Sec1p Binds to SNARE Complexes and Concentrates at Sites of Secretion

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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 syntaxin and 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.

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).

A 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 GTPases (Sagaard et al., 1994; Haas et al., 1995; Grote, E., and P.J. Novick, manuscript in preparation). Rabbs 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). Rabbs 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, Rabbs 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 Rabbs 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 (Sölörner et al., 1993b), which separate v-SNAREs after assembly, promoting high-fidelity 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; García 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; García et al., 1994; Pevsner et al., 1994a; Sagaard et al., 1994; Grubowski and Allwitz, 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 (Schultz 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 Sudhof, 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 peptatin 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 (c creatine kinase, creatine phosphate, ATP, and MgCl2), the detergent NP-40 (also called IGEPA L CA-630), and the protease inhibitors antipain, aprotinin, leupeptin, chymostatin, and PMSF were purchased from Sigma Chemical Co. Protein G-Sepharose and the pGEX-4T1 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 Amer sham Corp. Flu-
Amino-terminally tagged MYC-
for auxotrophic requirements, as described (Guthrie and Fink, 1991).
For c-myc–tagged carboxyl terminus of a gene, as described previously (Schneider et al.,
lows for amplification of either a triple HA or a triple c-myc epitope
and standard molecular biological procedures (Sambrook et al., 1989). First, a PCR product containing the
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 YEp plasmid with vector SEC1, from S. K. eränen, VTT, Biotechnological Laboratory, Espoo, Finland) into pGEX 4T1. The Sec1p-GST fusion protein used to immunize rabbits was purified using glutathione-Sepharose resin and the recombinant GST protein was affinity-purified with glutathione-agarose resin. Biotinylated anti-Sso protein for immunoblotting was prepared using NIH-SC-L-Biotin (Pierce) according to the manufacturer’s protocol. The Snp antisera is described elsewhere (Rossi et al., 1997). The Sec9p antisera was a gift from P. Brennwald (Cornell University Medical School, New York, NY). Pepsip antisera was a gift from R. Piker (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 provided by Jackson ImmunoResearch Labs, Inc. A nitobides against green fluorescent protein (GFP) were from Clontech.

**Antibodies**

The monoclonal anti-MYC antibody (9E 10) was prepared by the Ponocon Rabbit Farm and Laboratory Inc. The monoclonal 12CA 5 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 YEp plasmid with vector SEC1, from S. K. eränen, VTT, Biotechnological Laboratory, Espoo, Finland) into pGEX 4T1. The Sec1p-GST fusion protein used to immunize rabbits was purified using glutathione-Sepharose resin and the recombinant GST protein was affinity-purified with glutathione-agarose resin. Biotinylated anti-Sso protein for immunoblotting was prepared using NIH-SC-L-Biotin (Pierce) according to the manufacturer’s protocol. The Snp antisera is described elsewhere (Rossi et al., 1997). The Sec9p antisera was a gift from P. Brennwald (Cornell University Medical School, New York, NY). Pepsip antisera was a gift from R. Piker (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 provided by Jackson ImmunoResearch Labs, Inc. A nitobides against green fluorescent protein (GFP) were from Clontech.

**Yeasts**

*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, SOS2, and SEC2 used a PCR method that allows for amplification of either a triple HA or a triple c-myc epitope linked to UR A3 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 (NY169, referred to in the text as GFP-SEC1 throughout the text), primers were designed for homologous recombination in the trypsin of MYC-epitope plus UR A3 at the carboxyl terminus of genomic SEC1 (forward primer 23315: GCGTTTTTTTGACACTCGAGGTTCTTGAAAAGAAAAATCTCACCATGATAAAAGG-GATTAGCGTTGCTCATTAGGGCGAATTGGGTACC). MYC-30299: TTCATAGTTTTCAGCGTACGGATTGTTATTCTCATAAGAAATATTGCAACAGGGAACAAAAGCTGG, reverse primer 30298: GTAATACTTTACATTTGAAAACTGCCCATACACGCAC-

**Table I. S. cerevisiae Strains**

| Strain                | Genotype                      |
|----------------------|-------------------------------|
| NY13                 | MATa ura3-52                  |
| NY28                 | MATa ura3-52 sec4-8           |
| NY179                | MATa leu2-3,112 ura3-52       |
| NY1491               | MATa ura3-52 leu2-3,112 trpl1 his3Δ200 |
| NY1642               | MATa ura3-52 leu2-3,112 SNC1::URA3 SNC2::GAL1p HA,SNC2 LEU2 |
| NY1689               | MATa ura3-52 leu2-3,112 trpl1 his3Δ200 SEC1::SEC1MYC UR A3 sec4-8 |
| NY1690               | MATa ura3-52 trpl1 his3Δ200 SEC1::SEC1MYC UR A3 sec18-1 |
| NY1691               | MATa ura3-52 trpl1 his3Δ200 SEC1::SEC1MYC UR A3 sec18-1 |
| NY1692               | MATa ura3-52 trpl1 his3Δ200 SEC1::SEC1MYC UR A3 sec18-1 |
| NY1693               | MATa leu2-3,112 SSO1::LEU2 SSO2::HA SSO2 sec4-8 |
| NY1694               | MATa ura3-52 leu2-3,112 SSO1::LEU2 SSO2::HA SSO2 sec4-8 |
| NY1697               | MATa ura3-52 leu2-3,112 ura3-52 SEC1::SEC1GFP UR A3 sec4-8 |
| NY1698               | MATa ura3-52 SEC1::SEC1GFP UR A3 sec4-8 |
| NY1699               | MATa ura3-52 leu2-3,112 SEC1::SEC1GFP UR A3 |
| NY1700               | MATa ura3-52 leu2-3,112 SSO1::LEU2 SSO2::MYC SSO2 |
| NY1701               | MATa ura3-52 sec4-8           |
| NY1702               | MATa ura3-52 leu2-3,112 his3Δ200 SNC1::URA3 SNC2::GAL1p HA,SNC2 LEU2 SEC1::SEC1MYC UR A3 |
| NY1704               | MATa leu2-3,112 his3Δ200 SSO1::LEU2 SSO2::MYC SSO2 |

**Immunoprecipitation (IP)**

Strains used for IP experiments were grown overnight in YPD at 25°C to an absorbance at 600 nm (A600) of typically 0.6–1.0. Cells were harvested and resuspended in YPD at a concentration of 15 A600 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 A TP, and inhibit membrane fusion, the cultures were diluted 10-fold into ice-cold wash buffer (20 mM Tris, pH 7.5, 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 PM SF). 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 Bioruptor Products). For lysates prepared in the presence of A TP, NaF, and NaF were omitted from the wash buffer. EDTA was omitted from the IP buffer, and an A TP-regeneration system was added to a final concentration of 10 μg/ml creatine kinase, 5 mM creatine phosphate, 1 mM 3′GTP, and 1 mM MgCl2. 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-

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Carr et al. Sec1p and SNARE Complexes in Secretion

335
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 specifically bound to the resin, protein 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 monochlonal antibody 12CA5 was used at 1:1,000 dilution to specifically precipitate HA-tagged species, and the Snp1 antisera was also used at a 1:1,000 dilution in Snp1 IPs. A 1 h of rocking at 4°C, the beads and bound proteins were pelleted by centrifugation for 10 s at 4°C, and the 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. An antibody to 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 antisera against the protein of interest: Sec1p, Sncp, Pep12p, Sec22p, and Bos1p. Sec1p and MYC-Sec1p were detected with affinity-purified Sec1p antibodies. Biotinylated, affinity-purified anti-Sso1 antibodies and peroxidase-conjugated avidin were used to detect Sso1 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 a reference ratio of Sso1 to Snpc in SNARE complexes the ratio of Ssop:ATM to Sncp:ATM in purified, soluble SNARE complexes (Sso1 and Snc1; Rice et al., 1997). A sing densitometry, the band intensities of Sso1 and Snpc coprecipitated with MYC-Sec1p were compared with the band intensities of Sso1 and Snpc in yeast cell lysates and 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 Sec1p, Snpc, and the SNA-P-25 domain of Sec9p have been described previously (Nicholson et al., 1998). Sec1p was modified for use in binding reactions by fusing the maltose-binding protein to the amino terminus (MBP-Ssec1p), using the pMAL-C2 system described above. MBP-Sso1p (and carboxyl-terminal truncation products) was purified and bound to amylose resin. A 10 μl aliquot of resin-bound MBP-Sso1p was assembled into SNARE complexes by first adding an excess of the SNA-P-25 domain of Sec9p (Sec9CT). These binary complexes were formed at 18°C by first adding an excess of the SNAP-25 domain of Sec9p (Sec9CT). 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-Sso1p or MBP-SNA-P-RE complex (MBP-Sso1p:Sec9CT-Snc2p) 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% gels for Sec1p blots and 13% 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 YP 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 SEC1 Δ strains. Expression levels of GFP-Sec1p were identical in NY 1697, NY 1698, and NY 1699, 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 A xiphot microscope equipped with a 100× oil immersion objective (1.3 NA) and a fluorescence filter (FITC, excitation 480 nm, emission 535 nm, dichroic BS 505). Images were recorded on Kodak TMAX100 (ASA 400) 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 Sso1, we looked for specific coprecipitation between Sec1p and Sso1 in yeast cells. A n 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 Sso1 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 Sso1 antibody, we were able to detect a band corresponding to Sso1 from the MYC-SEC1 strain, but no Sso1 band from the untagged strain, confirming a specific interaction between Sec1p and Sso1 in yeast cell lysates. By comparing the intensity of the Sso1 band in MYC-SEC1p IPs to the intensity of the Sso1 band in threefold serial dilutions of the lysate, we estimate that ~0.2% of the total Sso1 is bound to MYC-SEC1p.

Because Sso1 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 Sso1. 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 cell 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. 1B), 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.

A further 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
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 SNA P-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 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...
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 complex 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 NaN₃ 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 NaN₃ 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 A₆₀₀) before lysis. The monoclonal HA antibody was used to precipitate HA-Ssop 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,
are two distinct cross-reacting bands detected in the Western
these mixing experiments HA-Ssop runs as a doublet, and there
shown represent 1% of the protein used for the IPs. Note that in
anti-MYC IPs were detected with the Ssop antibody. Lysates
lysis (MIX). Sso proteins coprecipitated in the
of the protein used for the IPs. (B) Sec1p binds to Ssop in yeast
detected with the Ssop antibody. Lysates shown represent
lysis (MIX). Sso proteins coprecipitated in the anti-HA IPs were
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
because the interaction between Sec1p and Ssop requires
SNARE complexes, and SNARE complexes are pre-
formed 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 pre-
viously unavailable pool of SNARE complexes becomes
exposed during the experiment.
From the results of these mixing experiments, we con-
clude 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 predic-
tion, 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 indi-
cate 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 -Sso
and an unchanged level of untagged Ssop coprecipitation
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 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 (NY 1642) and MYC -SSO (NY 1704) 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 (NY 1689) and HA -SSO (NY 1692) 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 ab-
ence of MYC -Ssop in the HA -Sscp IPs from the mixed
sample indicates that the SNARE complexes detected in
these experiments are formed exclusively in vivo. No fur-
ther 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 exper-
iments, 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, produc-
ing a strain with HA -Ssop and untagged Sec1p. The cul-
tures 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
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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-Sso1 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 strain. In contrast, MYC-Sec1p from the sec18-1 strain is unaltered in its ability to coprecipitate Sso1.

The results of mixing experiments with SEC + and sec strains support the conclusion that Sec1p binds to Sso1 after it is assembled into SNARE complexes. As predicted, the interaction between MYC-Sec1p and Sso1 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 Sso1 co-precipitated with MYC-Sec1p, as indicated by the ability of MYC-Sec1p from one strain to coprecipitate both wild-type levels of Sso1 from the SEC + strain plus enhanced levels of Sso1 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 Sso1 in the lysates. We cannot exclude the possibility that Sec1p also functions before SNARE complex assembly by binding transiently or with low affinity to Sso1. 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 Sso1 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 SNARE complexes 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-Sso1 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...
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 Snrp 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 Snrcp.

Several observations suggest that Sec1p preferentially coprecipitates with SNAREs assembled into SNARE complexes. The ratio of Ssop to Snrcp in Sec1p IPs resem-
bles the 1:1 ratio of these two proteins in purified S N A R E complexes. This finding indicates a significant enrichment of S N A R E complexes in the Sec1p IPs, because only ~1% of the exocytic S N A R E s 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 S N A R E complexes in the assembly mutant sec4-8 and in the disassembly mutant sec18-1, as predicted if Sec1p binds assembled S N A R E complexes.

C oprecipitation of Sec1p and Ssop is highly sensitive to disassembly of S N A R E complexes by the ATPase activity of Sec18p; therefore, it is only observed in the absence of a TP 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 S N A R E 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 S N A R E s (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 S N A R E s and S N A R E complexes. Furthermore, our findings emphasize that care must be taken to prevent S N A R E complex disassembly in order to examine interactions between Sec1 proteins and S N A R E complexes. Results from recent studies of Sec18p illustrate the importance of inhibiting ATPase activity for the recovery of S N A R E complexes from lysates (Uengermann et al., 1998a), and suggest a reexamination of earlier experiments, in which a TP was present.

C urrent models propose that Sec1 proteins regulate S N A R E complex assembly by binding the uncomplexed syntaxin homologues, either to prevent S N A R E 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 S N A R E complexes. The results of mixing experiments and binding studies with purified S N A R E complexes establish that the association of Sec1p with preassembled S N A R E complexes does not require an interaction between Sec1p and S N A R E components before complex assembly. Conversely, failure of S N A R E s 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 S N A R E complexes from SEC + strains is unaltered. These experiments do not formally rule out the possibility that Sec1p interacts with Ssop before S N A R E complex assembly. However, we favor the position that Sec1p functions after assembly, because the level of S N A R E 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 S N A R E complexes.

A n 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 S N A R E complexes? In one study, pairwise binding experiments revealed that the high-affinity n-Sec1/syntaxin interaction prevents association of either the v-S N A R E V A M P or the other t-S N A R E S N A P-25 with syntaxin in vitro (Pevsner et al., 1994b). However, neither the ability of n-Sec1 to prevent assembly of the complete ternary S N A R E complex nor the failure of n-Sec1 to bind to preassembled S N A R E complexes was demonstrated by these studies. In Drosophila, overexpression of either the Sec1 homologue R O P or syntaxin causes a decrease in neurotransmitter release that is relieved when syntaxin and R O P are co-overexpressed (Wu et al., 1998), suggesting that excess R O P can block neurotransmission by titrating 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 S N A R E complexes either from neuronal systems (Hata et al., 1993; Söllner et al., 1993a; García et al., 1994; Pevsner et al., 1994b; Wu et al., 1998) or from yeast extracts (Brenwald et al., 1994; Segaard 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-S N A R E , a conformation that is only present in S N A R E 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; Fleig 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 S N A R E s should resolve some of these issues and reveal more about the function of Sec1 proteins in secretion.

A re S N A R E complexes receptors for Sec1? The localization of GFP-tagged Sec1p in intact cells coincides with sites of vesicle docking and exocytosis, where productive S N A R E complexes are believed to assemble and function in membrane fusion. While it remains possible that other factors in addition to S N A R E complexes are required for the localization of Sec1p to sites of secretion, the notion that S N A R E 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 S N A R E complex assembly and a corresponding defect in the association between Sec1p and S N A R E complexes. Likewise, the robust localization of GFP-Sec1p in sec18-1 correlates with the increased abundance of S N A R E complexes that accumulates due to a defect in complex disassembly and a corresponding increase in the amount of S N A R E complexes recovered in Sec1p IPs.

S N A R E s were originally identified as receptors for α-S N A P and NSF in neurons (Söllner et al., 1993a; McMahon and Sudhof, 1995). The binding of Sec17p and Sec18p to S N A R E s results in the disassembly of S N A R E complexes.
plexes formed on opposing membranes as well as those in the same membrane, as demonstrated by reconstitution studies with yeast vacuoles (Ungeremann et al., 1999b). 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 complex is more resistant to 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 Sec65 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. A nother 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., 1994) 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 other proteins that stimulate exocytosis. A central tenet of these models is that all Sec1 proteins bind to their cognate other proteins that stimulate exocytosis.

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The Journal of Cell Biology, Volume 146, 1999 344