Sec1p Binds to SNARE Complexes and Concentrates at Sites of Secretion

Chavela M. Carr,* Eric Grote,* Mary Munson,‡ Frederick M. Hughson,‡ and Peter J. Novick*

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and ‡Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

Abstract. Proteins of the Sec1 family have been shown to interact with target-membrane t-SNAREs that are homologous to the neuronal protein syntaxin. We demonstrate that yeast Sec1p coprecipitates not only the syntaxin homologue Ssop, but also the other two exocytic SNAREs (Sec9p and Sncp) in amounts and in proportions characteristic of SNARE complexes in yeast lysates. The interaction between Sec1p and Ssop is limited by the abundance of SNARE complexes present in *sec* mutants that are defective in either SNARE complex assembly or disassembly. Furthermore, the localization of green fluorescent protein (GFP)-tagged Sec1p coincides with sites of vesicle docking and fusion where SNARE complexes are believed to assemble and function. The proposal that SNARE complexes act as receptors for Sec1p is supported by the mislocalization of GFP-Sec1p in a mutant defective for SNARE complex assembly and by the robust localization of GFP-Sec1p in a mutant that fails to disassemble SNARE complexes. The results presented here place yeast Sec1p at the core of the exocytic fusion machinery, bound to SNARE complexes and localized to sites of secretion.

Key words: Sec1 proteins • syntaxin proteins • SNARE complex • secretion • yeast

VESICLE trafficking ensures high-fidelity transport of materials to specific destinations inside the cell, or to the cell surface. Cells as distantly related as yeast and neurons share homologous proteins such as SNAREs, Rabs, and members of the Sec1 family, which are believed to work together for efficient docking and fusion of transport vesicles with their target membranes (for review see Bennett and Scheller, 1993).

At the center of the vesicle docking and membrane fusion reaction is a complex of membrane-associated proteins known as SNAREs. The SNARE complex was first isolated from brain extracts and named for its ability to bind the chaperone-like α-SNAP/NSF complex (Söllner et al., 1993a). SNARE complexes form when integral-membrane proteins on the vesicle (v-SNAREs;[1] in neurons VAMP, or synaptobrevin) bind to their cognate protein complexes on the target membrane (t-SNAREs; in neurons a heterodimer comprising the integral-membrane protein syntaxin and a membrane-associated protein SNAP-25). Based on a variety of results, the assembly of SNAREs into complexes is believed to link two bilayers for membrane fusion and cargo transfer (for review see Skehel and Wiley, 1998). After membrane fusion, the SNARE complexes are disassembled and recycled for the next round of vesicle docking and fusion.

In yeast, the SNAREs required for post-Golgi vesicle trafficking assemble to form exocytic SNARE complexes between vesicles and the plasma membrane. The yeast exocytic SNARE complex is analogous to that formed between synaptic vesicles and the presynaptic plasma membrane in the neuron (Rossi et al., 1997). The redundant genes *SSO1* and *SSO2* (or simply, *SSO*) encode the yeast t-SNAREs homologous to syntaxins (Aalto et al., 1993). The product of the *SEC9* gene shares homology with the t-SNARE SNAP-25 (Brennwald et al., 1994), and the redundant genes *SNC1* and *SNC2* (or simply, *SNC*) encode the v-SNAREs homologous to the neuronal SNARE VAMP, or synaptobrevin (Protopopov et al., 1993).

Originally, the SNARE hypothesis provided a model for the specificity of vesicle targeting (Rothman, 1994). SNARE complexes were not only considered a component of the fusion machinery, but the preference of certain v-SNAREs for certain t-SNAREs was believed to be sufficient for correct docking of vesicles to their target membranes (Calakos et al., 1994). The subsequent identification of SNARE complexes associated with various steps of vesicle transport in yeast has revealed that some SNAREs are shared between SNARE complexes at different steps (for review see Gotte and von Mollard, 1998). Furthermore, the affinity of various SNARE-complex combinations formed in vitro does not correlate with the specificity

Address correspondence to Peter J. Novick, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510. Tel.: (203) 785-5871. Fax: (203) 785-7226. E-mail: peter.novick@yale.edu

1. *Abbreviations used in this paper:* GFP, green fluorescent protein; IP, immunoprecipitation; t-SNARE, target SNARE; v-SNARE, vesicle SNARE.

of SNARE complexes found in vivo (Yang et al., 1999). These results indicate that the specificity of vesicle targeting cannot be conferred exclusively by SNAREs. Therefore, while most current models assign a membrane fusion function to SNARE complexes, it is clear that other factors are required to maintain the fidelity of vesicle targeting and membrane fusion.

Accurate vesicle targeting requires multiple layers of regulation, at the donor compartment during formation of transport vesicles, in the cytoplasm as vesicles are directed toward their destination, and at the target membrane where appropriate vesicles are docked for membrane fusion. Because membrane fusion is irreversible, it is reasonable to propose that a final safeguard is especially critical to prevent missorting of cargo and loss of distinction between membrane compartments. The best candidates for a final safeguard are factors that act before membrane fusion, at vesicle docking sites. Such factors might include those that regulate the assembly and disassembly of SNARE complexes (Pfeffer, 1996).

Assembly of SNARE complexes requires a variety of trafficking factors as well as flux through the secretory pathway. Among those factors are the Rab family of small Ras-like GTPases (Søgaard et al., 1994; Haas et al., 1995; Grote, E., and P.J. Novick, manuscript in preparation). Rabs undergo a chemical cycle between a GDP and a GTP state and a physical cycle between specific target and donor membranes (for reviews see Novick and Brennwald, 1993; Schimmoller et al., 1998). Rabs and other factors have been implicated in tethering vesicles to their target membranes (Barroso et al., 1995; Mayer and Wickner, 1997; Cao et al., 1998; Guo et al., 1999). However, Rabs alone are not sufficient for specificity of vesicle targeting, as demonstrated by the ability of a Sec4p-Ypt1p chimera to act as a functional Rab at two steps of secretion without missorting of cargo (Brennwald and Novick, 1993). Therefore, it is likely that some other factor, or some combination of factors including Rabs and SNAREs (Lupashin and Waters, 1997; Christoforidis et al., 1999; Grote, E., and P.J. Novick, manuscript in preparation), is necessary for accurate SNARE complex assembly.

Disassembly of SNARE complexes is achieved through the chaperone-like ATPase activity of the NSF homologues (Söllner et al., 1993b), which separate v-SNAREs from t-SNAREs for sorting and recycling before the next round of vesicle docking and fusion (Mayer et al., 1996; Ungermann et al., 1998a). More than one homologue of NSF has been identified in yeast (for review see Patel and Latterich, 1998). Nonetheless, loss of NSF function in the yeast mutant *sec18-1* results in a block at multiple steps in secretion (Graham and Emr, 1991), indicating that Sec18p function is not limited to a specific step of vesicle transport. Therefore, if the fidelity of membrane fusion is controlled by disassembly of incorrect SNARE complexes, some other factor or factors must also contribute to the editing process.

Members of the Sec1 family have been described both as activators and inhibitors of SNARE complex assembly (for review see Halachmi and Lev, 1996). Loss-of-function mutants of Sec1 homologues in *Saccharomyces cerevisiae* (Novick and Scheckman, 1979; Robinson et al., 1988; Wada et al., 1990; Ossig et al., 1991; Cowles et al., 1994), *Drosophila melanogaster* (Harrison et al., 1994), and *Caenorhabditis elegans* (Hosono et al., 1992) accumulate vesicles that are blocked at specific steps in secretion, indicating that Sec1 function is essential for vesicle consumption. Furthermore, the results of binding and localization studies have implicated mammalian Sec1 homologues in secretion and neurotransmission (Hata et al., 1993; Garcia et al., 1994, 1995; Hodel et al., 1994; Pevsner et al., 1994a). In yeast there are four discernible Sec1 homologues, although more than four vesicle trafficking steps have been identified. Therefore, although essential, a distinct Sec1 protein may not be specifically required at every step in secretion.

In addition to a positive role, an inhibitory role has been suggested by the finding that Sec1 proteins bind to t-SNAREs (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994a; Søgaard et al., 1994; Grabowski and Gallwitz, 1997; Nichols et al., 1998) and can prevent pairwise SNARE interactions in vitro (Pevsner et al., 1994b). Furthermore, overexpression of the Sec1 homologue Rop blocks exocytosis in *D. melanogaster*, and this block is relieved by co-overexpression of syntaxin (Schultze et al., 1994; Wu et al., 1998), supporting the conclusion that Sec1 proteins block SNARE complex assembly by binding to syntaxin homologues. The identification of novel Sec1-interacting proteins (Aalto et al., 1997; Okamoto and Südhof, 1997; Verhage et al., 1997) has inspired models that reconcile this apparent paradox by proposing stepwise interactions between syntaxin homologues, Sec1 proteins, and postulated docking proteins, culminating in the assembly of the SNARE complex (for review see Schimmoller et al., 1998).

Our approach to understanding Sec1p function makes use of the secretory mutants (*sec*) available in yeast. We found alterations in protein interactions and Sec1p localization in two *sec* mutants: one that prevents SNARE complex assembly (*sec4-8*) and one that blocks SNARE complex disassembly (*sec18-1*). Specifically, we present evidence that Sec1p binds to assembled SNARE complexes and localizes to sites of exocytosis. In contrast to models that propose a regulatory role for Sec1 proteins in SNARE complex assembly, we speculate that Sec1p acts after assembly, promoting high-fidelity vesicle docking and fusion by specifically binding to productive SNARE complexes.

Materials and Methods

Materials

Oligonucleotides used in this study were prepared by M. Talmor (Department of Pathology, Yale University, New Haven, CT). Vent and *Taq* polymerases used for PCR and pepstatin A were purchased from Boehringer Mannheim. Restriction enzymes, the pMAL-C2 vector, and amylose resin were purchased from New England Biolabs. Plasmid and PCR purification was performed using Qiagen reagents. The components of the ATP-regeneration system (creatine kinase, creatine phosphate, ATP, and $MgCl_2$), the detergent NP-40 (also called IGEPAL CA-630), and the protease inhibitors antipain, aprotinin, leupeptin, chymostatin, and PMSF were purchased from Sigma Chemical Co. Protein G–Sepharose and the pGEX4T1 vector were from Pharmacia Biotech. The protein assay reagent and chemicals used for SDS-PAGE were purchased from Bio-Rad Laboratories. Rainbow molecular weight markers and reagents for enhanced chemiluminescence were purchased from Amersham Corp. Fluo-

rography was performed using a Kodak X-OMAT film processor and X-OMAT AR or X-OMAT BMR film.

Antibodies

The monoclonal anti-MYC antibody (9E10) was prepared by the Pocono Rabbit Farm and Laboratory Inc. The monoclonal 12CA5 antibody was purchased from Boehringer Mannheim. For Sec1p antibodies, the 174 carboxyl-terminal amino acids of Sec1p were fused in frame with glutathione-S-transferase protein by subcloning the BamHI-EcoRI fragment of Sec1p (pNB680, a Yep24 vector with SEC1, from S. Keränen, VTT, Biotechnical Laboratory, Esposo, Finland) into pGEX4T1. The Sec1p-GST fusion protein used to immunize rabbits was purified using glutathione-Sepharose resin, as instructed by the manufacturer (Pharmacia Biotech, Inc.). Sec1p-GST antibodies were purified from rabbit antiserum (Cocalico) on amylose resin prebound to the same fragment of Sec1p, which was produced as a maltose-binding protein conjugate using pMAL-C2. Antiserum against purified Sso1p (a gift from A. Brünger) was generated by Cocalico. The Ssop antiserum was affinity-purified using GST-Sso1p (Rice et al., 1997) bound to glutathione agarose resin. Biotinylated anti-Ssop for immunoblotting was prepared using NHS-LC-Biotin (Pierce) according to the manufacturer's protocol. The Sncp antiserum is described elsewhere (Rossi et al., 1997). The Sec9p antiserum was a gift from P. Brennwald (Cornell University Medical School, New York, NY). Pep12p antiserum was a gift from R. Piper (University of Iowa, Iowa City, IA). Sec22p and Bos1p antisera were gifts from S. Ferro-Novick (Yale Medical School, New Haven, CT). Peroxidase-conjugated avidin was from Amersham Life Sciences, and peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Labs, Inc. Antibodies against green fluorescent protein (GFP) were from Clontech.

Yeast Strains

S. cerevisiae strains used in this study are listed in Table I. Cells were grown in a rich medium (YPD) or in a minimal medium, supplemented for auxotrophic requirements, as described (Guthrie and Fink, 1991). Epitope tagging of *SEC1*, *SSO2*, and *SNC2* used a PCR method that allows for amplification of either a triple HA or a triple c-myc epitope linked to *URA3* marker, which can be integrated directly at the amino or carboxyl terminus of a gene, as described previously (Schneider et al., 1995). For c-myc–tagged *SEC1* (NY1689, referred to as MYC-*SEC1* throughout the text), primers were designed for homologous recombination of the triple-MYC epitope plus *URA3* at the carboxyl terminus of genomic *SEC1* (forward primer 23314: GAAGAAACGTAGCAAATTCTCGAGGTTCTTGAAAAGAAAATCTCACCATGATAAAAGGGAACAAAAGCTGG, reverse primer 23315: GCGTTTTTTTGACAAGAGCAAGTGATTTTTTTTATCACTGTCTTCTTTAAGGGATCACTATAGGGCGAATTGG). The strain referred to as HA-*SSO* (NY1692) was constructed by amplification of a triple HA epitope plus *URA3*, with primers designed to integrate the tag by homologous recombination at the amino terminus of the endogenous *SSO2* (forward primer 30298: GTAATACTTTACATTTGAAAACTGCCCATACACGCACAAATATTGCAGCAACAGGGAACAAAAGCTGG, reverse primer 30299: TTCATAGTTTTCAGCGTACGGATTGTTATTCTCATAAGGATTAGCGTTGCTCATTAGGGCGAATTGGGTACC). MYC-*SEC1* and HA-*SSO* were independently crossed with *sec4-8* and *sec18-1* strains to construct strains NY1690 (MYC-*SEC1 sec4-8*), NY1691 (MYC-*SEC1 sec18-1*), NY1693 (HA-*SSO sec4-8*), and NY1694 (HA-*SSO sec18-1*). Amino-terminally tagged MYC-*SSO* (NY1704) was similarly constructed using primers 30298 and 30299 by homologous recombination of the triple MYC epitope plus *URA3*, which was subsequently looped out by selection on 5-fluoro-orotic acid (Guthrie and Fink, 1991), regenerating the *ura3-52* auxotrophy. HA-*SNC2* (NY1642) has been described previously (Abeliovich et al., 1998).

For localization studies, the *SEC1* coding sequence was replaced with *SEC1* in frame with the coding sequence of the *mut3* version of *A. victoria* GFP (S65G, S72A; Cormack et al., 1996) by homologous recombination (NY1699, referred to in the text as GFP-*SEC1*). The integration vector containing the GFP-*SEC1* DNA (pNB828) was constructed by a three-step process, using the *Escherichia coli* strain DH5α and standard molecular biological procedures (Sambrook et al., 1989). First, a PCR product containing the carboxyl-terminal, 42-bp fragment of *SEC1* in frame with the GFP coding sequence was ligated to a PCR product containing the 538-bp *SEC1* terminator sequence. Second, this PCR ligation product was subcloned into pNB680 at the XhoI and SphI sites. From this vector, a 1,040-bp carboxyl-terminal AvrII-SalI fragment of SEC1-plus-GFP-plus-SEC1 terminator was subcloned into the SpeI-SalI sites of the URA3 integration vector pRS306 (Sikorski and Hieter, 1989). The correct nucleotide sequence of the SEC1 fragment-GFP-SEC1 terminator was determined by microsequencing (W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University). The final integration vector, pNB879, was digested with EcoRI before transformation into NY179, in order to initiate recombination at a single crossover point (near the carboxyl terminus of *SEC1*). To amplify the GFP coding sequence in frame with the carboxyl-terminal 42-bp segment of SEC1, a plasmid containing the *mut3* GFP sequence was used as a template with the forward primer 25412 (carboxyl-terminal *SEC1* fragment + GFP): GCGTTCTCGAGGTTCTTGAAAAGAAAATCTCACCATGATAAAATGAGTAAAGGAGAAGAACTTTTC, and a GFP reverse primer 24968: GAGCGGCCGCTCTAGCCC. For amplification of the *SEC1* terminator sequence, pNB680 was used as a template with the forward primer 25413 (carboxyl-terminal GFP+*SEC1* terminator): GCGGCGGGCATGGATGAACTATACAAATAATGATCCCTTAAAGAAGACAGTGATAAA, and the *SEC1* terminator reverse primer 25414: CATCGAGCATGCCAGATTACTACCAGGA.

Table I. S. cerevisiae Strains

Strain	Genotype
NY13	*MATa ura3-52*
NY28	*MATα ura3-52 sec4-8*
NY179	*MATa leu2-3,112 ura3-52*
NY1491	*MATα ura3-52 leu2-3,112 trp1 his3Δ200*
NY1642	*MATa ura3-52 leu2-3,112 SNC1::URA3 SNC2::GAL1p HA_3SNC2 LEU2*
NY1689	*MATα ura3-52 leu2-3,112 trp1 his3Δ200 SEC1::SEC1MYC_3URA3*
NY1690	*MATα ura3-52 trp1 his3Δ200 SEC1::SEC1MYC_3URA3 sec4-8*
NY1691	*MATα ura3-52 leu2-3,112 trp1 his3Δ200 SEC1::SEC1MYC_3URA3 sec18-1*
NY1692	*MATα ura3-52 leu2-3,112 SSO1::LEU2 SSO2::HA_3SSO2*
NY1693	*MATα leu2-3,112 SSO1::LEU2 SSO2::HA_3SSO2 sec4-8*
NY1694	*MATα ura3-52 leu2-3,112 SSO1::LEU2 SSO2::HA_3SSO2 sec18-1*
NY1697	*MATa ura3-52 leu2-3,112 ura3-52 SEC1::SEC1GFP URA3 sec4-8*
NY1698	*MATa ura3-52 SEC1::SEC1GFP URA3 sec18-1*
NY1699	*MATa ura3-52 leu2-3,112 SEC1::SEC1GFP URA3*
NY1700	*MATa ura3-52*
NY1701	*MATa ura3-52 sec4-8*
NY1702	*MATa ura3-52 leu2-3,112 his3Δ200 SNC1::URA3 SNC2::GAL1pHA_3SNC2 LEU2 SEC1::SEC1MYC_3URA3*
NY1704	*MATa leu2-3,112 his3Δ200 SSO1::LEU2 SSO2::MYC_3SSO2*

Immunoprecipitation (IP)

Strains used for IP experiments were grown overnight in YPD at 25°C to an absorbance at 600 nm (A_{600}) of typically 0.6–1.0. Cells were harvested and resuspended in YPD at a concentration of 15 A_{600} units in 5 ml, then incubated at 25°C or 37°C for 10 min, as indicated. To stop the temperature shift, deplete the cells of ATP, and inhibit membrane fusion, the cultures were diluted 10-fold into ice-cold wash buffer (20 mM Tris, pH 7.5, 20 mM NaN_3, and 20 mM NaF). Washed cells were pelleted at 4°C and resuspended in 1 ml of ice-cold IP buffer (50 mM Hepes, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.5% NP-40), supplemented with protease inhibitors (10 μM antipain, 1 μg/ml aprotinin, 30 μM leupeptin, 30 μM chymostatin, 1 μM pepstatin A, and 1 mM PMSF). Cells and IP buffer were transferred to 2-ml, conical, screw-capped tubes with 2 g of 1 mm zirconia-silica beads and lysed in a Mini-beadbeater-8 at full power for 4 min at 4°C (beads and instrument from Biospec Products). For lysates prepared in the presence of ATP, NaN_3 and NaF were omitted from the wash buffer, EDTA was omitted from the IP buffer, and an ATP-regeneration system was added to a final concentration of 10 μg/ml creatine kinase, 5 mM creatine phosphate, 1 mM ATP, and 1 mM $MgCl_2$. The concentration of protein in the supernatant fraction of the lysates was determined by the Bio-Rad protein assay, using IgG as a protein concentra-

tion standard. The samples were adjusted to 4 mg/ml total protein with ice-cold IP buffer plus protease inhibitors. To minimize recovery of products that adhere nonspecifically to the protein G–Sepharose beads, 1.2-ml samples were incubated with rocking for 30 min at 4°C with 30 μl of a 50% protein G–Sepharose slurry in IP buffer. The beads, debris, and nonspecifically bound products were pelleted for 15 min at ~13,000 *g* in a microcentrifuge at 4°C. For each sample, 1 ml of the supernatant fraction was transferred to a clean tube on ice to which antibody was added for IP. The monoclonal antibody 9E10 was used at a 1:300 dilution to specifically precipitate MYC-tagged species. Efficiency of MYC-Sec1p recovery was typically 50%. The monoclonal antibody 12CA5 was used at 1:1,000 dilution to specifically precipitate HA-tagged species, and the Sncp antiserum was also used at a 1:1,000 dilution in Sncp IPs. After 1 h of rocking at 4°C, the beads and bound proteins were pelleted by centrifugation for 10 s at 4°C, and each sample was washed five times with 1 ml IP buffer. Proteins were eluted from the beads by boiling them in SDS sample buffer (60 mM Tris, pH 6.8, 100 mg/ml sucrose, 2% SDS, 0.05 mg/ml bromophenol blue, and 100 mM DTT) for 5 min. For stoichiometry measurements, proteins were eluted with 0.2% SDS in PBS for 5 min at 42°C to minimize elution of a cross-reacting contaminant from protein G–Sepharose beads. Proteins from the IPs were separated by SDS-PAGE on 12% minigels, and 15% minigels were used for the stoichiometry measurements. The proteins were then transferred from the gels to nitrocellulose membranes by electrophoresis for ~12 h at 25 mA per gel. Rainbow molecular weight markers aided the sectioning of nitrocellulose membranes according to the molecular weight of the proteins of interest. Each section was probed by Western blot analysis (using a blocking buffer of 0.5% Tween and 5% milk in PBS, pH 7.4) with antiserum against the protein of interest: Sec9p, Sncp, Pep12p, Sec22p, and Bos1p. Sec1p and MYC-Sec1p were detected with affinity-purified Sec1p antibodies. Biotinylated, affinity-purified anti-Ssop antibodies and peroxidase-conjugated avidin were used to detect Ssop amid the antibody heavy and light chains, which would otherwise cross-react with secondary antibodies. In all other cases, peroxidase-conjugated secondary antibodies (anti–mouse or anti–rabbit) were used. A chemiluminescent peroxidase substrate was used in conjunction with fluorography to reveal the presence or absence of the proteins in the IPs. For stoichiometry measurements, we used as a reference for the ratio of Ssop to Sncp in SNARE complexes the ratio of SsopΔTM to SncpΔTM in purified, soluble SNARE complexes (Sso1 and Snc1; Rice et al., 1997). Using densitometry, the band intensities of Ssop and Sncp coprecipitated with MYC-Sec1p were compared with the band intensities of SsopΔTM and SncpΔTM in twofold serial dilutions of the purified SNARE complexes.

Binding Experiments with Recombinant SNAREs

Binding studies were performed with yeast lysates prepared exactly as for the IP experiments. The recombinant, cytoplasmic domains of Sso1p, Sncp, and the SNAP-25 domain of Sec9p have been described previously (Nicholson et al., 1998). Sso1p was modified for the binding reactions by fusing the maltose-binding protein to the amino terminus (MBP-Sso1), using the pMAL-C2 system described above. MBP-Sso1 (and carboxyl-terminal truncation products) was purified and bound to amylose resin. An aliquot of resin-bound MBP-Sso1 was assembled into SNARE complexes by first adding an excess of the SNAP-25 domain of Sec9p (Sec9CT). These binary complexes were formed at 18°C for 48 h and the cytoplasmic domain of Snc2p (Snc2) was added 1 h before the binding experiment (Nicholson et al., 1998). The final recombinant protein-resin mixture was washed three times with ice-cold IP buffer before the binding reactions, to remove unbound proteins. Lysates were prepared from NY13 transformed with pNB680 for high levels of Sec1p. The supernatant fraction of the 13,000 *g* spin was diluted to 4 mg/ml (protein concentration) and incubated with 240 nM of resin-bound MBP-Sso1 or MBP-SNARE complex (MBP-Sso1:Sec9CT:Snc2) in 1-ml reactions. Samples were incubated with rocking for 1 h at 4°C. The resin was washed three times with ice-cold IP buffer and proteins were eluted by boiling for 5 min in sample buffer. Proteins were separated by SDS-PAGE on 10% minigels for Sec1p blots and 15% minigels for Coomassie-stained gels. Western blot analysis was performed with the Sec1p antibody, as described above.

Localization of GFP-Sec1p

For localization studies of GFP-Sec1p, 5 A_{600} units of early log phase cultures (A_{600} = 0.1 in YPD at 25°C) were incubated at 25°C or shifted to 37°C for 10 min, as indicated. Cells were washed with ice-cold wash buffer, fixed in methanol at −20°C for 10 min, washed with acetone at −20°C, and washed three times with ice-cold PBS, pH 7.4. The fixation protocol was necessary to enhance the very faint GFP-Sec1p fluorescence detected in living *SEC+* strains. Expression levels of GFP-Sec1p were identical in NY1697, NY1698, and NY1699, as judged by Western blot analysis of twofold, serial dilutions from samples of equal protein concentration, using the GFP antibody.

Epifluorescence microscopy was performed on a Zeiss Axiophot microscope equipped with a 100× oil-immersion objective (1.3 NA) and a fluorescein filter (FITC, excitation 480 nm, emission 535 nm, dichroic BS 505). Images were recorded on Kodak TMAX100 (ASA400) film with 30-s exposure times. Several different clones of each strain were examined to confirm the reproducibility of the observed localization of GFP-Sec1p.

Results

Sec1p Binds to Exocytic SNAREs

To test whether Sec1p binds to the exocytic t-SNARE Ssop, we looked for specific coprecipitation between Sec1p and Ssop in yeast cells. An IP from a strain in which Sec1p was tagged with a triple MYC epitope at its carboxyl terminus (MYC-*SEC1*) was compared with an IP from an isogenic strain with untagged Sec1p (Untagged) for the presence of a coprecipitating Ssop band by Western blot analysis (Fig. 1 A). The monoclonal MYC antibody specifically precipitated MYC-Sec1p with an efficiency of typically 50%, whereas no Sec1p was precipitated from the untagged strain. Using the Ssop antibody, we were able to detect a band corresponding to Ssop from the MYC-*SEC1* strain, but no Ssop band from the untagged strain, confirming a specific interaction between Sec1p and Ssop in yeast cell lysates. By comparing the intensity of the Ssop band in MYC-Sec1p IPs to the intensity of the Ssop band in threefold serial dilutions of the lysate, we estimate that ~0.2% of the total Ssop is bound to MYC-Sec1p.

Because Ssop is known to associate with the other exocytic t-SNARE Sec9p and the exocytic v-SNARE Sncp, we used Western blot analysis to probe for the presence of these SNARE proteins in the MYC-Sec1p IP. This analysis revealed the presence of both Sncp and Sec9p (Fig. 1 A) in addition to Ssop. By comparing the intensities of Sncp and Sec9p bands in the IPs to the intensity of threefold serial dilutions of the lysate, we estimate that ~0.4% of Sncp and 2% of the Sec9p is bound to MYC-Sec1p, assuming minimal degradation of these proteins during lysis. This small percentage of the total SNARE proteins present in the MYC-Sec1p IPs is reasonable, when compared with the 1% of total SNAREs assembled into exocytic SNARE complexes in yeast lysates (Grote, E., and P.J. Novick, manuscript in preparation).

To confirm the presence of Sncp in the MYC-Sec1p IPs, we looked directly for coprecipitation of MYC-Sec1p and HA-Sncp from a strain expressing both epitope-tagged proteins (MYC-*SEC1* HA-*SNC*). MYC-Sec1p and HA-Sncp were coprecipitated using either the monoclonal MYC antibody or the monoclonal HA antibody (Fig. 1 B), while neither antibody precipitated Sncp or Sec1p from an untagged strain. The detection of Sncp in addition to the t-SNAREs in the MYC-Sec1p IPs suggests that these proteins are assembled as SNARE complexes.

Another interpretation of the result that all three of the exocytic SNAREs coprecipitate with MYC-Sec1p is that Sec1p binds nonspecifically to all SNAREs. To address

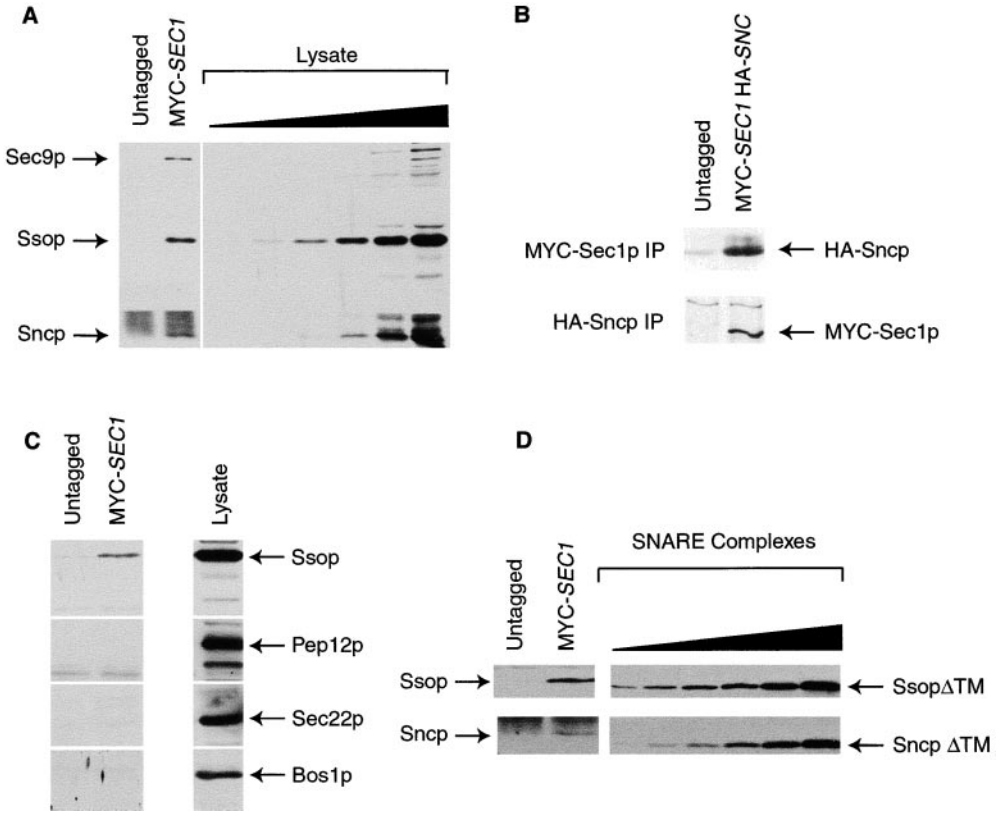

Figure 1. MYC-Sec1p binds to all three exocytic SNAREs. (A) Ssop, Sec9p, and Sncp coprecipitate with MYC-Sec1p. 15 A_{600} units of either untagged (NY1491) or MYC-*SEC1* (NY1689) cells were harvested and diluted 10-fold into ice-cold wash buffer (20 mM Tris-Cl, pH 7.5, plus 20 mM NaN_3, and 20 mM NaF) to inhibit membrane fusion and to deplete intracellular ATP. Cells were lysed in ice-cold IP buffer (50 mM Hepes, pH 7.4, 150 mM KCl, 1 mM EDTA, 0.5% NP-40 plus protease inhibitors) to solubilize proteins and inhibit ATP hydrolysis. Anti-MYC IPs were probed for coprecipitated SNARE proteins with antibodies to Sec9p, Ssop, and Sncp using standard Western blot analysis. (B) MYC-Sec1p coprecipitates with HA-Sncp. Lysates of untagged (NY1491) and MYC-*SEC1* HA-*SNC* (NY1702) were prepared as in A. To detect coprecipitation of HA-Sncp with MYC-Sec1p, MYC-Sec1p IPs were blotted for HA-Sncp using the monoclonal HA antibody. To detect coprecipitation of MYC-Sec1p with HA-Sncp, HA-Sncp IPs were blotted for MYC-Sec1p using the Sec1p antibody. (C) Non-exocytic SNAREs do not coprecipitate with MYC-Sec1p. IPs from lysates of untagged (NY1491) and MYC-*SEC1* (NY1689) strains were divided into four samples and analyzed for coprecipitated SNARE proteins with antibodies to Ssop, Pep12p, Sec22p, and Bos1p. The lysate shown is 2% of the protein used for the IP. (D) Stoichiometry of SNARE proteins bound to MYC-Sec1p. IPs from lysates of untagged (NY1491) and MYC-*SEC1* (NY1689) were probed with antibodies to Ssop and Sncp. The ratio of Ssop to Sncp coprecipitated with MYC-Sec1p was compared with twofold serial dilutions of purified, soluble SNARE complexes, in which the ratio of soluble SNAREs, SsopΔTM to SncpΔTM to Sec9p (SNAP-25–like domain), is 1:1:1.

this possibility, MYC-Sec1p IPs were probed with antisera specific to SNAREs that function at other steps in vesicle trafficking. No coprecipitation of Sec22p, Pep12p, or Bos1p was detected in MYC-Sec1p IPs, when compared with MYC IPs from an untagged strain (Fig. 1 C), in spite of the fact that each antibody easily detected <0.2% of its antigen from the lysate (2% shown). These findings support the conclusion that Sec1p binds specifically to exocytic SNAREs in yeast cell lysates.

While the recovery of all three exocytic SNAREs in MYC-Sec1p IPs suggests an interaction between MYC-Sec1p and SNARE complexes, it remains possible that Sec1p predominantly binds to one of the proteins (for example, Ssop) which is not assembled into SNARE complexes. To address this possibility, we compared the ratio of Ssop to Sncp in the MYC-Sec1p IPs with the 1:1 ratio of Ssop to Sncp in purified yeast exocytic SNARE complexes (Rice et al., 1997). The purified SNARE complexes consist of the cytoplasmic domains of Ssop (SsopΔTM) and Sncp (SncpΔTM) but only the region of Sec9p homologous to SNAP-25. Thus, we were able to probe for the Ssop and Sncp epitopes, but not the Sec9p fragment, with our antibodies (Fig. 1 D). The ratio of the SsopΔTM to SncpΔTM band intensities was 1.45 in the equimolar complex (an average of two measurements, 1.2 and 1.7; see Materials and Methods). The band intensity ratio of Ssop to Sncp in the IPs (an average of two measurements, 1.2 and 1.4) was 1.3. Thus, the ratio of SsopΔTM to SncpΔTM in the purified SNARE complexes closely resembles the ratio of Ssop to Sncp in the IPs, indicating that SNARE complexes are greatly enriched in the MYC-Sec1p IPs. These results are consistent with the notion that Sec1p interacts predominantly with SNARE complexes.

Effects of SNARE Complex Assembly and Disassembly on Interactions with Sec1p

Because SNARE complex assembly and disassembly are known to be defective in certain *sec* mutants, we asked how the association between Sec1p and Ssop is affected in these mutants. The Rab mutant *sec4-8* is defective in exocytic SNARE-complex assembly (Grote, E., and P.J. Novick, manuscript in preparation); accordingly, we detected little coprecipitating Ssop in a Sncp IP from a *sec4-8* strain shifted to the restrictive temperature (37°C) for 10 min. In the same experiment, we examined Ssop coprecipitated

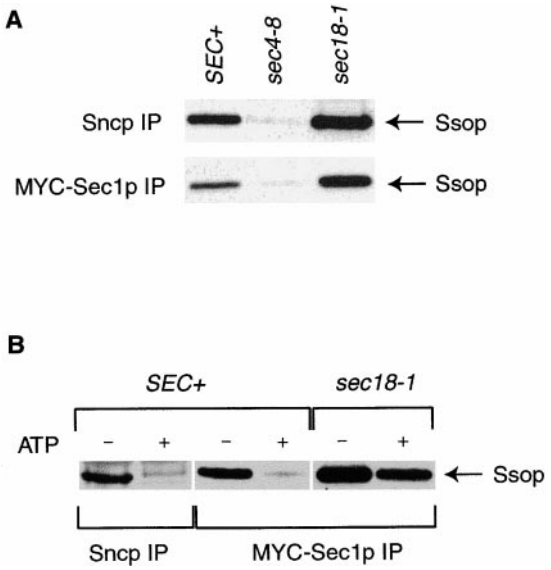

Figure 2. Conditions that alter the abundance of SNARE complexes also alter the Sec1p-Ssop interaction. (A) The interaction of Ssop with either MYC-Sec1p or Sncp is altered in *sec* mutants. *SEC+* (NY1689), *sec4-8* (NY1690), and *sec18-1* (NY1691) MYC-*SEC1* strains were shifted to 37°C for 10 min. The Ssop antibody was used to detect Ssop from the anti-Sncp and the anti-MYC IPs. (B) Sec18p disassembles SNARE complexes and disrupts the Sec1p-Ssop interaction. Lysates of *SEC+* (NY1689) and *sec18-1* (NY1691) MYC-*SEC1* strains were prepared either with ATP (+) or without ATP (−), and the anti-MYC or anti-Sncp IPs were probed for coprecipitated Ssop. When ATP was required, NaN_3 and NaF were omitted from the wash buffer, and EDTA was replaced by an ATP-regeneration system (10 μg/ml creatine kinase, 5 mM creatine phosphate, 1 mM ATP, and 1 mM $MgCl_2$) in the IP buffer.

with MYC-Sec1p from the *sec4-8* strain and found that the association between MYC-Sec1p and Ssop was also impaired, indicating that the defect in SNARE complex assembly is correlated with a defect in the Sec1p-Ssop interaction (Fig. 2 A, *sec4-8*). The mutant *sec18-1* is defective in SNARE complex disassembly, as reflected by an excess (two- to fivefold) of Ssop detected in Sncp IPs from a *sec18-1* strain shifted to the restrictive temperature (37°C) for 10 min. Under the same conditions, we observed enhanced coprecipitation of Ssop with MYC-Sec1p from the *sec18-1* mutant (Fig. 2 A, *sec18-1*), demonstrating a correlation between increased abundance of SNARE complexes and an increased amount of Ssop bound to MYC-Sec1p.

We then asked how the interaction between Sec1p and Ssop is affected by the disassembly of SNARE complexes in lysates. The ATPase activity of Sec18p is essential for SNARE complex disassembly (Brennwald et al., 1994); therefore, conditions that inhibit ATP hydrolysis preserve SNARE complexes in yeast lysates. For this reason, our standard IPs are performed with cells washed in the presence of NaN_3 and NaF (to deplete the cells of ATP) and lysed in the presence of EDTA (to chelate magnesium) to minimize disassembly of SNARE complexes by Sec18p activity. Using these conditions for both Sncp and MYC-Sec1p IPs, we observed Ssop coprecipitated with both Sncp and MYC-Sec1p (Fig. 2 B, −ATP). This result indicates that Sec1p associates with Ssop under conditions that preserve SNARE complexes. Conversely, in the presence of ATP and magnesium, SNARE complexes in yeast lysates are effectively disassembled (Brennwald et al., 1994). Little Ssop coprecipitated with either Sncp or MYC-Sec1p if NaN_3 and NaF were absent from the wash buffer and cells were lysed in the presence of an ATP-regeneration system (Fig. 2 B, +ATP). This result indicates that the Sec1p-Ssop interaction is virtually eliminated under conditions that effectively disassemble SNARE complexes.

While ATP-dependent disassembly of SNARE complexes can be attributed to Sec18p, it is possible that other ATP-dependent processes could affect the association of Sec1p with Ssop. For example, phosphorylation of neuronal Sec1 protein was shown to inhibit its association with syntaxin (Fujita et al., 1996; Shuang et al., 1998). To address this possibility, we repeated the MYC-Sec1p IPs in the *sec18-1* background in the presence of an ATP source, or in its absence. Ssop coprecipitates with MYC-Sec1p in *sec18-1* under both of these conditions (Fig. 2 B, *sec18-1*). The partial effect of ATP seen in the MYC-Sec1p IP from *sec18-1* is also observed in Sncp IPs (data not shown) and is believed to be due to low levels of ATPase activity of the mutant Sec18p under the IP conditions. Thus, like SNARE complexes, the association between Ssop and MYC-Sec1p is disrupted by an ATP-dependent and Sec18p-dependent process.

Sec1p Binds to Preassembled SNARE Complexes

The association between Sec1p and Ssop is dependent on the same factors that affect SNARE complex assembly and disassembly, indicating that assembled SNARE complexes are required for an association between Ssop and Sec1p. However, the experiments described above do not address the order of assembly of Sec1p and SNAREs into Sec1p-bound SNARE complexes. Does Sec1p first bind to Ssop before it assembles into SNARE complexes, or does Sec1p bind to SNARE complexes after they are assembled? To determine the order of assembly, we performed "mixing" experiments, in which an association between proteins is detected by coprecipitation of a protein from one strain with a protein from another strain when the two strains are lysed together.

The mixing protocol was first used to ask if SNARE complexes assemble only in vivo, or if Ssop from one strain can assemble with Sncp from another strain in a mixed lysate (Fig. 3 A). In one strain (MYC-*SSO*), the only copy of Ssop was tagged with a triple-MYC epitope, producing a strain with MYC-Ssop, and untagged Sncp. In another strain (HA-*SNC*), the only copy of Sncp was tagged with a triple-HA epitope, producing a strain with HA-Sncp, and untagged Ssop. The cultures were mixed 1:1 (based on A_{600}) before lysis. The monoclonal HA antibody was used to precipitate HA-Sncp from lysates of MYC-*SSO*, HA-*SNC*, or a mixture of the two strains (MIX). In the HA IPs, the endogenous, untagged Ssop coprecipitated with HA-Sncp from HA-*SNC* lysates. No Ssop coprecipitated from MYC-*SSO*, due to the absence of HA-Sncp in that strain. In the mixed sample, untagged Ssop, but not MYC-Ssop, coprecipitated with HA-Sncp,

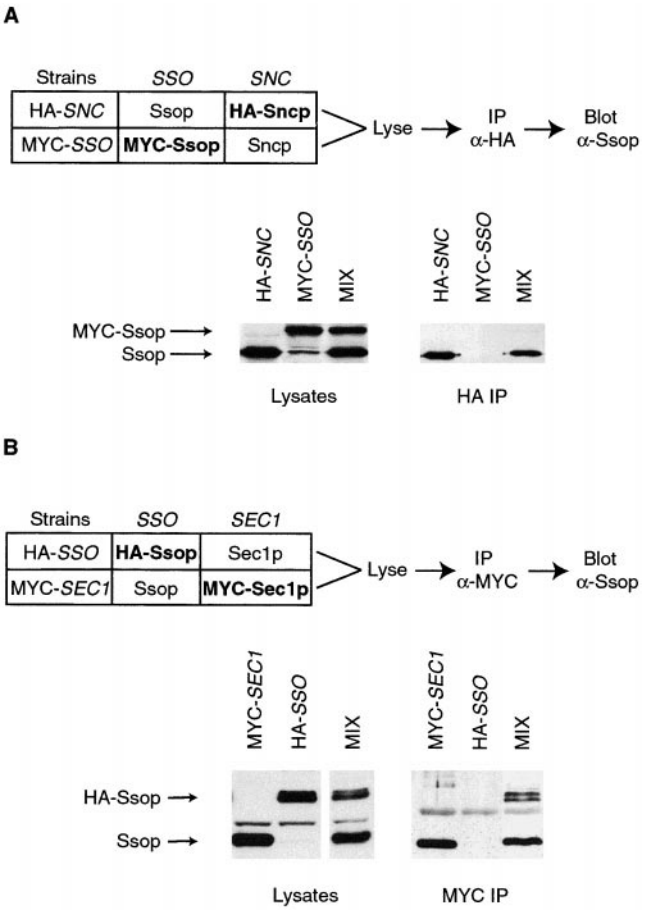

Figure 3. Exocytic SNARE complexes assemble only in vivo, but Sec1p can bind to SNARE complexes in lysates. For each experiment, a mixing protocol was used to test for the ability of epitope-tagged proteins expressed in two different strains to bind to each other. The strains that were mixed in each experiment are depicted schematically above the results, with the products of the relevant genes listed after the strain names. (A) Exocytic SNARE complexes assemble in vivo, not in yeast lysates. HA-*SNC* (NY1642) and MYC-*SSO* (NY1704) were mixed 1:1 before lysis (MIX). Sso proteins coprecipitated in the anti-HA IPs were detected with the Ssop antibody. Lysates shown represent ~1% of the protein used for the IPs. (B) Sec1p binds to Ssop in yeast lysates. MYC-*SEC1* (NY1689) and HA-*SSO* (NY1692) were mixed 1:1 before lysis (MIX). Sso proteins coprecipitated in the anti-MYC IPs were detected with the Ssop antibody. Lysates shown represent 1% of the protein used for the IPs. Note that in these mixing experiments HA-Ssop runs as a doublet, and there are two distinct cross-reacting bands detected in the Western blot, one in the lysate and one in the IPs.

despite its presence in the lysate used for the IP. The absence of MYC-Ssop in the HA-Sncp IPs from the mixed sample indicates that the SNARE complexes detected in these experiments are formed exclusively in vivo. No further assembly of SNARE complexes can be detected in the lysates.

Since SNARE complex assembly is completed in vivo, we could ask whether Sec1p binds to these preassembled SNARE complexes in lysates, using the mixing protocol to test for the association of Sec1p from one strain with Ssop from another strain (Fig. 3 B). For these mixing experiments, MYC-*SEC1* was used as the source of MYC-Sec1p plus untagged Ssop. In the other strain (HA-*SSO*) the sole copy of Ssop was tagged with a triple-HA epitope, producing a strain with HA-Ssop and untagged Sec1p. The cultures were mixed 1:1 before lysis. The monoclonal MYC antibody was used to precipitate MYC-Sec1p from lysates of MYC-*SEC1*, HA-*SSO* or a mixture of the two strains (MIX). In the MYC IPs, untagged Ssop coprecipitated with MYC-Sec1p from the MYC-*SEC1* strain. No Ssop protein coprecipitated from HA-*SSO*, due to the absence of MYC-Sec1p. In the mixed sample, not only untagged Ssop, but also HA-Ssop, coprecipitated with MYC-Sec1p. The presence of the HA-Ssop band in the IP of the mixed sample indicates that Sec1p can bind to Ssop in lysates. Because the interaction between Sec1p and Ssop requires SNARE complexes, and SNARE complexes are preformed in vivo, we conclude that Sec1p can bind to SNAREs after they are assembled into SNARE complexes. Furthermore, the results of these mixing experiments indicate either that the binding of Sec1p to SNARE complexes has a significant rate of exchange, or that a previously unavailable pool of SNARE complexes becomes exposed during the experiment.

From the results of these mixing experiments, we conclude that Sec1p binds to preassembled SNARE complexes. If this conclusion is correct, then the interaction between Sec1p and Ssop should be limited by the abundance of SNARE complexes present in the lysate, not by the total amount of Sec1p or SNAREs. To test this prediction, we repeated the mixing experiment, exploiting the *sec* mutants for their altered levels of SNARE complexes. In this experiment, HA-*SSO* and MYC-*SEC1* strains with either a *SEC*+ or *sec* genotype were mixed and shifted to the restrictive temperature for 10 min before lysis (Fig. 4). The MYC IP from the *SEC*+ mixture (Fig. 4, lane 1) indicates HA-Ssop bound to MYC-Sec1p, as shown in Fig. 3 B. If HA-*SSO* carried a *sec4-8* mutation, untagged Ssop, but not HA-Ssop, coprecipitated with MYC-Sec1p (Fig. 4, lane 2). However, if MYC-*SEC1* carried the *sec4-8* mutation, HA-Ssop, but none of the untagged Ssop, coprecipitated with MYC-Sec1p (Fig. 4, lane 4). These results indicate that the Ssop from a *sec4-8* strain is not competent to bind to MYC-Sec1p, even with wild-type Sec4p in the mixed lysate. In contrast, the MYC-Sec1p from a *sec4-8* strain does bind the Ssop contributed from a *SEC*+ strain.

Similar mixing experiments were performed between the disassembly mutant, *sec18-1*, and *SEC*+ strains. If HA-*SSO* carried a *sec18-1* mutation, an excess of HA-Ssop and an unchanged level of untagged Ssop coprecipitated with MYC-Sec1p (Fig. 4, lane 3), when compared with the levels of HA-Ssop and Ssop coprecipitated from a mixture of the two *SEC*+ strains (Fig. 4, lane 1). Likewise, if MYC-*SEC1* carried the *sec18-1* mutation, an excess of untagged Ssop and an unchanged level of HA-Ssop (Fig. 4, lane 5) coprecipitated with MYC-Sec1p, when compared with the levels of these proteins coprecipitated from a mixture of the two *SEC*+ strains. HA-Ssop did not precipitate with the MYC antibody in the absence of MYC-Sec1p (Fig. 4, lane 6), as shown previously (Fig. 3 B). These results indicate that elevated levels of Ssop coprecipitated with MYC-Sec1p only when the Ssop was from a *sec18-1*

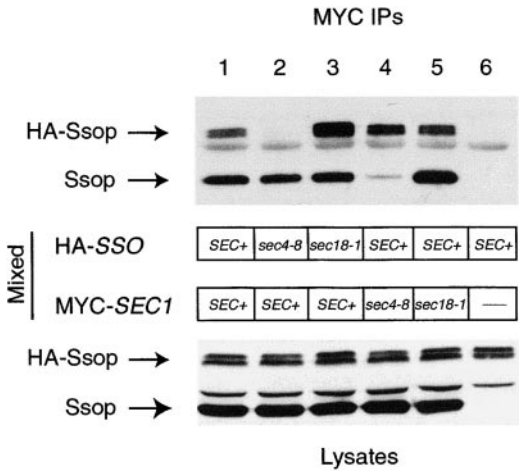

Figure 4. The interaction between Sec1p and Ssop is limited by the abundance of SNARE complexes recovered from *sec* mutants. HA-*SSO* and MYC-*SEC1* strains were mixed and lysed to look for coprecipitation of HA-Ssop with MYC-Sec1p as in Fig. 3 B, except that *SEC+* cells were mixed with either *sec4-8* or *sec18-1* cells. For each pair of strains mixed, the *sec* genotypes are indicated in boxes between the MYC IPs and the lysates. In lanes 1–3, MYC-*SEC1 SEC+* (NY1689) was mixed 1:1 with HA-*SSO SEC+* (NY1692), HA-*SSO sec4-8* (NY1693), and HA-*SSO sec18-1* (NY1694), respectively. In lanes 4–6, HA-*SSO SEC+* (NY1692) was mixed with MYC-*SEC1 sec4-8* (NY1690), MYC-*SEC1 sec18-1* (NY1691), and lysis buffer (—), respectively. Mixed cultures were shifted to 37°C for 10 min. Sso proteins coprecipitated with MYC-Sec1p were detected with the Ssop antibody. Lysates shown represent 1% of the protein used for the IPs.

strain. In contrast, MYC-Sec1p from the *sec18-1* strain is unaltered in its ability to coprecipitate Ssop.

The results of mixing experiments with *SEC+* and *sec* strains support the conclusion that Sec1p binds to Ssop after it is assembled into SNARE complexes. As predicted, the interaction between MYC-Sec1p and Ssop is limited by the abundance of SNARE complexes present in the mixed lysates. In contrast, the concentration of MYC-Sec1p in the lysate does not limit the amount of Ssop coprecipitated with MYC-Sec1p, as indicated by the ability of MYC-Sec1p from one strain to coprecipitate both wild-type levels of Ssop from the *SEC+* strain plus enhanced levels of Ssop from the *sec18-1* strain. Furthermore, neither the *sec4-8* nor the *sec18-1* mutant causes an irreversible change in MYC-Sec1p that affects its ability to associate with Ssop in the lysates. We cannot exclude the possibility that Sec1p also functions before SNARE complex assembly by binding transiently or with low affinity to Ssop. However, the observations that SNARE complexes are preformed in vivo and that Sec1p can bind to preassembled SNARE complexes from another strain demonstrate that prior association with Ssop is not required for Sec1p to bind to SNARE complexes.

Sec1p Binds Recombinant SNARE Complexes

In light of the results from IP experiments, we were prompted to examine whether Sec1p can interact with purified, recombinant SNARE complexes. Complete reconstitution of Sec1p-bound SNARE complexes from purified components was impossible, because recombinant Sec1p aggregates irreversibly (Munson, M., and F. Hughson, unpublished observations). Therefore, we used resin-bound, recombinant SNAREs to recover Sec1p from yeast lysates. Soluble, recombinant Sso1 protein was fused to the carboxyl terminus of maltose-binding protein (MBP-Sso1). As a source of Sec1p, we prepared a lysate from a yeast strain overexpressing Sec1p. Binding reactions were prepared with either resin-bound, uncomplexed MBP-Sso1 or resin-bound MBP-Sso1:Sec9CT:Snc2, prepared as has been previously described (see Materials and Methods; Nicholson et al., 1998). In spite of the fact that equimolar amounts of MBP-Ssop and MBP-SNARE complexes were used in the binding reactions, Sec1p bound preferentially to the ternary complex, with minimal binding to MBP-Sso1 alone (Fig. 5). While Sec1p recovery was maximal from lysates with high levels of Sec1p, the same results were obtained with lysates from strains expressing endogenous levels of Sec1p (data not shown). In each case, the amount of Sec1p bound to recombinant SNARE complexes was too low to detect by Coomassie stain. This substoichiometric binding suggesting that only a fraction of the Sec1p molecules or SNARE complexes was competent for binding. Alternatively, the affinity or association/dissociation rates prevented quantitative recovery of Sec1p with the recombinant proteins.

GFP-Sec1p Is Concentrated at Sites of Secretion

Because Sec1p binds to exocytic SNARE complexes, we predicted that these proteins would colocalize at sites of secretion. Secretion is localized to specific sites in the yeast

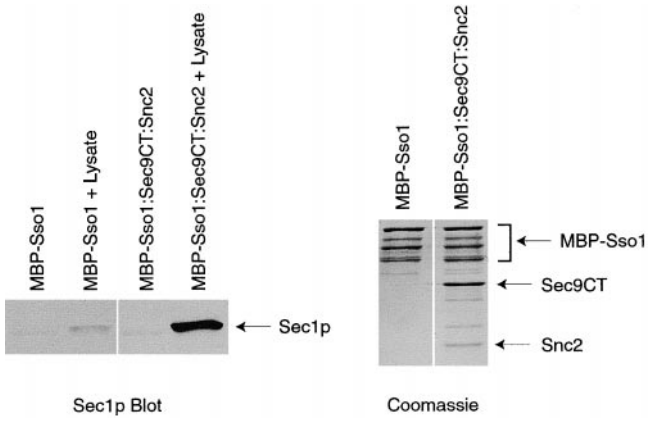

Figure 5. Recombinant SNARE complexes bind Sec1p from yeast lysates. Amylose resin-bound MBP-Sso1 and MBP-Sso1:Sec9CT:Snc2 complex were used at 240 nM in 1-ml binding reactions with a lysate from a strain overexpressing Sec1p (NY13 transformed with pNB680). After washing, resin with MBP-Sso1 alone, MBP-Sso1 bound to lysate, MBP-Sso1:Sec9CT:Snc2 alone, and MBP-Sso1:Sec9CT:Snc2 bound to lysate were separated by 10% SDS-PAGE gels and blotted with the Sec1p antibody. A 15% SDS-PAGE Coomassie-stained gel indicates 25% of the resin-bound recombinant proteins used in each of the binding reactions. Note that carboxyl-terminal truncation products of MBP-Sso1 are also bound to the amylose resin and that Snc2 does not stain well with Coomassie (Nicholson et al., 1998).

cell, such as the site of the emerging daughter cell (bud), the tip of the growing bud, and the junction, or "neck," between the mother and daughter cells during cytokinesis (Lew and Reed, 1995). Previous studies have shown that the t-SNAREs Ssop and Sec9p are distributed over the entire plasma membrane (Brennwald et al., 1994), and that Sncp is enriched in vesicle fractions from yeast lysates (Protopopov et al., 1993). SNARE complexes have not been localized in yeast cells; however, they are predicted to assemble at sites of secretion, based on their proposed function in vesicle docking and membrane fusion.

To observe the localization of Sec1p in yeast cells we created a Sec1p-green fluorescent protein chimera (GFP-Sec1p) by gene replacement, and we used fluorescence microscopy to detect sites of concentrated GFP-Sec1p (Fig. 6). GFP-*SEC1* was introduced into *SEC+*, *sec4-8*, and *sec18-1* strains, and the resulting strains are described in Table I. The temperature sensitivity of the *SEC+* and *sec18-1* strains was unaffected by the presence of GFP-*SEC1*, but the *sec4-8* strain displayed modestly slower growth in the presence of GFP-*SEC1*. The expression level of GFP-Sec1p was identical in all three strains, as determined by Western blot analysis of serial dilutions of the lysates (data not shown). In *SEC+* cells incubated at either 25°C or 37°C, GFP-Sec1p could be detected in some cells as faint fluorescence, concentrated at the tips of small buds, or at mother-daughter necks. However, in *sec4-8* cells, GFP-Sec1p localization was not apparent in any of the cells incubated at either 25°C or 37°C. The autofluorescence detected at both temperatures was also observed in *sec4-8* cells with an untagged *SEC1* gene (No GFP). In the *sec18-1* cells incubated at 25°C, fluorescent GFP-Sec1p was observed concentrated at bud tips and mother-daughter necks. The GFP-Sec1p fluorescence was more intense and more easily detected in *sec18-1* cells incubated at 37°C than in *SEC+* cells incubated at either temperature. Although in these experiments we have not ruled out the possibility that other factors are required for Sec1p localization, the localization of Sec1p in *SEC+* and *sec* mutant cells is consistent with the proposal that Sec1p binds to assembled SNARE complexes at sites of secretion.

Discussion

In agreement with binding studies of syntaxin homologues and members of the Sec1 family from various systems (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994b; Søgaard et al., 1994; Grabowski and Gallwitz, 1997; Nichols et al., 1998), we observe the syntaxin homologue Ssop bound to Sec1p in immunoprecipitates from yeast lysates. However, while others have found an interaction between Sec1 homologues and syntaxin homologues in the absence or even to the exclusion of other SNARE proteins, we observe that Sec1p coprecipitates all three components of the exocytic SNARE complex, Ssop, Sec9p, and Sncp.

Several observations suggest that Sec1p preferentially coprecipitates with SNAREs assembled into SNARE complexes. The ratio of Ssop to Sncp in Sec1p IPs resem-

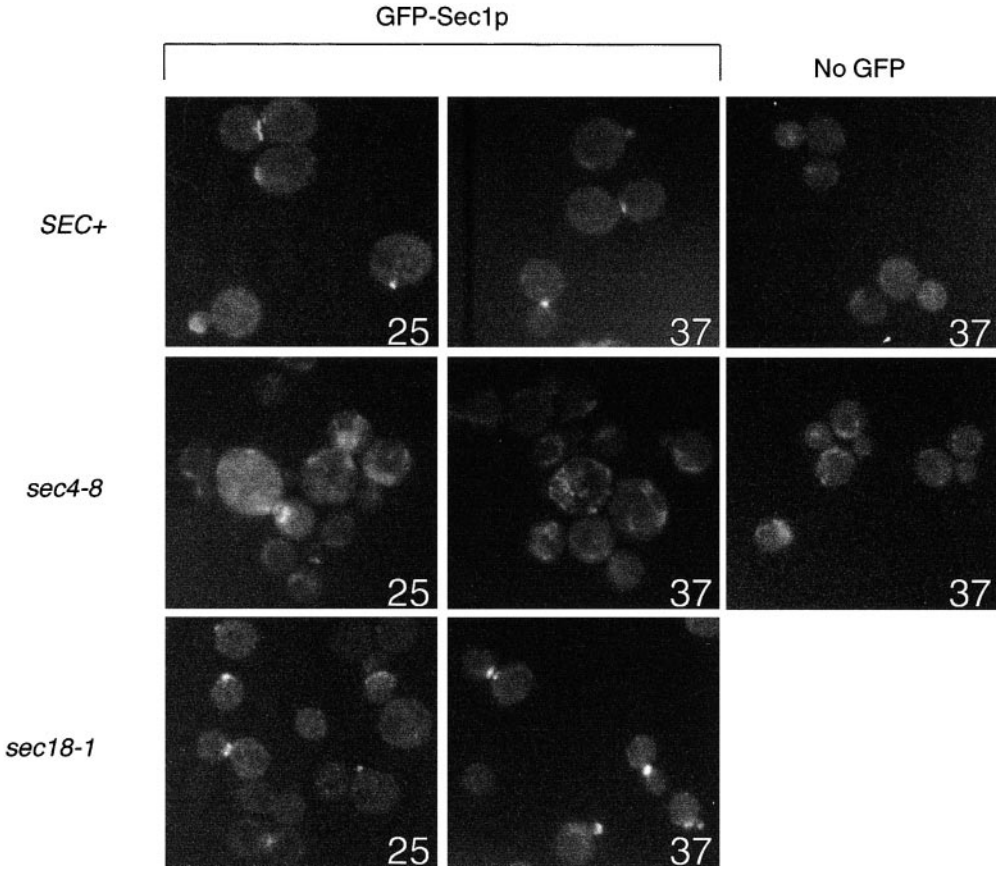

Figure 6. The localization of GFP-Sec1p to sites of exocytosis is altered in *sec* mutants. *SEC+* GFP-Sec1p (NY1699), *SEC+*; No GFP (NY 1700), *sec4-8* GFP-Sec1p (NY1697), *sec4-8*; No GFP (NY1701) and *sec18-1* GFP-Sec1p (NY1698) were grown to early log phase. 5 A_{600} units were concentrated and incubated at 37°C for 10 min (or incubated at 25°C), then diluted 10-fold into ice-cold wash buffer. Cells were fixed with methanol and acetone, washed with ice-cold PBS, and visualized by epifluorescence microscopy.

bles the 1:1 ratio of these two proteins in purified SNARE complexes. This finding indicates a significant enrichment of SNARE complexes in the Sec1p IPs, because only ~1% of the exocytic SNAREs are assembled into complexes (Grote, E., and P.J. Novick, manuscript in preparation). Moreover, the extent of coprecipitation of Ssop with Sec1p depends on the abundance of assembled SNARE complexes in the assembly mutant *sec4-8* and in the disassembly mutant *sec18-1*, as predicted if Sec1p binds assembled SNARE complexes.

Coprecipitation of Sec1p and Ssop is highly sensitive to disassembly of SNARE complexes by the ATPase activity of Sec18p; therefore, it is only observed in the absence of ATP or in a *sec18-1* mutant. These results are consistent with an earlier observation concerning the ER-to-Golgi trafficking step in yeast. The Sec1 homologue Sly1p was found to coprecipitate with the ER-to-Golgi SNARE complex (Sed5p, Bos1p, Bet1p, and Sec22p), but this interaction was observed only in the *sec18-1* mutant (Søgaard et al., 1994). By contrast, the interaction between Sed5p (the ER-to-Golgi syntaxin homologue) and Sly1p was not dependent on the *sec18-1* mutation (see also Lupashin and Waters, 1997). Furthermore, Sly1p has a high affinity for Sed5p in the absence of other SNAREs (Grabowski and Gallwitz, 1997), as observed for neuronal Sec1 and syntaxin. Taken together, these results suggest that Sec1 homologues may display a range of affinities for unassembled SNAREs and SNARE complexes. Furthermore, our findings emphasize that care must be taken to prevent SNARE complex disassembly in order to examine interactions between Sec1 proteins and SNARE complexes. Results from recent studies of Sec18p illustrate the importance of inhibiting ATPase activity for the recovery of SNARE complexes from lysates (Ungermann et al., 1998a), and suggest a reexamination of earlier experiments, in which ATP was present.

Current models propose that Sec1 proteins regulate SNARE complex assembly by binding the uncomplexed syntaxin homologues, either to prevent SNARE complex formation or to stimulate it. Unexpectedly, we observed little recovery of Sec1p bound to uncomplexed Ssop either in IPs, or in binding experiments with purified Ssop. Instead, we found that Sec1p binds to preassembled SNARE complexes. The results of mixing experiments and binding studies with purified SNARE complexes establish that the association of Sec1p with preassembled SNARE complexes does not require an interaction between Sec1p and SNARE components before complex assembly. Conversely, failure of SNAREs to form a complex is not the result of an irreversible defect in Sec1p function in *sec4-8* strains, because the interaction between Sec1p from those strains and SNARE complexes from *SEC+* strains is unaltered. These experiments do not formally rule out the possibility that Sec1p interacts with Ssop before SNARE complex assembly. However, we favor the position that Sec1p functions after assembly, because the level of SNARE complexes recovered by IP from two loss-of-function alleles of *sec1* is unaltered (data not shown), as predicted if Sec1p function is not required for the assembly of SNARE complexes.

An abundance of data from yeast and other systems indicates that Sec1 proteins bind syntaxin proteins, but do these observations rule out interactions between Sec1 proteins and SNARE complexes? In one study, pairwise binding experiments revealed that the high-affinity n-Sec1/syntaxin interaction prevents association of either the v-SNARE VAMP or the other t-SNARE SNAP-25 with syntaxin in vitro (Pevsner et al., 1994b). However, neither the ability of n-Sec1 to prevent assembly of the complete ternary SNARE complex nor the failure of n-Sec1 to bind to preassembled SNARE complexes was demonstrated by these studies. In *Drosophila*, overexpression of either the Sec1 homologue ROP or syntaxin causes a decrease in neurotransmitter release that is relieved when syntaxin and ROP are co-overexpressed (Wu et al., 1998), suggesting that excess ROP can block neurotransmission by titering syntaxin in vivo. Similar studies in yeast reveal no deleterious effect of overexpression of Sec1p or Ssop; on the contrary, overexpression of these proteins suppresses several secretory mutants (Aalto et al., 1993). Furthermore, attempts to copurify Sec1 proteins with syntaxin homologues or with SNARE complexes either from neuronal systems (Hata et al., 1993; Söllner et al., 1993a; Garcia et al., 1994; Pevsner et al., 1994b; Wu et al., 1998) or from yeast extracts (Brennwald et al., 1994; Søgaard et al., 1994; Grabowski and Gallwitz, 1997; Lupashin and Waters, 1997; Nichols et al., 1998) have yielded mixed results.

The apparent discrepancies raised by these studies may reflect a fundamental difference in function between Sec1 homologues. Alternatively, Sec1 homologues may share an affinity for a specific conformation of the t-SNARE, a conformation that is only present in SNARE complexes in the case of Ssop, but present in other syntaxin homologues, even in their uncomplexed form. In this regard, recent structural studies of syntaxin (Fernandez et al., 1998) and Ssop (Nicholson et al., 1998; Fiebig et al., 1999) support previous conclusions that these proteins can adopt alternate conformations (Calakos et al., 1994; Hanson et al., 1995). Future study of the interactions between Sec1 homologues and SNAREs should resolve some of these issues and reveal more about the function of Sec1 proteins in secretion.

Are SNARE complexes receptors for Sec1? The localization of GFP-tagged Sec1p in intact cells coincides with sites of vesicle docking and exocytosis, where productive SNARE complexes are believed to assemble and function in membrane fusion. While it remains possible that other factors in addition to SNARE complexes are required for the localization of Sec1p to sites of secretion, the notion that SNARE complexes act as receptors for Sec1p is supported by the altered pattern of GFP-Sec1p fluorescence in *sec* mutants. The mislocalization of GFP-Sec1p in *sec4-8* correlates with the defect in SNARE complex assembly and a corresponding defect in the association between Sec1p and SNARE complexes. Likewise, the robust localization of GFP-Sec1p in *sec18-1* correlates with the increased abundance of SNARE complexes that accumulates due to a defect in complex disassembly and a corresponding increase in the amount of SNARE complexes recovered in Sec1p IPs.

SNAREs were originally identified as receptors for α-SNAP and NSF in neurons (Söllner et al., 1993a; McMahon and Sudhof, 1995). The binding of Sec17p and Sec18p to SNAREs results in the disassembly of SNARE com-

plexes formed on opposing membranes as well as those in the same membrane, as demonstrated by reconstitution studies with yeast vacuoles (Ungermann et al., 1998b). However, disassembly may be undesirable when SNARE complexes are required in vivo, such as during vesicle docking or membrane fusion. Under these circumstances, the binding of NSF and α-SNAP homologues to SNARE complexes may be prevented. In addition, NSF and α-SNAP homologues may be dissociated from SNARE complexes by conditions that inactivate the ATPase in vitro. For example, the Sec17p/Sec18p bound to a subset of SNARE complexes in vivo may be displaced by lysis in EDTA solutions, releasing a free pool of unbound SNARE complexes. This may explain the ability of preassembled SNARE complexes to bind Sec1p from another strain in the mixing experiments. In this regard, it may be relevant that α-SNAP can compete with neuronal Sec1 for binding to syntaxin (Hayashi et al., 1995). However, results from another in vitro experiment indicate that the binding of Sec17p and Sly1p to Sed5p is not mutually exclusive (Kosodo et al., 1998). Whether or not Sec1p and Sec17p/Sec18p compete for binding to SNARE complexes remains to be determined.

Our results place Sec1p at the core of the exocytic fusion machinery, bound to SNARE complexes, and localized to sites of secretion. We speculate that Sec1p functions to promote exocytosis after SNARE complexes are assembled. One model for Sec1p function is as a passive shield, protecting correct SNARE complexes from disassembly by Sec18p. In this model, Sec1p and Sec18p binding is mutually exclusive; thus, productive SNARE complexes bound by Sec1p are permitted to carry out their postulated role as the membrane fusion machinery. Another model considers an active role for Sec1p in promoting membrane fusion. Fusion of liposomes reconstituted with purified SNARE proteins is unphysiologically slow (Weber et al., 1998) and may require other factors, such as Sec1p, to stimulate rearrangement of SNARE complexes into an efficient fusion-active machine. This model predicts that addition of Sec1p to reconstituted liposomes would increase the rate of membrane fusion in vitro. We must also consider the possibility that Sec1p functions with other factors to promote exocytosis. The binding of Sec1p to SNARE complexes may displace regulatory factors, or recruit other proteins that stimulate exocytosis. A central tenet of these models is that all Sec1 proteins bind to their cognate SNARE complexes. A future challenge is not only to test this hypothesis, but also to establish the function of Sec1p bound to SNARE complexes.

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