Ordering the Final Events in Yeast Exocytosis

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Abstract. In yeast, assembly of exocytic soluble N-ethylmaleimide–sensitive fusion protein (NSF) attachment protein receptor (SNARE) complexes between the secretory vesicle SNARE Sncp and the plasma membrane SNAREs Ssop and Sec9p occurs at a late stage of the exocytic reaction. Mutations that block either secretory vesicle delivery or tethering prevent SNARE complex assembly and the localization of Sec1p, a SNARE complex binding protein, to sites of secretion. By contrast, wild-type levels of SNARE complexes persist in the sec1-1 mutant after a secretory block is imposed, suggesting a role for Sec1p after SNARE complex assembly. In the sec18-1 mutant, cis-SNARE complexes containing surface-accessible Sncp accumulate in the plasma membrane. Thus, one function of Sec18p is to disassemble SNARE complexes on the postfusion membrane.

Key words: NSF • membrane fusion • SNAREs • exocyst • Sec1

Introduction

The secretory pathway carries newly synthesized integral membrane and secretory proteins from the ER to the cell surface. This pathway involves a series of vesicle budding and fusion events to carry cargo forward, and it is coordinated with a retrograde transport pathway that serves to recycle membrane components and soluble resident proteins. Each stage of transport involves several steps. First, proteins are collected into a transport compartment that buds from a donor membrane. Second, the transport compartment is transported to an acceptor site. Finally, the transport compartment is tethered to an appropriate target organelle, and the two membranes fuse.

Molecular dissection of membrane trafficking in yeast began with the identification of temperature-sensitive sec mutants defective in secretion (Novick and Schekman, 1979; Novick et al., 1980). In 10 complementation groups of these sec mutants, there is an accumulation of 100-nm secretory vesicles containing fully processed invertase (Novick et al., 1981). The proteins encoded by the corresponding late-acting SEC genes were therefore deduced to function in the fusion of post-Golgi secretory vesicles with the plasma membrane. Additional proteins required for exocytic fusion are general factors that also function at several other membrane transport steps, including transport through the early secretory pathway (Novick et al., 1981).

Yeast secretory vesicles derived from the trans-Golgi network are transported along actin cables to exocytic sites at the tips of newly emerging buds and at mother–daughter necks in dividing cells (Finger and Novick, 1998). Mutations that inhibit vesicle transport result in secretory vesicle accumulation in the mother cell rather than in the bud. One protein required for vesicle transport is Sec2p, the nucleotide exchange factor for Sec4p (Walch-Solimena et al., 1997). Activated GTP-Sec4p is thought to promote myosin (Myo2)-dependent motility of secretory vesicles to exocytic sites (Govindan et al., 1995; Schott et al., 1999).

After secretory vesicles have been transported to the bud, a complex of proteins known as the exocyst is required for an event leading to fusion with the plasma membrane. The exocyst contains six late-acting Sec proteins, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, and Sec15p, and two other proteins essential for exocytosis, Exo70p and Exo84p (TerBush et al., 1996; Guo et al., 1999a). One exocyst component, Sec15p, can bind to GTP-Sec4p on secretory vesicles (Guo et al., 1999b). Another exocyst component, Sec3p, is localized at exocytic sites even in the absence of membrane traffic (Finger et al., 1998). Thus, assembly of the exocyst may tether secretory vesicles to the plasma membrane. The interaction of at least two exocyst components, Sec15p and Exo70p, with activated GTPases suggests that the exocyst may also be a regulator of secretion that integrates cellular signaling pathways (Adamo et al., 1999; Guo et al., 1999b; Robinson et al., 1999).

Soluble N-ethylmaleimide–sensitive fusion protein (NSF) attachment protein receptors (SNAREs) are required at the membrane fusion step of most, if not all, intracellular
transport events (Ferro-Novick and Jahn, 1994; Rothman and Warren, 1994). For fusion to occur, a trans-SNARE complex must assemble between SNARE proteins integrated in the two fusing membranes (Nichols et al., 1997; Weber et al., 1998). SNARE proteins within a single membrane can form cis-SNARE complexes (Otto et al., 1997; Swanton et al., 1998; Ungermann et al., 1998a). These cis-complexes may represent intermediates in the fusion mechanism (Price et al., 2000a), nonproductive intermediates or remnants of prior fusion events. The SNAREs for yeast exocytosis are Snep on secretory vesicles and Ssop and Sec9p on the plasma membrane (Aalto et al., 1993; Protopopov et al., 1993; Brennwald et al., 1994). The Snc proteins are encoded by two functionally redundant genes: SNC1 and SNC2. Similarly, SSO1 and SSO2 are functionally redundant genes that encode Sso proteins. There are a large number of proteins in the SNAres family, and individual family members are concentrated on distinct classes of transport vesicles and organelles within the cell (Ferro-Novick and Jahn, 1994; Rothman and Warren, 1994). This diversity suggested that SNAres may regulate the specificity of membrane fusion. Current evidence, however, indicates that the interactions between SNARE proteins are promiscuous (Gotte and von Mollard, 1998; Grote and Novick, 1999; Yang et al., 1999).

Structural and biochemical data suggest that trans-SNARE complexes catalyze the merger of lipid bilayers during intracellular membrane fusion. SNARE proteins assemble into a four-stranded, parallel α-helical bundle with two or more transmembrane segments protruding from its COOH terminus (Hanson et al., 1997; Lin and Scheller, 1997; Sutton et al., 1998). Topological similarity between the structures of assembled SNARE complexes and the fusion active conformation of viral fusion proteins supports the model that SNARE complexes are the fusion proteins for intracellular fusion (Skelhel and Wiley, 1998; Hughson, 1999). In fact, membrane fusion has been reconstituted with purified SNARE proteins incorporated into liposomes (Weber et al., 1998). However, it has also been reported that disassembly of trans-SNARE complexes does not prevent subsequent membrane fusion (Coorssen et al., 1998; Ungermann et al., 1998b).

Sec1p, another factor required for exocytosis, binds to assembled exocytic SNARE complexes, but not to free Ssop (Carr et al., 1999). Green fluorescent protein (GFP)-Sec1p is concentrated at sites of secretion, and this localization correlates with the abundance of SNARE complexes in two mutant strains: sec4-8, a SNARE complex–assembly mutant, and sec18-1, a SNARE complex–disassembly mutant. Based on these observations, we proposed that Sec1p stimulates fusion after SNARE complex assembly or regulates the fidelity of SNARE complex interactions (Carr et al., 1999). In contrast to yeast Sec1p, a rat brain Sec1p homologue has been proposed to regulate SNARE complex assembly because it binds with nanomolar affinity to the plasma membrane SNARE syntaxin, but not to assembled SNARE complexes (Pevsner et al., 1994; Yang et al., 2000).

Since loss of function point mutations in ROP, a Drosophila Sec1p homologue, can either enhance or inhibit synaptic vesicle fusion, it is possible that Sec1p proteins have both positive and negative regulatory roles in membrane fusion (Wu et al., 1998). One of these functions may be the transmission of signals from Rab proteins since an activating mutation in Sly1p, the Sec1 homologue for ER to Golgi transport in yeast, suppresses a deletion of the Rab GTPase Ypt1p (Dascher et al., 1991; Ossig et al., 1991).

Sec18p, the yeast homologue of NSF, binds to SNARE complexes in the presence of its partner Sec17p (Sollner et al., 1993b; Ungermann et al., 1998a). A temperature-sensitive mutation in Sec18p results in inhibition of membrane transport at several stages of the secretory pathway after shifting to 37°C (Graham and Emr, 1991). NSF has ATP-dependent SNARE complex–disassembly activity in vitro (Sollner et al., 1993a). It was originally proposed that disassembly of SNARE complexes might trigger fusion by exposing the amphipathic helicies of v- and t-SNAREs and thereby destabilizing the opposing membranes. More recently, Sec18p/NSF has been shown to act at a priming stage before membrane fusion in homotypic vacuolar fusion, chromaffin granule exocytosis, and synaptic transmission (Banerjee et al., 1996; Mayer et al., 1996; Kawasaki et al., 1998; Littleton et al., 1998; Schweizer et al., 1998; Xu et al., 1999). Experiments using an in vitro assay for vacuolar fusion suggest that disassembly of intravacuolar cis-SNARE complexes by Sec18p frees SNARE proteins to assemble into trans-SNARE complexes bridging two vacuoles (Ungermann et al., 1998a). Thus, after NSF is inactivated, there is an activity-dependent delay before membrane fusion is blocked that may correspond to the fusion of membranes containing previously primed SNAREs (Kawasaki et al., 1998; Littleton et al., 1998; Schweizer et al., 1998; Sanyal et al., 1999; Xu et al., 1999).

In addition to its interaction with SNAREs, NSF also has an ATPase-independent activity necessary for fusion of postmitotic Golgi vesicles (Muller et al., 1999). We have quantified the binding of Ssop to Snep in sec mutant strains in order to incorporate SNARE complex assembly and disassembly into the sequence of events occurring in the later stages of the exocytic pathway. Our results suggest that Sec2p and the exocyst are required before SNARE complex assembly, whereas Sec1p acts after SNARE complex assembly. In addition, we have found that cis-SNARE complexes containing Snep in the plasma membrane accumulate in a sec18-1 mutant. This result demonstrates that SNARE complexes are disassembled by Sec18p after membrane fusion.

Materials and Methods

Strains and Growth Conditions

The strains used in this study are listed in Table I. The sec1Δ smc2Δ strain NY2201 is a spontaneous revertant of JG8 (Protopopov et al., 1993; David et al., 1998) that grows on YPD and no longer carries the GAL1p-TSN1 balancer plasmid. The HA-SSO2, myc-SEC1, GFP-SEC1, and Gallp-SN1,2-HA strains in sec mutant backgrounds were constructed by standard genetic crosses from previously described strains (Abeliovich et al., 1998; Carr et al., 1999; Grote and Novick, 1999). Although the expression level of GFP-Sec1p is similar to wild-type in the temperature-sensitive exocyst mutants, expression of GFP-SEC1 as the sole copy of SEC1 slightly lowers the restrictive temperature. Cells expressing hemagglutinin (HA)-Sec2p from the GALI promoter were grown first in YP/2% raffinose/0.75% galactose overnight and then shifted for 1 h to YPD. Under these conditions, the cells grow rapidly, and the expression level of HA-Sec2p is similar to the expression level of the Snc proteins in wild-type cells (Abeliovich et al., 1998).

Quantitative SNARE Complex Coimmunoprecipitation Assay

Lysis, immunoprecipitation, and Western blotting conditions have been described previously (Grote and Novick, 1999). In brief, 30 A600 units of
yeast cells were collected and washed with ice-cold TAF (20 mM Tris, pH 7.5, 20 mM NaNO₃, 20 mM NaF) to deplete intracellular ATP and arrest membrane traffic. A cleared lysate was prepared in HKDNE buffer (20 mM Hepes, 150 mM KCl, 1 mM DTT, 0.5% IPGAL [NP-40] to deplete intracellular ATP and arrest membrane traffic. A cleared lysate was prepared in HKDNE buffer (20 mM Hepes, 150 mM KCl, 1 mM DTT, 0.5% IPGAL [NP-40]) to deplete intracellular ATP and arrest membrane traffic. A cleared lysate was prepared in HKDNE buffer (20 mM Hepes, 150 mM KCl, 1 mM DTT, 0.5% IPGAL [NP-40]) to deplete intracellular ATP and arrest membrane traffic. A cleared lysate was prepared in HKDNE buffer (20 mM Hepes, 150 mM KCl, 1 mM DTT, 0.5% IPGAL [NP-40]). The lysate was cleared of cellular debris by centrifugation for 10 min at 10,000 g and then fractionated into pellet and supernatant fractions by centrifugation for 10 min at 10,000 g. NP-40 was added to the supernatant to a final concentration of 0.5%. The pellet was resuspended in 600 μl complete IP buffer with 0.5% NP-40. A 30-μl aliquot of each sample was reserved to examine the intracellular distribution of Snp and Ssop. The remainder was immunoprecipitated with anti-Snp antibody.

Surface Iodination

Cell-surface proteins were iodinated by a modification of the method of Payne and Schekman (1989). In brief, 20 Adg units of cells were resuspended in 300 μl PBS, 1 mM EDTA, incubated with three iodobeads (Pierce Chemical Co.) and 300 μCi Naⁱ²³I for 15 min on ice, washed twice with PBS/EDTA, then processed for immunoprecipitation.

Results

Exocytic SNARE Complex Immunoprecipitation

To measure exocytic SNARE complexes, Ssop bound to Snpc was detected by probing a Western blot of an anti-Snp immunoprecipitate with antibodies against Ssop (Grote and Novick, 1999). The amount of Ssop in the immunoprecipitate was quantified by densitometry and compared with a standard curve prepared by diluting total cell lysate to calculate the percentage of total Ssop bound to Snpc. Procedures for the anti-HA and anti-myc immunoprecipitations have also been described previously (Carr et al., 1999).

Table I. Strains Used in this Study

| Strain | Genotype |
|-------|----------|
| NY15  | MAa ura3-52 |
| NY2201| MAa ura2-1::URA3 snc2::ADE6 sup ura3 ade6 leu2 trp1 his3 |
tagged proteins are expressed in different populations of cells that are lysed together (Carr et al., 1999). Third, there is a significant reduction in the amount of Ssop bound to Sncp if transport through the early secretory pathway is inhibited by temperature-sensitive sec mutations that interfere with budding from the ER or Golgi complex (Grote and Novick, 1999).

The amount of Ssop bound to Sncp is influenced by the growth rate. If wild-type cells are grown in glycerol (a non-fermentable carbon source) instead of glucose, their doubling time increases from 2.1 to 7.3 h and there is a 60% reduction in the amount of Ssop bound to Sncp (data not shown). This effect is likely to result from a reduction in the secretion rate, because a major function of the secretory pathway is to deliver membrane and cell wall components needed for growth. Because of the positive correlation between the amount of exocytic SNARE complexes and the growth rate, mutant strains that grow slowly at their permissive temperature were avoided in this study.

**Exocytic SNARE Complex Assembly Requires Vesicle Transport**

We have previously reported that exocytic SNARE complexes do not assemble at 37°C in sec4-8 mutant yeast. Therefore, we proposed that the Sec4p Rab-GTPase acts upstream of SNARE complex assembly (Grote and Novick, 1999). To determine whether Sec4p must be in its active, GTP-bound conformation to promote SNARE complex assembly, we measured the binding of Ssop to Sncp in the sec2-41 mutant. In this strain, deletion of a COOH-terminal targeting domain from Sec2p results in Sec2p mislocalization, and thereby prevents Sec2p from activating Sec4p (Nair et al., 1990; Walch-Solimena et al., 1997; Elkind et al., 2000). An advantage of the sec2-41 strain is that, unlike sec4-8, the cells divide at the same rate as wild-type cells and have wild-type levels of SNARE complexes at 25°C. After shifting sec2-41 cells to 37°C for 10 min, there was an 80% reduction in the amount of Ssop coprecipitated with Sncp compared with wild-type cells at 37°C or sec2-41 cells at 25°C (Fig. 1, a and b). This observation suggests that Sec2p acts upstream of SNARE complex assembly.

We next compared the secretion rates of wild-type and sec2-41 cells at 37°C to examine the relationship between the reduction in SNARE complex levels and the rate of exocytosis (Fig. 1, c and d). It is well established that secretion of invertase is significantly reduced in all of the sec mutants (Novick et al., 1980). However, secretion was

**Figure 1. SNARE complex assembly and secretion in sec2 mutant yeast.** (a and b) SNARE complex assembly. Wild-type (NY13) and sec2-41 (NY130) yeast were grown to early log phase at 25°C. An aliquot of each strain was shifted to 37°C for 10 min. Ssop coimmunoprecipitating with Sncp from a detergent-solubilized lysate was observed by Western blotting (a) and quantified by densitometry (b). The steady-state amount of SNARE complexes in wild-type cells at 25°C was defined as 100%. (c and d) Secretion rate. Cells were grown at 25°C, pelleted, and resuspended in [35S]methionine labeling medium prewarmed to 37°C. At the indicated times (in minutes), cells were pelleted from an aliquot and media proteins were collected by TCA precipitation. The media proteins and 5% of a total cell lysate from the 20-min time point were run on a 5% polyacrylamide gel and detected by autoradiography (c). A 16-h exposure for the secreted proteins and a 30-min exposure for the total cell lysates are presented. Secretion of the 150-kD protein (marked with an arrow in c) was quantified using a PhosphorImager (d).
used an [35S]methionine labeling assay to quantify the secretion rate during the first 16 min after shifting to 37°C. The rate of secretion was linear for the next 12 min, and then reached a plateau, possibly due to depletion of [35S]methionine precursors (Fig. 1 d). By extrapolating from the secretion curve, we calculate that <4 min is required for [35S]methionine uptake from the medium, protein synthesis, and transit of several proteins through the entire secretory pathway in wild-type yeast at 37°C. In contrast to wild-type cells, almost no secretion was observed from sec2-41 cells even though there was no difference between the levels of total protein synthesis in the two strains. The observation that both secretion and SNARE complex levels are reduced at an early time point after shifting to 37°C argues against the possibility that the reduction in SNARE complexes levels is an indirect effect of the sec2-41 mutation. Since secretory vesicles are not transported to sites of secretion in the sec2-41 mutant (Walch-Solimena et al., 1997), we propose that GTP-Sec4p-dependent transport of vesicles to fusion sites is essential before SNARE complexes can assemble. However, we cannot exclude the possibility that Sec4p has an additional activity that activates SNARE complex activity more directly.

**The Exocyst Has a Function before SNARE Complex Assembly**

To determine whether the exocyst is required for SNARE complex assembly, binding of Ssop to Sncp was measured in several temperature-sensitive exocyst mutant alleles. There was a clear reduction in the amount of Ssop bound to Sncp after 10 min at 37°C in sec5-24, sec6-4, and sec15-1. However, near wild-type levels of Ssop remained bound to Sncp in sec3-2, sec8-9, and sec10-2. These results suggested that components of the exocyst might function both before and after SNARE complex assembly. To examine this interpretation in more detail, we measured the amount of Ssop bound to Sncp at 3, 10, and 30 min after shifting to 38°C and compared these results with the severity of the secretion block (Fig. 2). A transient twofold increase in the amount of Ssop bound to Sncp was observed in the wild-type strain at the 3-min time point (Fig. 2 a). This temporary increase in the abundance of SNARE complexes may reflect an increase in the secretion rate resulting from the temperature shift. To emphasize the effects of mutations in the exocyst components (rather than general effects of the temperature shift), the data on Ssop binding to Sncp in the exocyst mutant strains are expressed as a percentage of the amount of Ssop bound to Sncp in wild-type cells at each time point (Fig. 2 b). In addition, the amount of SNARE complexes present at the 10-min time point is compared with the secretion rate between 4 and 12 min after the addition of [35S]methionine at 38°C (Fig. 2 c).

The results show a reduction in SNARE complex levels after 30 min at 38°C in all of the mutant strains. However, at this late time point, indirect effects resulting from an absence of flux through the secretory pathway may complicate the interpretation of the results. At the 3- and 10-min time points, reduced binding of Ssop to Sncp was observed in the sec5-24, sec6-4, and sec15-1 mutants and also in sec8-6,
which is a more tightly blocked SEC8 allele than sec8-9. Only a 25% reduction in the abundance of SNARE complexes was observed in the sec10-2 mutant at the early time points. However, the secretion rate in sec10-2 was reduced by only 50%. Thus, there is a positive correlation between the reduction in the abundance of SNARE complexes at the 10-min time point and the severity of the secretion block (Fig. 2 c). sec3-2 was excluded from this analysis because its secretion rate was reduced by 20%, 16 min after shifting to 37°C (data not shown). We conclude that Sec5p, Sec6p, Sec8p, and Sec15p are required before SNARE complex assembly. No firm conclusions can be made concerning the time of action of Sec3p, Sec10p, or the remaining exocyst components, Exo70p and Exo84p, because alleles with a fast-acting, conditional secretory block are not available.

Sec1p Functions after SNARE Complex Assembly

To evaluate the effect of Sec1p on SNARE complex levels, the amount of Sso2 bound to Sncp and the secretion rate was measured after shifting sec1-1 mutant cells to 37°C. The percentage of Sso2 bound to Sncp in the sec1-1 mutant was similar to wild-type for the first 10 min and then declined to 30% of wild-type levels after 30 min at 37°C (Fig. 3 a). By contrast, secretion was inhibited by >95% within 4 min after shifting to 37°C (Fig. 3 b). We conclude that Sec1p is not likely to be required for SNARE complex assembly because secretion was blocked but SNARE complexes remained assembled at the 10-min time point.

Sec1p Binding to Sso2 and the Polarized Localization of GFP-Sec1p Correlate with the Abundance of SNARE Complexes in sec Mutant Cells

We have previously reported that Sec1p from yeast lysates binds to preassembled SNARE complexes but not to free Sso2 (Carr et al., 1999). To address the possibility that other Sec proteins are required for the interaction between Sec1p and SNARE complexes, we used immunoprecipitation to examine this interaction in sec mutants incubated at 37°C for 10 min. For these experiments, epitope-tagged HA-Sso2 or myc-Sec1 proteins were immunoprecipitated with monoclonal anti-HA or anti-myc antibodies. To test for Sec1p binding to Sso2, we probed for Sec1p in the HA-Sso2p immunoprecipitates (Fig. 4 a) and probed for Sso2 in the myc-Sec1 immunoprecipitates (Fig. 4 b). We also measured the abundance of SNARE complexes in each mutant by probing for Sncp in the HA-Sso2p immunoprecipitates and by probing for Sso2 in an anti-Sncp immunoprecipitate from the myc-Sec1p lysates. Both precipitations were specific, because neither Sso2 nor Sec1p was present in immunoprecipitations from un-
tagged control strains. Except for the sec1-1 strain, there was a positive correlation between the amounts of Sec1p and Sncp bound to Ssop. Thus, the association of Sec1p with Ssop appears to be limited by the abundance of SNARE complexes in these sec mutants. The reduced binding of mutant Sec1-1p from the sec1-1 strain may result either from the reduced abundance of Sec1-1p in the lysate or because the mutant Sec1-1p is defective in SNARE binding.

We also previously reported a positive correlation between SNARE complex abundance and the concentration of GFP-Sec1p at exocytic sites (Carr et al., 1999). Here, we examine the localization of GFP-Sec1p in additional sec mutants (Fig. 5). In wild-type cells, GFP-Sec1p is concentrated in the vicinity of the nuclear envelope. At 25°C, the percentage of cells with polarized GFP-Sec1p was as follows: wild-type, 39 ± 6% (n = 826); sec2-41, 30 ± 14% (n = 569); sec3-2, 14 ± 6% (n = 753); sec5-24, 52 ± 9% (n = 409); sec6-4, 44 ± 9% (n = 232); sec8-6, 39 ± 9% (n = 708); sec8-9, 40 ± 10% (n = 410); sec10-2, 38 ± 7% (n = 396); and sec15-1, 18 ± 10% (n = 351). At 37°C, the percentage of cells with polarized GFP-Sec1p was as follows: SEC+, 18 ± 5% (n = 1,056); sec2-41, 8 ± 7% (n = 722); sec3-2, 10 ± 8% (n = 945); sec5-24, 6 ± 15% (n = 462); sec6-4, 0.8 ± 0.25% (n = 403); sec8-6, 8 ± 4% (n = 816); sec8-9, 3 ± 2% (n = 419); sec10-2, 16 ± 8% (n = 307); and sec15-1, 0 (n = 554).

Figure 5. Localization of GFP-Sec1p in sec mutants. (a) Fluorescent images of GFP-Sec1p localization in wild-type (SEC+, NY1696), sec2-41 (NY2222), sec3-2 (NY2214), sec 5-24 (NY2215) sec6-4 (NY2216), sec8-6 (NY2217), sec8-9 (NY2218), sec10-2 (NY2219), and sec15-1 (NY2220) cells at 25°C and after a 10-min incubation at 37°C. Cells were fixed and viewed by epifluorescence microscopy. (b) Quantitation of GFP-Sec1p localization in wild-type and sec mutant cells. The average of duplicate experiments (except for wild-type, which was done in triplicate) is followed by a range that reflects the variability between experiments performed on different days. At 25°C, the percentage of cells with polarized GFP-Sec1p was as follows: wild-type, 39 ± 13% (n = 826); sec2-41, 30 ± 14% (n = 569); sec3-2, 14 ± 6% (n = 753); sec5-24, 52 ± 9% (n = 409); sec6-4, 44 ± 9% (n = 232); sec8-6, 39 ± 9% (n = 708); sec8-9, 40 ± 10% (n = 410); sec10-2, 38 ± 7% (n = 396); and sec15-1, 18 ± 10% (n = 351). At 37°C, the percentage of cells with polarized GFP-Sec1p was as follows: SEC+, 18 ± 5% (n = 1,046); sec2-41, 8 ± 7% (n = 722); sec3-2, 10 ± 8% (n = 945); sec5-24, 6 ± 15% (n = 462); sec6-4, 0.8 ± 0.25% (n = 403); sec8-6, 8 ± 4% (n = 816); sec8-9, 3 ± 2% (n = 419); sec10-2, 16 ± 8% (n = 307); and sec15-1, 0 (n = 554).
The amount of Ssop bound to Sncp was quantified at the indicated points in the experiment (Steel et al., 1999). A threefold increase was observed in the amount of Ssop bound to Sncp after shifting to 37°C, consistent with the possibility that SNARE proteins must be primed by Sec18p for assembly into trans-SNARE complexes (Ungermann et al., 1998a). However, further SNARE complex assembly is also expected to be inhibited in the sec18-1 mutant as a consequence of the block in transit through the Golgi complex (Grote and Novick, 1999).

In an attempt to determine whether Sec18p functions before or after the late-acting Sec proteins, SNARE complex levels were measured in double mutant strains created by crossing sec18-1 to other temperature-sensitive sec mutants. An increase in Sec18p binding to Sncp after shifting to 37°C was observed in every double mutant tested, suggesting that Sec18p acts upstream of the other Sec proteins in the SNARE complex assembly-disassembly cycle. However, a trivial explanation for these results is that Sec18p is inactivated more rapidly than the other mutant Sec proteins.

To measure the rate of secretion in sec18-1 cells during the first 5 min after shifting to 37°C, cells were pulse labeled with [35S]methionine for 5 min at 25°C, pelleted, and resuspended in 37°C chase medium. Aliquots were collected at the indicated times, and secreted [35S]-p150 was quantified as in the legend to Fig. 1 d.

**Fig. 6.** SNARE complex accumulation and secretion in sec18-1 mutant yeast. (a) Kinetics of SNARE complex accumulation. Wild-type (NY13) and sec18-1 (NY431) cells were grown at 25°C. The amount of Ssop bound to Sncp was quantified at the indicated times after shifting to 37°C as in the legend to Fig. 1 b. (b) Onset of secretion at 37°C. Cells were labeled with [35S]methionine for 5 min at 25°C, pelleted, and resuspended in 37°C chase medium. Aliquots were collected at the indicated times, and secreted [35S]-p150 was quantified as in the legend to Fig. 1 d.
However, at early time points, there appears to be a more complete inhibition of SNARE complex disassembly than of membrane fusion. These results are consistent both with the relatively rapid inhibition (<1 min) of intra-Golgi transport reported for sec18-1 and the activity-dependent delay observed for NSF phenotypes in other systems (Graham and Emr, 1991; Kawasaki et al., 1998; Littleton et al., 1998; Schweizer et al., 1998; Sanyal et al., 1999).

To determine the site of action of Sec18p, we examined where SNARE complexes accumulate in the sec18-1 mutant. Cis-SNARE complexes have been reported between synaptobrevin and syntaxin in synaptic vesicles (Otto et al., 1997). To test for cis-SNARE complexes in yeast secretory vesicles, sec18-1 yeast maintained at 25°C or shifted to 37°C for 10 min were lysed in detergent-free buffer and separated into 10,000-g pellet and supernatant fractions (Fig. 7 a). At 10,000 g, most secretory vesicles remain in the supernatant, but the plasma membrane and docked secretory vesicles pellet (Goud et al., 1988). Sncp was present in both pellet and supernatant fractions as expected (Protopopov et al., 1993). Ssop, which is concentrated on the plasma membrane (Aalto et al., 1993; Brennwald et al., 1994), was primarily found in the pellet fraction, but ~5% of the Ssop remained in the supernatant. This small pool of Ssop may represent newly synthesized Ssop in transit to the plasma membrane, vesiculated fragments of the plasma membrane, or Ssop that has recycled via endocytosis. Sncp was immunoprecipitated from both fractions to determine the localization of SNARE complexes. As expected, there was an increase in the amount of Ssop bound to Sncp after shifting to 37°C. Most of the Ssop coprecipitating with Sncp was in the pellet fraction, but there was also a small amount of Ssop bound to Sncp in the supernatant fraction. Nevertheless, the amount of SNARE complexes in the supernatant increased less than twofold after shifting to 37°C. Therefore, we conclude that exocytic SNARE complexes are primarily associated with the plasma membrane, and that the SNARE complexes that accumulate in sec18-1 are not enriched in free secretory vesicles.

The SNARE complexes that pellet at 10,000 g might be either trans-SNARE complexes between the plasma membrane and docked secretory vesicles or cis-SNARE complexes where the transmembrane anchors of both Sncp and Ssop are in the plasma membrane. To address this issue, a surface iodination assay was used to quantify the amount of cis-SNARE complexes. For this experiment, COOH-terminally HA-tagged Sncp was expressed in wild-type and sec18-1 mutant cells. The triple-HA tag includes nine tyrosines that are accessible to surface iodination only if HA-Sncp is on the plasma membrane (Fig. 7 b). To test the specificity of iodination for HA-Sncp on the cell surface, iodination reactions were performed on intact cells and cells that had been homogenized with glass beads.
before labeling. Despite the accessibility of vesicular HA-Snc2p in the homogenized cells, the total amount of $^{125}$I incorporated into HA-Snc2p was reduced 20-fold in the homogenized cells compared with the intact cells due to competition from cytosolic proteins (data not shown). Thus, even if $^{125}$I crossed the plasma membrane of a small number of broken cells during the iodination procedure, labeling of vesicular HA-Snc2p would be minimal. Therefore, iodination is specific for surface-accessible HA-Sncp.

As previously observed for unlabeled cells (Fig. 6 a), there was a modest increase (fivefold) in the percentage of total HA-Snc2p bound Ssop when the sec18-1 strain was incubated at 37°C for 10 min before surface iodination (Fig. 7 c). Strikingly, there was a 50-fold increase in the amount of $^{125}$I-HA-Snc2p bound to Ssop (Fig. 7 c). Therefore, we conclude that cis-SNARE complexes on the plasma membrane are enriched in sec18-1 cells at 37°C.

Conversely, under permissive conditions, we surmise that trans-SNARE complexes between Sncp in secretory vesicles and Ssop on the plasma membrane are enriched over cis-SNARE complexes on the plasma membrane because there are 50-fold fewer cis-SNARE complexes, but the total amount of SNARE complexes (cis plus trans) is reduced by only fivefold.

As a control for the surface iodination experiment, a second immunoprecipitate was collected from the lysate of sec18-1 cells shifted to 37°C using anti-Tlg2p antibodies. Tlg2p is an Ssop homologue that binds to Sncp in vivo and is localized primarily on endosomal and trans-Golgi membranes (Abeliovich et al., 1998). 10-fold less $^{125}$I-HA-Snc2p was bound to Tlg2p than to Ssop, consistent with the primary localization of Tlg2p on intracellular membranes (Fig. 7 c). However, 2% of the $^{125}$I-HA-Snc2p co-precipitated with Tlg2p, suggesting that a fraction of the Tlg2p is located on the plasma membrane and capable of binding to Sncp under steady-state conditions. $^{125}$I-HA-Snc2p was not detected in a Tlg2p immunoprecipitate from sec18-1 cells maintained at 25°C (data not shown). Thus, a small amount of cis-SNARE complexes between Tlg2p and HA-Snc2p also accumulate on the plasma membrane after shifting sec18-1 cells to 37°C.

**Discussion**

**Events Leading to Membrane Fusion**

Assembly of exocytic SNARE complexes between Sncp, Ssop, and Sec9p is a tightly regulated process dependent on functions provided by Sec2p and several components of the exocyst. Sec2p, the nucleotide exchange factor for the Sec4p GTPase, is required for transport of vesicles along actin microfilaments into the bud (Walch-Solimena et al., 1997). Thus, a failure to transport secretory vesicles to exocytic sites can explain the SNARE complex assembly defect in sec2-41 cells. The heterooligomeric structure of the exocyst suggests that it has multiple functions, but the activity most likely to be required for SNARE complex assembly is vesicle tethering. Vesicle tethering has also been proposed as a precondition for SNARE complex assembly in ER to Golgi transport and homotypic vacuole fusion (Sapperstein et al., 1996; Mayer and Wickner, 1997; Cao et al., 1998). Tethering by the exocyst may be mediated by the binding of Sec15p to Sec4p on secretory vesicles (Guo et al., 1999b) and the subsequent assembly of Sec15 with Sec3p on the plasma membrane at sites of secretion (Finger et al., 1998). The instability of the exocyst observed in exocyst mutant strains (TerBush et al., 1996) may interfere with tethering and thereby prevent SNARE complex assembly. Conversely, increasing SNARE assembly by over-expressing a component of the SNARE complex may reduce the requirement for tethering and thereby explain the suppression of several exocyst mutants by Ssop overexpression (Aalto et al., 1993). The reduction in SNARE complex levels in exocyst mutants indicates that the exocyst acts upstream of SNARE complex assembly, but does not provide evidence for a physical interaction between the exocyst and SNARE proteins. Although binding of the rat neuronal homologues of the exocyst and Ssop has been reported (Kee et al., 1997), we have been unable to detect a similar interaction between the analogous yeast proteins (Grote, E., and D. TerBush, unpublished observation).

Proteins in the Sec1 family have been proposed to regulate SNARE complex assembly because the rat brain Sec1p homologue binds to syntaxin, an Ssop homologue, but not to an assembled SNARE complex (Pevsner et al., 1994; Yang et al., 2000). If Sec1p is essential for SNARE complex assembly, we would expect to find a reduction in the amount of Ssop bound to Snpc in the sec1-1 mutant as we found in the sec2-41 and exocyst mutants. Instead, we observed wild-type levels of SNARE complexes after secretion was blocked. Thus, if Sec1p is required for SNARE complex assembly, there must be a simultaneous block in SNARE complex disassembly such that assembled SNARE complexes are trapped for a period of time after Sec1p inactivation. The possibility that Sec1p has two activities is consistent with the observation from Drosophila that different ROP alleles have opposing effects on synaptic vesicle exocytosis (Wu et al., 1998).

Since yeast Sec1p binds to SNARE complexes but not to free Ssop, we favor the alternative model that Sec1p acts exclusively after SNARE complex assembly (Carr et al., 1999). However, an accumulation of SNARE complexes might be expected if fusion is blocked after SNARE complex assembly. We can suggest two possible explanations for the lack of complex accumulation in the sec1-1 mutant. Either the block in membrane fusion indirectly inhibits further SNARE complex assembly, or trans-SNARE complexes continue to assemble and disassemble in a futile cycle that is independent of fusion. Although our assay for SNARE complex abundance does not distinguish between static and dynamic SNARE complexes, the possibility that trans-SNARE complexes can be disassembled by Sec18p without membrane fusion is supported by observations in the in vitro vacuolar fusion system (Ungermann et al., 1999a).

The steps in the mechanism of membrane fusion that occur after SNARE complex assembly have only recently come under investigation. Experiments with purified SNARE proteins incorporated into liposomes suggest that a SNARE complex is the minimal machinery required to catalyze membrane fusion (Weber et al., 1998). However, even after preassembly of trans-SNARE complexes in this system, the half-time of fusion is longer than the time required for transport of proteins through the entire yeast.
Sec18p and SNARE Complex Recycling

Sec18p/NSF has been proposed to directly stimulate membrane fusion (Rothman and Warren, 1994) or, alternatively, to prime SNARE proteins for subsequent assembly into trans-SNARE complexes (Ungermann et al., 1998a). To identify the site of Sec18p action in yeast, we determined where SNARE complexes accumulate in the sec18-1 mutant. Our data show that SNARE complexes are predominantly associated with large membranes that pellet at 10,000 g, both before and after inactivating Sec18p. This result is consistent either with a trans-SNARE complex linking secretory vesicles to the plasma membrane or a cis-SNARE complex within the plasma membrane. Experiments in the comatose (NSF) mutant of Drosophila have produced conflicting results concerning the site of SNARE complex accumulation. One group reported that SNARE complexes accumulate in a 15,000-g supernatant fraction enriched in synaptic vesicles (Littleton et al., 1998), whereas a second group demonstrated that SNARE complexes are associated with large membranes that sediment more rapidly than synaptic vesicles on a glycerol gradient (Tolar and Pallanck, 1998). In more recent experiments on rat synaptic terminals, highly stable SNARE complexes were detected in a 15,000-g pellet containing the plasma membrane, but not in rapidly purified synaptic vesicles (Leveque et al., 2000). In agreement with the latter two groups, we conclude that SNARE complexes are associated with docked vesicles and/or the plasma membrane.

We used surface iodination of HA-Snc2p to mark cis-SNARE complexes on the plasma membrane. Our results revealed a 50-fold increase in the abundance of cis-SNARE complexes between HA-Snc2p and Ssop when sec18-1 cells were shifted to 37°C. Since the amount of total SNARE complexes (cis plus trans) increased by only 5-fold, the ratio of cis-complexes on the plasma membrane to total complexes increased 10-fold. Assuming that all of the SNARE complexes from the 37°C cells are cis-SNARE complexes, a maximum of 10% of the SNARE complexes can be cis-complexes before the temperature shift. Therefore, our results suggest a shift in SNARE complexes from docked secretory vesicles to the plasma membrane upon Sec18p inactivation, and also imply that >90% of the SNARE complexes recovered from wild-type cells are trans-complexes. We used the same protocol to explore the possibility that Sec1p also acts after fusion. The results showed a 2.9 ± 1.2-fold increase (n = 2) in the fraction of SNARE complexes containing 125I-Snc2-HA in sec1-1 cells after shifting to 37°C compared with the 10-fold increase observed in sec18-1 cells (our unpublished observation). Although we cannot rule out any postfusion role for Sec1p, the modest level of cis-SNARE complexes we detected excludes the possibility that Sec1p acts exclusively in SNARE complex disassembly. Exocytic SNARE complex assembly is dependent on upstream events mediated by Sec2p and the exocyst. Therefore, the cis-SNARE complexes that accumulate in the sec18-1 mutant are likely to be fusion remnants rather than complexes that spontaneously assembled between HA-Snc2 and Ssop proteins that were present on the plasma membrane before the temperature shift. Thus, we conclude that once trans-SNARE complexes have assembled, disassembly by Sec18p is not required for the completion of fusion.

Postfusion disassembly of SNARE complexes is essential to maintain the steady-state localization of SNAREs within a cell. After fusion, Snup recycles to the Golgi complex by endocytosis for subsequent rounds of secretion (Lewis et al., 2000; Grote et al., 2000), whereas Ssop and Sec9p remain on the plasma membrane. SNARE complexes must be disassembled before their constituent proteins can be segregated. Further support for the proposal that Sec18p/NSF facilitates SNARE recycling comes from the observation that NSF is concentrated at sites adjacent to active zones in the frog neuromuscular junction that may be specialized for endocytosis (Boudier et al., 1996; Roos and Kelly, 1999). Because NSF/Sec18p is not required for the budding of primary endocytic vesicles from the plasma membrane (Hicke et al., 1997), endocytosis of SNARE complexes under conditions of limited NSF activity can explain the presence of t-SNAREs and SNARE complexes in coated vesicles and synaptic vesicles (Walch-Solimena et al., 1995; Otto et al., 1997; Swanton et al., 1998).

Since SNARE proteins are continually recycled, our conclusion that Sec18p disassembles cis-SNARE complexes after fusion is consistent with the current consensus that Sec18p/NSF functions at a priming step before fusion. In the case of homotypic fusion, there is no distinction between postfusion disassembly and prefusion priming because the individual SNARE proteins are not segregated into separate compartments before the next round of fusion (Price et al., 2000b). However, since Sec18p/NSF interacts with both SNARE complexes and free t-SNAREs (Sollner et al., 1993a; Hanson et al., 1995; Nichols et al., 1997), Sec18p/NSF may function at several stages in the cycle to maintain SNAREs in a primed state. An alternative possibility is that disassembly of SNARE complexes by NSF immediately after fusion produces primed SNAREs that are stabilized until the next fusion event.

It has been suggested that assembly of cognate SNARE pairs plays a role in mediating the specificity of vesicle targeting (Rothman and Warren, 1994). For SNAREs to regulate the fidelity of vesicle targeting, there must be a mechanism to inactivate SNAREs that are mislocalized, including newly synthesized SNAREs in transit through the secretory pathway, v-SNAREs recycling from a target organelle back to a donor compartment, and t-SNAREs missorted due to insufficient NSF activity (Pfeffer, 1996). Surprisingly, we have found a small amount of the endosomal/trans-Golgi t-SNARE Tlg2p bound to surface-accessible HA-Snc2p in sec18-1 cells at 37°C (Fig. 7c). This result suggests that Tlg2p cycles through the plasma membrane, and more importantly, that Tlg2p is active for SNARE
Figure 8. Model of the secretory pathway.

complex assembly even when present on the plasma membrane. The apparent lack of specificity in the location of Tlg2p activation does not support the hypothesis that SNAREs or SNARE-activating proteins are determinants of vesicle targeting.

Analysis of the abundance of exocytic SNARE complexes in sec mutant yeast strains has allowed us to further refine the order of events in the secretory pathway (Fig. 8). In our model, SNARE complexes assemble after secretory vesicles are targeted to exocytic sites and tethered to the plasma membrane. Sec1p then binds to the assembled SNARE complexes, followed by membrane fusion. After fusion is complete, SNARE complexes are disassembled by Sec18p and the SNARE proteins are recycled. Although our experiments reveal the order of action of the Sec proteins, they do not address their precise biochemical activities. Thus, it will be interesting to discover how vesicle tethering and SNARE complex assembly are coordinated, and how the interaction between Sec1p and SNARE complexes stimulates membrane fusion.

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