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 secl0-2 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), 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 synaptaxin 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...
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: Snc1 and 2 are homologous to synaptobrevin and are localized on the surface of secretory vesicles (Gerst et al., 1992; Protopopov et al., 1993); Sso1 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 communoprecipitate with Sec9 from detergent solubilized 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 (Sgaard 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 sec24-1, 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 immunoseparated from radiolabeled strains containing the sec-3, sec-5, and sec-10 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 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 Nutridima-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 SEC1 (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 XLlBlue 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 and gel purified. pNB328 (contains SEC8 as a 4.4-kb Smal-Sall fragment in YEP24 vector) (Carlson and Botstein, 1982; Bowser et al., 1992) was digested with Sall and gel purified. The 4.4-kb Smal-Sall, SEC8-containing fragment and the Sall digested pBluescript II KS (+) were ligated and transformed into TGI cells. The plasmid (pNB504) was recovered and the construction confirmed by digestion with Smal, Sall, EcoRI, HindIII, and Xhol. pNB504 was transformed into CJI236 (dar- ura-) and ssDNA was isolated.

**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 and subsequently treated with dNTPs and T4 polynucleotide kinase to form blunt-ended DNA. The product was gel purified, ligated, and transformed into TGI cells to yield pNB499 (which lacks the BamHI site in the polylinker region). pNB499 was digested with Sall and gel purified. The Sall-Sam fragment containing SEC8-c-myc, SEC8-6-his, and SEC8-c-myc-6-his from pNB650, pNB561, and pNB555, respectively, were gel purified. The Smal-Sall digested pNB499 and was mixed respectively with the Sall-Sam fragments containing SEC8-c-myc, SEC8-6-his, and SEC8-c-myc-6-his from pNB650, pNB561, and pNB555, respectively, were gel purified. The Smal-Sall digested pNB499 and was mixed respectively with the Sall-Sam fragments containing SEC8-c-myc, SEC8-6-his, and SEC8-c-myc-6-his, ligated, and transformed into TGI cells. The constructions of the recovered plasmids (pNB557, pNB558, and pNB593, 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.

**Fractionation of Lysates**

Competent NY813 cells (MATa/a, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC8::c-myc::pNB338URA3) were prepared by the alkali cation method (Ito et al., 1983) and transformed with BstXI-digested pNB557, pNB558, and pNB593, respectively. Transformants were plated on selective medium at 25°C. Tetrad from positive transformants were dissected and the haploid progeny were analyzed for the presence of the LEU2 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.

**S-500 Column Fractionation and Ni²⁺ Resin Column Fractionation of Lysates**

Overnight cultures (300 ml) of NY13 (wild-type Sec8) or NY1012 (6-histidine-tagged Sec8) were grown to an A900 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 Na3HPO4, 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 0.125-0.62 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) were 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 Sec6, Sec9, and Sec15 were pooled and adjusted to 20 mM imidazole. The pooled fractions were applied to a 2-ml Ni²⁺-immobilidate (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 Ni²⁺-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 125I-protein A (Salminen and Novick, 1989). The primary antibodies used to detect the transfected proteins included rabbit anti-SEC15-241 (1:1,000) (Salminen and Novick, 1989), rabbit anti-See9582-6S1 (1:1,000) (Bowser and Novick, 1991), rabbit anti-See95-724 (1:1,000) (Pozena et al., 1992), rabbit anti-See20-350 (1:1,000) (Nair et al., 1990), rabbit anti-See50-62 (1:2,000) (see above), rabbit anti-See4-215 (1:1,000) (Groud et al., 1988), rabbit anti-Gdi1191-451 (1:5,000) (Garrett et al., 1994), and rabbit anti-See92-401 (1:250) (Brennwald et al., 1994). In some experiments, fractions from the Ni²⁺-column fractionation were blotted for GFP-binding proteins using a [32P]GFP (Amerham 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 preclarified 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 Ni²⁺ column fractions and in addition antibodies generated in rabbits against Myo2p (1:50 dilution) and Smy1p (1:1,000 dilution) (Lillie and Brown, 1994).

**c-myc Immunoprecipitation from [35S]Methionine/Cysteine-labeled Yeast**

50 ml cultures of NY13 and NY1115 were grown overnight to an A900 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 A900. 10 μl of label mix (label mix contains 14.3 μCi/μl of total labeled [35S]methionine/cysteine, Amerahm Corp., Arlington Heights, IL) was added for each 0.75
**Table 1. Yeast Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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</tr>
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**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 A599 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 from each strain was 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. The gradients contained 1 mM DTF (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. The protein concentration was then adjusted to ~10 mg/ml with lysis buffer and Triton X-100 was added to 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 (Bowers 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) 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 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 (Bowers 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) 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 1%.
ting and counting the amount of 32P-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 A599 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 7.5, 1.6 ml of 37% formaldehyde. The yeast was gently rocked for 20 min and then pelleted. The pellet was resuspended in 10 ml of freshly prepared 4% formaldehyde (Robert 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/PPI 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 A599 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 (Axiohot; 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 Cofractionation on an Ni2⁺ 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 Ni2⁺ resin column fractionation.

The initial approach was to fractionate lysates made from yeast strains containing or lacking 6-histidine-tagged Sec8 on an Ni2⁺ 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 immunoreactivity by Western blot. For both strains, Sec8 and Sec15 were found in the same fractions with a peak of immunoreactivity 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 Ni2⁺ 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, 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 Ni2⁺ IDA column fractions were also analyzed for proteins which bind [α-32P] GTP. Seven proteins which bind [α-32P]GTP could be detected in the pooled S-500 column fractions.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY13: 1023-NKRYTEALEKLNSLEKEQSKGARTKIGKLKSLNAVHTANEK-1065 (wild-type Sec8)</td>
<td></td>
</tr>
<tr>
<td>NY1008: 1023-NKRYTEALEKLNSLEKEQSKGARTKIGKLEQKLISEEDIHTANEK-1068 (c-myc-tagged Sec8; the codon for lysine-1053 was deleted in the mutagenesis reaction)</td>
<td></td>
</tr>
<tr>
<td>NY1012: 1023-NKRYTEALEKLNSLEKEQSKGARTKIGKLKKAHHHHHTANEK-1065 (6-histidine–tagged Sec8)</td>
<td></td>
</tr>
<tr>
<td>NY1115: 1023-NKRYTEAEOKLISEEGLOSKSGARTKIGKLKKAHHHHHTANEK-1065 (c-myc- and 6-histidine–tagged Sec8)</td>
<td></td>
</tr>
</tbody>
</table>
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 Ni2÷ IDA column. The flow-through was passed over the Ni2+ 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 NaPO4, 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, Gd1, or Sec4. The results are representative of two or more experiments for each protein analyzed.

and the Ni2+ 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 Ni2÷ 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 Ni2+ IDA columns were 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 Ni2+ 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 Ni2+ IDA columns were 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.

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 Secl5 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).
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 Smyl 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 immunoslolated 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 NaC1, 0.5% Tween 20, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 2 X 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 [\(\alpha^{32}\)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 immunosolated 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 form a multisubunit complex as indicated by Methods. After the final wash, the beads were then boiled with sample buffer, pellet, and the supernatant analyzed by SDS-PAGE and Western blot for Sec8, Sec6, Sec15, Sec2, and Sec1. The results are representative of two or more experiments for each protein blotted.

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 Sec8, Sec6, Sec15, Sec2, and Sec1. The results are representative of two or more experiments for each protein blotted.
proteins 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 Ni²⁺ 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 Ni²⁺ column fractions by SDS-PAGE and silver stain (see above).

**Assignment of the Sec6, Sec8, and Sec15 Proteins in the Immunisolated 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 ³⁵S-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 a), 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.
NY1183 (SEC8-c-myc-6-his, sec2-41, 2.0 \times 10^6 \text{ cpm/IP}), NY1184 (SEC8-c-myc-6-his, sec6-4 1.8 \times 10^6 \text{ cpm/IP}), and NY1185 (SEC8-c-
myc-6-his, sec15-1, 1.8 \times 10^6 \text{ 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.

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_{999} of each yeast strain were pelleted and resuspended in 1 ml of fresh SD plus uracil. To each tube, 10 \muL of 14.3 \muCi/\muL of [35S]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 \times 10^6 \text{ cpm/IP}),

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 35S-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 lystate 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 sec-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:
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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$_{600}$ 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 [35S]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.

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

<table>
<thead>
<tr>
<th>Band</th>
<th>kD</th>
<th>Wild type</th>
<th>sec3-2</th>
<th>sec5-24</th>
<th>sec6-4</th>
<th>sec10-2</th>
<th>sec15-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>144</td>
<td>+</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>b (Sec8)</td>
<td>121</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c (Sec15)</td>
<td>113</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
<td>107</td>
<td>+</td>
<td>±</td>
<td>-</td>
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<tr>
<td>e</td>
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<td>+</td>
</tr>
<tr>
<td>g (Sec6)</td>
<td>88</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>h</td>
<td>70</td>
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<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

*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.

Sec8 (Fig. 7, D and F) or, lacking c-myc 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 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) is 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).
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 A₅₉₀ 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

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 A₅₉₀ of 0.7 and 0.8, respectively. The cultures were spun down and resuspended in YPD, pH 4.0, at an A₅₉₀ 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.
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 Ni²⁺ 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 (Bowers et al., 1992). Sec6 cofractionates with Sec8/15 by immobilized metal affinity chromatography, Sephacyr 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 MgCl₂, 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 coatamer.

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-

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