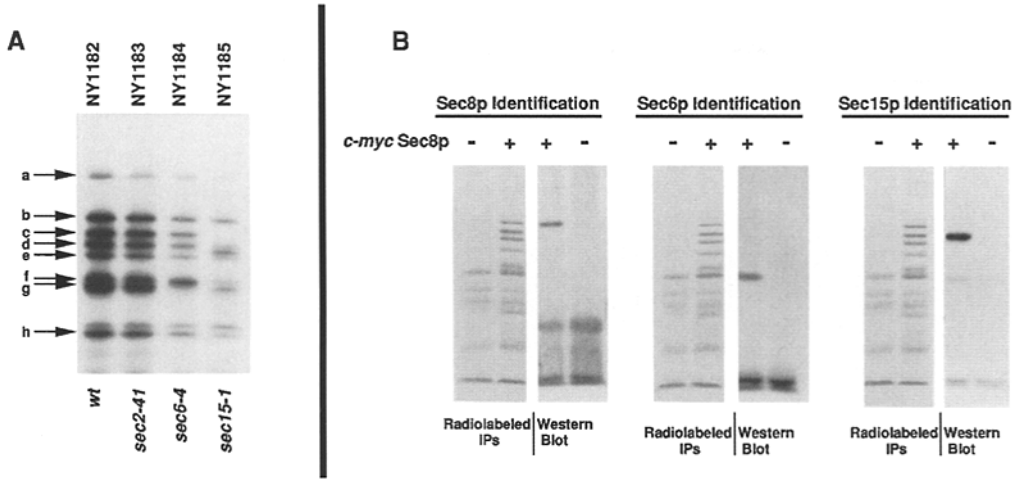


Figure 5. Identification of protein bands corresponding to Sec8, Sec6, and Sec15. Cultures of various yeast strains were grown overnight in SD medium supplemented with uracil. For each gel lane, 0.75 A_{599} U of each yeast strain were pelleted and resuspended in 1 ml of fresh SD plus uracil. To each tube, 10 μl of 14.3 μCi/μl of [^{35}S]methionine/cysteine label mix was added and the cultures incubated for 2 h at 25°C (*A*) or for 2.5 h at 30°C (*B*). (*A*) Lysates from radiolabeled NY1182 (*SEC8-c-myc-6-his*, 2.3 × 10^8 cpm/IP), NY1183 (*SEC8-c-myc-6-his*, *sec2-41*, 2.0 × 10^8 cpm/IP), NY1184 (*SEC8-c-myc-6-his*, *sec6-4* 1.8 × 10^8 cpm/IP), and NY1185 (*SEC8-c-myc-6-his*, *sec15-1*, 1.8 × 10^8 cpm/IP) were immunoprecipitated overnight according to Methods. The immunoprecipitated proteins were separated by SDS-PAGE on 5–15% acrylamide gradient gels, dried, and exposed to film for autoradiography. The results are representative of three experiments. (*B*) Lysates from radiolabeled NY13 (Sec8) or NY1115 (c-*myc*– and 6-histidine–tagged Sec8) were immunoprecipitated overnight according to Methods. The immunoprecipitated proteins were separated by SDS-PAGE on 7% acrylamide gels and then transferred to Immobilon P nylon membranes (Millipore Corp.) overnight. The nylon membranes were exposed to film to give an exposure of the radiolabeled polypeptides. The nylon membranes were then Western blotted with antibodies to Sec8, Sec6, or Sec15 and the specific bands determined by the Rad-Free chemiluminescent detection system. Exposure times were 2 min for Sec8, 15 min for Sec6, and 1 min for Sec15. The figure shows the pattern of radiolabeled proteins transferred to the membrane on the left and the specific band identified by Western blot, which corresponds to Sec8, Sec6, or Sec15 on the right. In the figure, the images of the Western blots have been flipped around their vertical axes to place the protein detected by chemiluminescent detection adjacent to the corresponding radiolabeled band from the immunoprecipitation. The results are representative of two experiments.

fects of the various *sec* mutations on the stability of the Sec6/8/15 complex, ^{35}S-labeled lysates of each strain were prepared and incubated with 9E10 antibody. The resulting immunoprecipitated proteins were separated on 5–15% gradient gels by SDS-PAGE and the radiolabeled proteins imaged by autoradiography (Fig. 5 *A*). All eight bands were immunoprecipitated from wild-type and *sec2-41* lysates although the bands were of slightly lower intensity from *sec2-41*. In contrast, the amount of the eight proteins which could be immunoprecipitated from lysates of the *sec6-4* and *sec15-1* strains were significantly reduced. In addition, the band at position *g* (Sec6 protein) shifted to 91 kD and overlaps band *f* in the immunoprecipitate from the *sec6-4* strain and the band at position *c* (Sec15 protein) shifted to 103 kD between bands *d* and *e* in the immunoprecipitate from the *sec15-1* strain. These observed shifts in the molecular masses of the Sec6-4 protein and the Sec15-1 protein were confirmed by Western blot of nonradioactive lysates (data not shown; Salminen and Novick, 1989).

Sec6/8/15 Complex Is Specifically Disrupted in sec3-2, sec5-24, and sec10-2 Strain Backgrounds

We extended the analysis of the effect of *sec* mutant backgrounds on the stability of the Sec6/8/15 complex by generating additional strains containing the *SEC8-c-myc-6-his* construct: NY1192 (*sec1-1*), NY1244 (*sec3-2*), NY1193 (*sec4-8*), NY1245 (*sec5-24*), NY1246 (*sec8-9*), NY1247 (*sec9-4*), NY1248 (*sec10-2*), NY1249 (*sec17-1*), and NY1250 (*sec18-1*). These strains were analyzed together with NY177 (wild type), NY1182 (wild type with *SEC8-c-myc-6-his*), NY1183 (*sec2-41*), NY1184 (*sec6-4*), and NY1185 (*sec15-1*) by preparing ^{35}S-labeled lysates of each strain (adjusted to contain equal cpms) and immunoprecipitating overnight with the 9E10 antibody. The resulting immunoprecipitated proteins were separated on 5–15% gradient gels by SDS-PAGE and the radiolabeled proteins imaged by autoradiography (Fig. 6). The effects of the *sec* backgrounds on the stability of the Sec6/8/15 complex can be divided into three groups. The first group includes *sec1-1*, *sec2-41*, *sec4-8*, *sec8-9*, *17-1*, and *18-1* strains which vary slightly in the amount of the Sec6/8/15 complex which can be specifically immunoprecipitated but all contain substantial amounts with normal subunit composition. The differences in the amount of the labeled complex which could be immunoprecipitated correlated with the slight variation in the expression of the Sec8-c-*myc*-6-his-tagged protein detected by Western blot (data not shown). Thus, the only effect of these *sec* mutations at 25°C, if any, might be on the expression of the Sec8-c-*myc*-6-his protein. No significant change in the composition of the complex was observed after a 30-min shift to 37°C before lysate preparation (not shown). The second group includes *sec6-4* and *sec15-1* which are integral members of the Sec6/8/15 complex but which have only a slight effect on the subunit composition at 25°C (Figs. 5 *A* and 6; Table III). After a shift to 37°C for 30 min the recovered complex was dramatically reduced to almost nondetectable levels from the *sec6-4* and the *sec15-1* mutant strains reflecting increased degradation and/or disassembly of the complex at the nonpermissive temperature (data not shown). The most striking effects on the subunit composition of the Sec6/8/15 complex occur in the mutant strains of the third group:

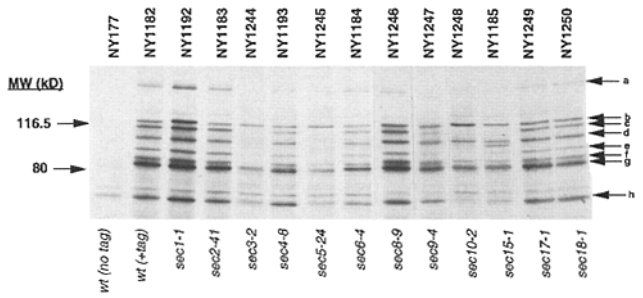


Figure 6. Composition of the Sec6/8/15 complex in various *sec* mutant strain backgrounds. Cultures of various yeast strains were grown overnight in SD medium supplemented for auxotrophic requirements. For each gel lane, 0.75 A_{599} U of each yeast strain were pelleted and resuspended in 1 ml of fresh, supplemented SD. To each tube, 10 μl of 14.3 μCi/μl of [^{35}S]methionine/cysteine label mix was added and the cultures incubated for 2 h at 25°C. The amount of lysate from the strains was adjusted with buffer so that each contained 4.7×10^7 cpm of radiolabeled protein in 1 ml and the Sec6/8/15 complex was then immunoprecipitated overnight according to Methods. The immunoprecipitated proteins were separated by SDS-PAGE on 5–15% acrylamide gradient gels, dried, and exposed to film for autoradiography. The results are representative of five experiments.

sec3-2, *sec5-24*, and *sec10-2*. The complex isolated in each of these *sec* mutant backgrounds has several bands specifically deleted or significantly decreased at 25°C. In *sec3-2*, six proteins are deleted (*a* and *f*) or significantly reduced (*c–e*, and *h*); in *sec5-24*, five proteins are deleted (*c–e*) or significantly reduced (*a* and *h*); and in *sec10-2*, four proteins are deleted (*c* and *e*) or significantly reduced (*a* and *h*). The results for bands *c* (Sec15) and *g* (Sec6) were confirmed by Western blot, i.e., the amount of the Sec6 protein was decreased for all three strains but was still detectable and the amount of the Sec15 protein was decreased in the *sec3-2* strain but was completely absent in the *sec5-24* and *sec10-2* strains (data not shown). The effects of these three strain backgrounds were evaluated in different clonal isolates and in multiple experiments, each with longer exposure times than that shown in Fig. 6 to pick up the presence of very faint bands. The results are summarized in Table III. The composition of the complex was found to be essentially the same after a shift of these mutants to 37°C for 30 min (not shown). We also transformed each strain with a wild-type copy of the defective gene on a *CEN* plasmid. In the presence of the wild-type gene, the subunit composition of the previously disrupted complex was restored for each strain indicating that the effects were specific to the *sec* mutations (data not shown). Sec10 probably is not an integral component of the complex since its predicted molecular mass differs from the nearest Sec6/8/15 complex subunit by 35 kD (Maurice, T., unpublished observations). Analysis of Sec3 and Sec5 is in progress.

c-myc Sec8 Is Localized to Small Bud Tips

Previous subcellular fractionation studies have shown that 20–25% of Sec8 and Sec15 are found in association with the plasma membrane with the remainder found in the cytosol (Bowser and Novick, 1991; Bowser et al., 1992). Immunofluorescence localization of Sec8, Sec6, and Sec15 has been problematic due to the poor utility of the rabbit polyclonal serum for this technique and the low abundance of the proteins in wild-type cells. The presence of the c-*myc* tag on Sec8 has facilitated the immunolocalization of Sec8 using the 9E10 mouse mAb. We used a sandwiching protocol with 9E10 antibody as primary, goat anti–mouse antibody as secondary, mouse anti–goat antibody as tertiary, and Texas red–conjugated donkey anti–mouse as the quaternary antibody. The fluorescence of several fields of unsynchronized yeast containing c-*myc* Sec8 (Fig. 7, *D* and *F*) or, lacking c-*myc* Sec8 (Fig. 7 *B*) are shown with their corresponding phase images just above (Fig. 7, *C*, *E*, and *A*, respectively). The fluorescence indicates that c-*myc* Sec8 specifically localizes as a patch of bright staining in the tips of small buds consistent with plasma membrane association at the site of rapid cell growth. There is also generally brighter cell body fluorescence in cells expressing c-*myc* sec8 (Fig. 7, *D* and *F*) compared with cells that express only Sec8 (Fig. 7 *B*) consistent with the large fraction of the complex which is cytosolic. Larger buds stain similarly to the cell bodies (data not shown). We also performed immunofluorescence on yeast synchronized at the small budded stage by α-mating factor arrest and subsequent release (Fig. 8, *A–D*). This enabled us to find multiple cells close to each other with their small buds in the same focal plane. Phase and fluorescence images of yeast strains containing Sec8 (Fig. 8, *A* and *B*) or c-*myc* Sec8 (Fig. 8, *C* and *D*) are shown. Four small buds are pictured in the control strain and four in the c-*myc* Sec8 strain. As was seen with the unsynchronized cells, the tips of small buds are brightly fluorescent only in the c-*myc* Sec8 strain (8 *D*). Sec8, and presumably the other

Table III. Proteins Associated with the Sec6/8/15 Complex in Different sec Mutant Backgrounds

Band	kD	Wild type	*sec3-2*	*sec5-24*	*sec6-4*	*sec10-2*	*sec15-1*
a	144	+	−	±	+	±	±
b (Sec8)	121	+	+	+	+	+	+
c (Sec15)	113	+	±	−	+	−	+*
d	107	+	±	−	+	+	+
e	100	+	±	−	+	−	+
f	91	+	−	+	+	+	+
g (Sec6)	88	+	+	+	+‡	+	+
h	70	+	≠	≠	±	≠	≠

*The Sec15-1 protein runs at 103 kD.
‡The Sec6-4 protein runs at 90–91 kD.
+, band is definitely present. −, band is definitely absent. ±, band is reduced but is evident upon longer exposure. ≠, band is probably absent but a nonspecific band makes a definitive determination difficult.

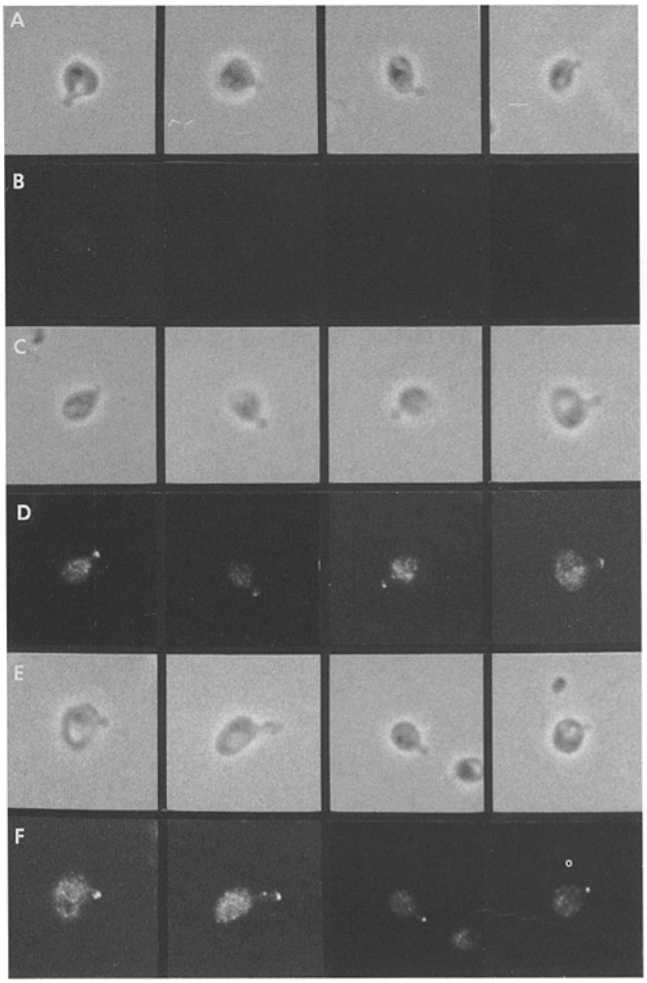
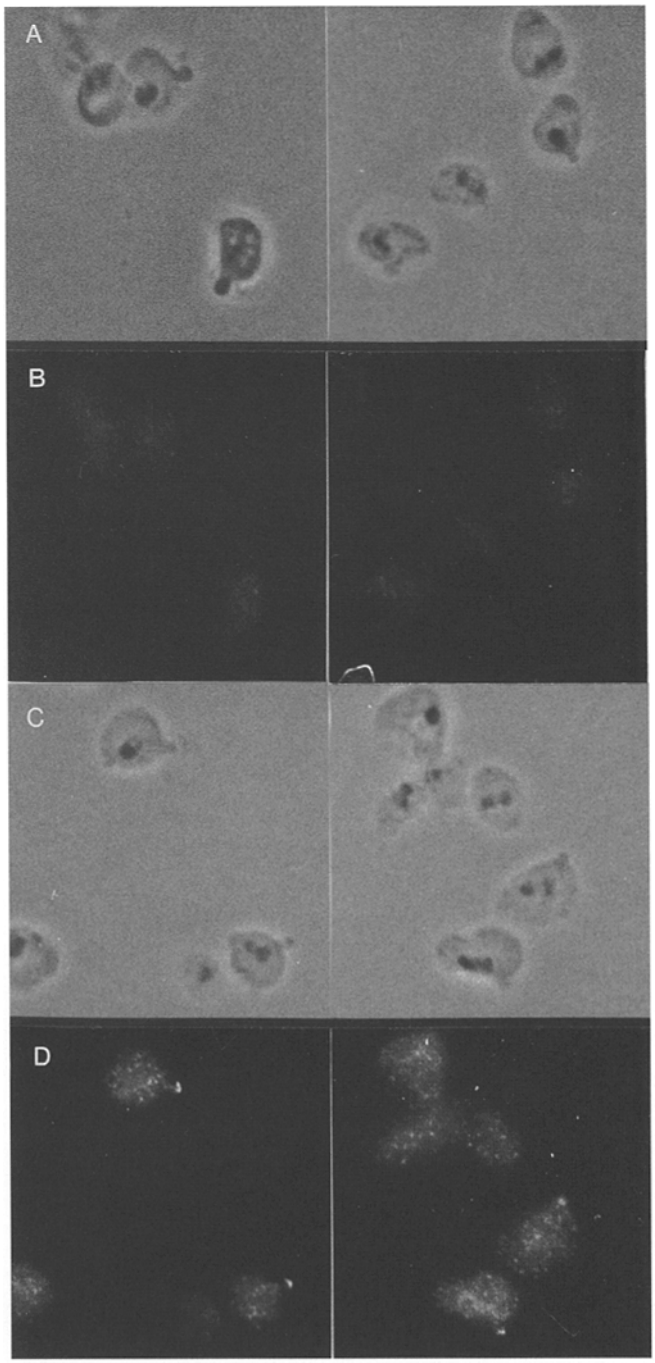


Figure 7. Indirect immunofluorescence on unsynchronized cultures of NY13 (wild-type Sec8) and NY1008 (c-*myc*–tagged Sec8). Overnight cultures of NY13 and NY1008 were grown in YPD at 25°C to a final A_{599} of 0.45 and 0.65, respectively. The yeast were fixed, spheroplasted, permeabilized and plated onto microwell slides as per Methods. All antibody incubations were for 1 h at room temperature and 20 μl in vol. The primary antibody (supernatant from 9E10 cells cultured with serum) was diluted 1:3 with PBT, the secondary antibody (precleared goat anti–mouse) and the tertiary antibody (precleared mouse anti–goat) were diluted 1:1 with PBT, and the quaternary antibody (Texas red–conjugated donkey anti–mouse) was diluted 1:500 with PBT. (*A*) Four fields of strain NY13 with small budded yeast. (*B*) The indirect immunofluorescence of the fields corresponding to A. (*C* and *E*) Eight fields of strain NY1008 with yeast showing small buds. (*D* and *F*) The indirect immunofluorescence of the fields corresponding to C and E. The results are representative of seven experiments.

components of the Sec6/8/15 complex, are thus localized to small bud tips which are sites of rapid cell growth. As the bud increases in size, the concentration of the complex appears to be diluted out until the staining pattern mimics that of the cell body. This pattern of localization is similar



spun down and resuspended in YPD, pH 4.0, at an A_{599} of 0.2. The yeast were synchronized by α mating factor arrest, released from arrest for 45 min, and then fixed, spheroplasted, permeabilized, and plated onto microwell slides as per Methods. All antibody incubations were for 1 h at room temperature and 20 μl in vol. The primary antibody (Nutridoma-cultured 9E10) was diluted 1:3 with PBT, the secondary antibody (precleared goat anti–mouse) was diluted 1:4 with PBT, the tertiary antibody (precleared mouse anti–goat) was diluted 1:30 with PBT, and the quaternary antibody (Texas Red conjugated donkey anti–mouse) was diluted 1:500 with PBT. (*A*) Two fields of strain NY13 with unbudded and small budded yeast. (*B*) The indirect immunofluorescence of the fields corresponding to A. (*C*) Two fields of strain NY1008 with unbudded and small budded yeast. (*D*) The indirect immunofluorescence of the fields corresponding to C. The results are representative of three experiments.

Figure 8. Indirect immunofluorescence on synchronized cultures of NY13 (wild-type Sec8) and NY1008 (c-*myc*–tagged Sec8). Overnight cultures of NY13 and NY1008 were grown in YPD at 25°C to a final A_{599} of 0.7 and 0.8, respectively. The cultures were

to that of several other proteins involved in polarized growth: Myo2, Smy1 (Lillie and Brown, 1994), calmodulin (Brockerhoff et al., 1994), and Cdc42 (Ziman et al., 1993) except that we do not see mother–bud neck staining or shmoo tip staining with c-*myc* Sec8 (data not shown). Extensive addition of membrane occurs at the mother–bud neck before cytokinesis and during shmoo formation before mating. We believe our inability to see c-*myc* Sec8 localization to these sites is because the concentration of the secretory components may be less than in small buds and we are below the limits of detection with our current immunofluorescent method. Immunofluorescent staining of Smy1 at the mother–bud neck and at shmoo tips was less intense than in small buds indicating the difficulty of detecting components involved in polarized growth in these regions (Brown, S., personal communication). Since the localization of Smy1 and Myo2 were similar to Sec8, we examined by Western blot both Ni^{2+} column fractions and immunoprecipitates from lysates containing 6-histidine–tagged Sec8. However, neither Myo2 nor Smy1 cofractionated with tagged Sec8 (data not shown). The staining is also somewhat similar to that of Spa2 and Sec4, except that they stain large bud tips as well as smaller buds (Snyder et al., 1991; Novick and Brennwald, 1993).

Discussion

In this study, we show that Sec6 is a component of the previously identified Sec8/15 complex (Bowser et al., 1992). Sec6 cofractionates with Sec8/15 by immobilized metal affinity chromatography, Sephacryl S-500 gel filtration, and sucrose velocity gradient centrifugation (Figs. 1 and 2). Sec6 and Sec15 are coimmunoprecipitated by the 9E10 antibody with c-*myc*–tagged Sec8 (Fig. 3). Together these data conclusively show that Sec6 is a stable component of the now renamed Sec6/8/15 complex. The association between Sec6 and Sec8/15 was missed in earlier experiments (Bowser et al., 1992) primarily because immunodetection of Sec6 by Western blot is severalfold less sensitive for a given amount of protein than is detection of either Sec15 or Sec8 by their respective polyclonal sera (see Fig. 2 where twice as much of each fraction was used to detect Sec6 compared with Sec8 or Sec15). The structure of the Sec6/8/15 complex is now shown to contain eight polypeptides ranging in molecular mass from 144 to 70 kD with a minimum total molecular mass of 834 kD (assuming a copy number of one for each polypeptide) (Fig. 4). The wild-type complex is stable during a 3-h incubation in 300 mM NaCl, but the complex is less stable when isolated from strains containing *sec6-4* or *sec15-1* mutant alleles (Fig. 5 *A*), and has several specific proteins deleted when isolated from strains containing *sec3-2*, *sec5-24*, and *sec10-2* (Fig. 6). The effects of the *sec3-2*, *sec5-24*, and *sec10-2* alleles on the stability of the complex is particularly striking in that each *sec* mutant causes a distinct pattern of proteins to be lost from the complex. This suggests that the proteins encoded by the *SEC3*, *SEC5*, and *SEC10* genes biochemically interact with distinct Sec6/8/15 complex proteins during or after complex assembly. In either case, their biochemical function is probably necessary for the activity of the Sec6/8/15 complex in the secretory pathway, and the block of secretion in the mutant alleles of these genes may be a consequence of preventing a sufficient quantity of the Sec6/8/15 complex to be maintained within the cell.

The immunofluorescent localization of the Sec6/8/15 complex may offer a clue to its function. While subcellular fractionation and immunolocalization experiments indicate that the complex is present in the cytosol, the most intense staining of the c-*myc*–tagged Sec8 protein is localized to the tip of the bud (Figs. 7 and 8), the predominant site of exocytosis in *S. cerevisiae*. Since the Sec6/8/15 complex immunoprecipitated from solubilized membranes contains all eight polypeptides, and we never observe monomeric Sec8, it is likely that the observed localization of Sec8 represents the fraction of the intact Sec6/8/15 complex which is associated peripherally with the plasma membrane. As the bud enlarges, the staining pattern for c-*myc*-Sec8 mimics that of the mother cell and the specific bud tip staining is lost. We believe that the complex is still localized to the plasma membrane of the larger buds but with our current immunofluorescent method we are unable to detect it. The specific localization of the complex to the tip of the bud could result from its recruitment to the site of exocytosis either before or after vesicle arrival. If the complex is localized to the site of exocytosis before the vesicle arrives, it may be involved in the targeting reaction, perhaps by readying the SNAREs for docking. If the complex is recruited to the site of exocytosis only after the SNAREs have interacted and the vesicle has been docked, it may be involved in a subsequent biochemical step leading to fusion. The Sec6/8/15 complex does not seem to ride on a secretory vesicle as it is transported from the *trans*-Golgi network to the bud tip. We have isolated secretory vesicles on an S-1000 column from a *sec1-1* yeast strain, since Sec1 is not part of the Sec6/8/15 complex (see Fig. 3), and blotted the vesicle fractions for Sec6, Sec8, and Sec15. Regardless of whether the vesicles were prepared in the presence or absence of GTPγS and $MgCl_2$, we were unable to detect any Sec6, Sec8, or Sec15 proteins associated with the isolated secretory vesicles (data not shown). Thus, the Sec6/8/15 complex does not appear to be stably associated with the surface of a secretory vesicle and it is unlikely to be part of a stable vesicle coat structure analogous to coatomer.

Analysis of genetic interactions can aid in understanding the functions of the encoded gene products. Overexpression of either *SEC9* or *SSO1* and *2* will suppress the temperature-sensitive phenotype of the *sec15-1* allele, and *sec8-9* is suppressed by overexpression of *SEC9* (Aalto et al., 1993; Brennwald et al., 1994). Furthermore, synthetic lethality is observed when *sec9-4* is combined with either *sec8-9* or *sec15-1*. Compensation for a decrease in the function of one protein by overexpression of another and the observed lethality upon combination of two otherwise viable alleles both suggest that the two gene products act in the same biochemical pathway (Rine, 1991). Since the Sso and Sec9 proteins are the t-SNARES that have been implicated in secretory vesicle targeting, the observed genetic interactions suggest that the Sec6/8/15 complex may also be involved in the targeting reaction.

The Sso and Sec9 proteins cannot solely specify the targeting of secretory vesicles since they are not localized exclusively to the site of exocytosis, but are found rimming the entire inner surface of the plasma membrane (Brenn-

wald et al., 1994). One attractive possibility is that the Sec6/8/15 complex is required in combination with Sso and Sec9 to target secretory vesicles to the site of exocytosis at the tip of the bud. If the Sec6/8/15 complex functions in targeting it might be expected that genes encoding the components of the complex would interact with genes encoding proteins associated with the cytoplasmic surface of the secretory vesicle, such as *SEC4*. In fact, duplication of *SEC4* suppresses the temperature-sensitive growth defect of *sec8-9* and *sec15-1*, and *sec4-8* is lethal in combination with both *sec8-9* and *sec15-1*. Genetic interactions are seen with genes encoding components thought to act both upstream (Sec4) and downstream (Sso and Sec9) in the vesicle targeting/docking reaction. Thus, genetic interaction and immunofluorescent localization data suggest that the Sec6/8/15 complex may function as a key discriminator which determines whether an "activated" secretory vesicle is allowed to dock. Final determination of the function of the Sec6/8/15 complex will await the identification of the five unknown polypeptides and further biochemical characterization of the purified complex. While there are presently no known homologues of Sec6, Sec8, or Sec15 in any other cell type, given the high degree of conservation of the other components of the secretory pathway (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994) it is likely that such homologues will be found. Determining the biochemical function of the Sec6/8/15 complex may therefore aid our understanding of protein transport in higher eukaryotes.

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