Geranylgeranylated SNAREs Are Dominant Inhibitors of Membrane Fusion

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Abstract. Exocytosis in yeast requires the assembly of the secretory vesicle soluble N-ethylmaleimide–sensitive factor attachment protein receptor (v-SNARE) Snclp and the plasma membrane t-SNAREs Ssop and Sec9p into a SNARE complex. High-level expression of mutant Snc1 or Sso2 proteins that have a COOH-terminal geranylgeranylation signal instead of a transmembrane domain inhibits exocytosis at a stage after vesicle docking. The mutant SNARE proteins are membrane associated, correctly targeted, assemble into SNARE complexes, and do not interfere with the incorporation of wild-type SNARE proteins into complexes. Mutant SNARE complexes recruit GFP-Sec1p to sites of exocytosis and can be disassembled by the Sec18p ATPase.

Introduction

Fusion of lipid bilayers is essential for a variety of fundamental biological processes including the entry of enveloped viruses into cells and intracellular membrane traffic. Since there is a substantial energy barrier preventing spontaneous membrane fusion under physiological conditions, biological membrane fusion is mediated by fusion proteins. Fusion proteins have been identified and extensively characterized for several enveloped viruses, but the identity of the fusion proteins for intracellular membrane fusion has not been conclusively established. Intracellular membrane fusion is a multistep process in which the two membranes must be correctly targeted and docked before fusion can occur. Many of the proteins implicated in the process of membrane fusion function at the targeting and docking stages. The most attractive candidate fusion proteins are the members of the soluble N-ethylmaleimide–sensitive factor (NSF)1 attachment protein (SNAP) receptor (SNARE) family (Sollner et al., 1993b; Skehel and Wiley, 1998; Weber et al., 1998).

SNAREs were originally identified as membrane proteins that bound to the ATPase NSF via its cofactor α-SNAP (Sollner et al., 1993b). For fusion to occur, a trans-SNARE complex must assemble between v-SNAREs on a transport vesicle and t-SNAREs on its fusion target (Nichols et al., 1997). cis-SNARE complexes have also been observed where both transmembrane domains are present in the same membrane (Otto et al., 1997). These complexes may be remnants of previous membrane fusion events or may have simply assembled nonproductively. ATP hydrolysis by NSF leads to the disassembly of SNARE complexes and primes t-SNAREs for subsequent assembly into trans-SNARE complexes (Sollner et al., 1993a; Mayer et al., 1996; Ungermann et al., 1998a). The core of a SNARE complex is a four-stranded parallel α-helical bundle (Sutton et al., 1998). Transmembrane domains extending from the COOH terminus of the bundle are anchored in both the vesicle and target membranes (Hanson et al., 1997). The core of the SNARE complex also includes helices from proteins such as SNAP-25 that do not have a transmembrane domain but are associated with membranes often via a covalently attached lipid (Hess et al., 1992). The fact that membranes must be closely apposed to be bridged by a trans-SNARE complex lends support to the

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notion that SNAREs function as fusion proteins. The data on this issue, however, are conflicting. SNARE-dependent fusion has been reconstituted with recombinantly expressed proteins incorporated into liposomes (Weber et al., 1998). However, it has also been reported that disassembly of trans-SNARE pairs by Sec18p, the yeast NSF, can occur before the completion of fusion in an in vitro assay of vacuole fusion (Uengerman et al., 1998b). To reconcile these observations, it has been suggested that the prefusion state of the SNARE complex has low affinity and is thus not detectable by standard assays (Chen et al., 1999).

For insight into how SNAREs might catalyze intracellular fusion, we have looked to the best understood form of membrane fusion, that mediated by the hemagglutinin (HA) protein of influenza virus (Bentz, 1993; White et al., 1996). Internalization of the virus to acidic endosomes triggers a conformational shift in HA2 resulting in insertion of the fusion peptide into the endosomal membrane. Fusion is thought to occur in two steps. First, the outer leaflets join to yield a hemifusion intermediate. Then, the inner leaflets join to form a fusion pore, which expands to allow entry of the viral core into the cytoplasm. The glycosphingolipid-dilysin (GPI)-HA mutant fusion protein is defective at the transition from the hemifusion intermediate to complete fusion (Kemble et al., 1993). In GPI-HA, the COOH-terminal transmembrane and cytoplasmic domains of HA have been replaced by a GPI membrane anchor. Unlike the COOH terminus of wild-type HA, the GPI anchor of GPI-HA is not inserted through the hydrophilic face of the cytoplasmic leaflet of the host cell. Thus, the GPI anchor can diffuse through the lipidd stall connecting the hemifused membranes. Addition of cholera toxin subunit α (CT), a membrane-permeant, inverted cone-shaped amphipath that preferentially accumulates in the cytoplasmic leaflet of the plasma membrane. In theory, adding cholera toxin increases the curvature of the HA2 lipidic stalk. This positive curvature then stabilizes small pores that form spontaneously, thereby increasing the probability that they will expand to complete fusion.

We have examined the role of SNARE transmembrane domains in membrane fusion by replacing the COOH-terminal transmembrane domains of the yeast exocytic SNAREs Sec2p and Sso2p (Sec9p has no transmembrane domain) with signals for addition of a geranylgeranyl lipid domain) with signals for addition of a geranylgeranyl lipid. High expression of geranylgeranylated SNAREs Snc2p and Sso2p (Sec9p has no transmembrane anchor. Unlike the COOH terminus of wild-type HA, the GPI anchor of GPI-HA is not inserted through the hydrophilic face of the cytoplasmic leaflet of the host cell. Thus, the GPI anchor can diffuse through the lipidd stalk connecting the hemifused membranes. Addition of cholera toxin subunit α (CT), a membrane-permeant, inverted cone-shaped amphipath that preferentially accumulates in the cytoplasmic leaflet of the plasma membrane. In theory, adding cholera toxin increases the curvature of the HA2 lipidic stalk. This positive curvature then stabilizes small pores that form spontaneously, thereby increasing the probability that they will expand to complete fusion.

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Materials and Methods

Plasmids and Strain Construction

The SNC-CIIl integrating plasmid pNB975 was constructed by PCR amplification of SNC1 using the reverse primer GCGGAAGCTTTACATA-GAATTATAACACATCTTTTATTAGATC to append sequences coding for CIll-Stop (underlined) after Cys95. The PCR product was inserted between the BamHI and HindIII sites of pNB529, an integrating vector with a GAL promoter, ADH1 terminator, and LEU2 selectable marker. The SSO-CIIl integrating plasmid pNB975 was constructed by PCR amplification of SSO2 using the reverse primer CCAAGCTTATCATAA-GATTACACATCATCTTTATTAGATC to append sequences coding for CIll-Stop (underlined) after Cys95. The PCR product was inserted between the BamHI and HindIII sites of pNB529, an integrating vector with a GAL promoter, ADH1 terminator, and LEU2 selectable marker. The SSO-CIIl integrating plasmid pNB975 was constructed by PCR amplification of SSO2 using the reverse primer CCAAGCTTATCATAA-GATTACACATCATCTTTATTAGATC to append sequences coding for CIll-Stop (underlined) after Cys95. The PCR product was inserted between the BamHI and HindIII sites of pNB529, an integrating vector with a GAL promoter, ADH1 terminator, and LEU2 selectable marker. The SSO-CIIl integrating plasmid pNB975 was constructed by PCR amplification of SSO2 using the reverse primer CCAAGCTTATCATAA-GATTACACATCATCTTTATTAGATC to append sequences coding for CIll-Stop (underlined) after Cys95. The PCR product was inserted between the BamHI and HindIII sites of pNB529, an integrating vector with a GAL promoter, ADH1 terminator, and LEU2 selectable marker.
expression was measured in the parent strain and in 60 spontaneous revertants were frequently observed. Snc-CIIL cose medium before plating on YP galactose plates, spontaneously revertants were frequently observed. Snc-CIIL was purified as an NH₂-terminal tagged GST fusion protein from E. coli. In the presence of lysozyme, wild-type yeast expressing type II geranylgeranyltransferase, [³H]geranylgeranyl phosphorylation was attached to the COOH terminus of Snc-CIIL, but not to the unmodified Snc2p cytoplasmic domain or to COOH-terminally truncated proteolytic fragments of Snc-CIIL (Fig. 1a).

The SNC-CIIL gene was inserted behind a GAL1 promoter in a yeast integrating vector and integrated at the LEU2 locus of SEC+ yeast. Expression of the native Snc1 and Snc2 proteins was not perturbed in the SNC-CIIL transformed strain (data not shown). To determine if replacing the transmembrane domain of Snc2p altered its intracellular targeting, we observed the intracellular distribution of Snc-CIIL by subcellular fractionation and immunofluorescent microscopy. In the first approach, wild-type control cells and cells expressing Snc-CIIL were lysed in detergent-free buffer, and the homogenates were fractionated by standard methods including differential centrifugation, velocity sedimentation in glycerol gradients, and sedimentation and flotation to equilibrium density on sucrose gradients. By all four fractionation methods Snc-CIIL, which migrates more rapidly than wild-type Sncp on polyacrylamide gels, was observed in the same fractions as the native Snc proteins. Furthermore, Snc-CIIL expression did not significantly alter the fractionation pattern of wild-type Sncp at an early time point after inducing expression by shifting to galactose medium (data not shown). Immunofluorescent staining of highly expressed Snc-CIIL with anti-Sncp antibodies was compared with the less intense staining pattern of Sncp in wild-type cells. In both cell types, labeling was observed on the cell surface and on punctate structures in the cytoplasm (data not shown). When sncΔ cells were stained with anti-Snc antibodies, only weak background fluorescence was observed. We conclude that geranylgeranylation of Sncp is sufficient for membrane attachment and does not disturb the normal targeting of Sncp to secretory vesicles and the plasma membrane. Snc-CIIL is probably transported to the plasma membrane via the conventional secretory pathway after posttranslational insertion into the ER like the prenylated protein N-Ras (Choy et al., 1999).

Induction of high-level Snc-CIIL expression with galactose significantly reduced the growth rate of SNC-CIIL cells compared with wild-type controls. However, if SNC-CIIL cells were grown for several generations in YP glucose medium before plating on YP galactose plates, spontaneous revertants were frequently observed. Snc-CIIL expression was measured in the parent strain and in 60 spontaneous revertants by Western blotting. In the parent strain, after 8 h induction with galactose the amount of Snc-CIIL expressed was at least five times greater than the combined expression level of the native Snc1 and Snc2 proteins. By contrast, Snc-CIIL expression was lost in 59 of 60 spontaneous revertants. The expression level of Snc-CIIL in the remaining revertant was reduced to a level equivalent to that of the native Snc proteins. Thus, Snc-CIIL is a dose-dependent inhibitor of growth.

In an attempt to identify Sncp interacting proteins, we screened a multicopy genomic library for genes that suppress SNC-CIIL when overexpressed. 10 suppressing plasmids were isolated. Five of these plasmids contained either the SNC1 or SNC2 genes. The remaining plasmids interfered with Snc-CIIL expression. We conclude that the criti...
We found that by overexpressing proteins likely to interact with Sncp and CIIL, we directly tested for suppression of the two mutants the mechanism of growth inhibition by Snc-CIIL and Sso-CIIL to wild-type Sncp expressed.

Sec4p overproduction, which suppresses most correction overexpression of other known SNARE complex interacting proteins, including Sec9p, Sec1p, Sec17p, or Sec18p, did not suppress either SNC-CIIL or SSO-CIIL. Thus, the ggSNAREs are not inhibiting growth by titrating out other components known to interact with exocytic SNARE proteins.

One caveat concerning the interpretation of these results is that overexpression of wild-type t-SNAREs inhibits its membrane transport in several other systems (Dascher et al., 1994; Bittner et al., 1996; Wu et al., 1998). Consistent with these reports, we found that massive overproduction of Sso2p from a multicopy plasmid suppressed the growth defect of an SSO-CIIL strain, wild-type Sso2p and Sso-CIIL must inhibit growth by distinct mechanisms.

**SNARE Complex Assembly with ggSNAREs**

We next tested whether the ggSNAREs assemble into SNARE complexes. Replacing the transmembrane domains of Sncp and Sso with lipid anchors does not alter the domains essential for SNARE complex assembly in vitro, but assembly in yeast is subject to additional levels of regulation. We have previously documented that ~1% of the total Sso in SEC+ cells coimmunoprecipitates with Sncp and a similar fraction of the total Snc coimmunoprecipitates with Sso (Grote and Novick, 1999). Coimmunoprecipitation is a valid measure of SNARE complex assembly during exocytosis because the amount of Sso coimmunoprecipitated with Snc is reduced in mutant yeast strains with defects that prevent secretory vesicle docking with the plasma membrane (Grote and Novick, 1999; Grote et al., 2000, 2001; Grote and Gruenberg, 2001; Gruenberg et al., 2001; Ourair et al., 2001; Ourair and Novick, 2001).
this issue). Immunoprecipitation of Ssop from cells expressing Snc-CIIL resulted in coimmunoprecipitation of both Snc-CIIL and wild-type Snop (Fig. 2 a). Similarly, immunoprecipitation of Snop from cells expressing Sso-CIIL resulted in coimmunoprecipitation of both Sso-CIIL and wild-type Ssop (Fig. 2 b). One way that ggSNAREs might inhibit secretion is by competing with wild-type SNARE proteins for assembly into SNARE complexes. Interestingly, Snc-CIIL expression did not affect the amount of native Snop associated with Ssop, and Sso-CIIL expression did not affect the amount of native Ssop associated with Snop. Thus, cells expressing ggSNAREs contain wild-type as well as mutant SNARE complexes.

The NSF ATPase disassembles SNARE complexes (Sollner et al., 1993a). In yeast, we and others have observed that addition of ATP and an ATP regenerating system to lysates induces disassembly of SNARE complexes by the NSF homologue Sec18p (Ungerermann et al., 1998a; Carr et al., 1999). To test whether geranylgeranylation of SNAREs inhibits SNARE complex disassembly, we prepared lysates with and without ATP from SNC-CIIL cells. Neither Snop nor Snc-CIIL coprecipitated with Snop in lysates prepared under SNARE disassembly (+ATP) conditions (Fig. 2 c). Therefore, the geranylgeranyl membrane anchor does not interfere with SNARE complex disassembly in lysates.

Exocytic SNARE complex assembly depends on continued traffic through the secretory pathway (Grote and Novick, 1999). Thus, SNARE complexes fail to assemble at 37°C in the sec5-24 temperature-sensitive mutant (Grote et al., 2000). Since Sec5p is a component of the exocyst complex that tethers secretory vesicles to the plasma membrane (TerBush et al., 1996; Guo et al., 1999), we concluded that vesicle tethering is required for exocytic SNARE complex assembly. To determine if vesicle tethering is also required for the assembly of SNARE complexes containing Sso-CIIL, we observed SNARE complexes in a sec5-24 SSO-CIIL strain. To avoid complications arising from the dominant-negative effects of high-level Sso-CIIL expression, the experiment was performed at an early time point after galactose addition before Sso-CIIL had a measurable effect on the growth rate. In addition, raffinose was included in the induction medium to reduce Sso-CIIL expression and minimize the disruptive effect of changing carbon sources. A 30% reduction in the binding of Snop to Ssop was observed in sec5-24 cells after shifting to 37°C in YP galactose plus raffinose. This effect is small compared with the 90% reduction in SNARE complex levels observed in the same cells grown in YP glucose (Grote et al., 2000). In the absence of glucose, a reduced growth rate and partial translocation of Snop from the plasma membrane to intracellular vesicles (data not shown) may contribute to the reduced response of SNARE complexes to the temperature shift. Nevertheless, when sec5-24 SSO-CIIL cells were shifted to 37°C, there was a reduction in binding of both Sso-CIIL and Snop to Snop, and this reduction was equivalent to the reduction in binding of Snop to Snop in sec5-24 cells not expressing Sso-CIIL. Furthermore, binding of Sso-CIIL to Snop was not reduced at 37°C in a SECS SSO-CIIL strain (Fig. 2 d). Because the sec5-24 mutation has equivalent effects on SNARE complexes containing Sso-CIIL and wild-type Snop, we conclude that tethering of secretory vesicles to the plasma membrane precedes the assembly of SNARE complexes between Snop and Sso-CIILp at the plasma membrane.

Sec1p binds to assembled Snop/Ssop/Sec9p SNARE complexes and is targeted to sites of exocytosis in the tips of emerging buds and at mother–daughter necks in dividing cells (Carr et al., 1999). The fluorescence pattern of GFP-Sec1p was observed in SNC-CIIL and SSO-CIIL cells to determine the effect of ggSNARE expression on Sec1p targeting. Expression of Snc-CIIL or Sso-CIIL did not interfere with the targeting of GFP-Sec1p to bud tips and necks. However, the region of bright GFP-Sec1p fluorescence extended over a larger area of the plasma membrane within the bud in ggSNARE-expressing cells (Fig. 3). Since the peak intensity was similar in wild-type and mutant cells, the total amount of GFP-Sec1p targeted to the plasma membrane in the bud appears to be greater in the mutant cells. Coimmunoprecipitation of Sec1p with SNARE complexes was also enhanced by ggSNARE expression (see below, and data not shown). Thus, Sec1p interacts with SNARE complexes containing ggSNAREs.

Figure 3. GFP-Sec1p fluorescence. GFP was integrated at the 5' end of the SEC1 gene in wild-type, SNC-CIIL, and SSO-CIIL cells. Transformants were grown to early log phase in YP raffinose and then transferred to YP galactose medium for 4 h at 30°C. The cells were fixed with methanol to enhance the GFP signal.
ter centrifugation at 300,000 g. 200 µl of a cleared lysate was loaded on the top of a 5–35% glycerol gradient prepared in 0.5% NP-40 lysis buffer. After centrifugation at 300,000 g for 5.3 h, fractions were collected from the bottom of the gradient. An aliquot of each gradient fraction was run on an SDS-PAGE gel and probed for Sec1p, Sso1p, and Sncp by immunoblotting. The upper band detected with the Sec1p antibody is not Sec1p. In an identical gradient run, HA-Sso2–bound Snep was enriched in the more rapidly sedimenting fractions, whereas the unbound Snep sedimented as monomer (Fig. 4 b). HA-Sso2p–bound Sec1p and Sso1p were also enriched in the more rapidly sedimenting fractions. Coprecipitation of untagged Sso1p with HA-Sso2p is especially informative because it suggests that the rapidly sedimenting SNARE proteins are components of higher-order SNARE multimers containing at least two SNARE heterotrimers. However, since these SNARE complexes sediment heterogeneously and have not been purified, it is impossible to determine the precise stoichiometry of each component or to exclude the possibility that other unidentified proteins may associate with this pool of SNAREs.

A small fraction of the Snep, Sec1p, and untagged Sso from the top fraction of the glycerol gradient coprecipitated with HA-Sso2p (Fig. 4 b). These proteins were not present in a control anti-HA precipitation from the top fraction of a gradient prepared from an untagged sec18-1 strain (data not shown). Since myoglobin, a 17-kD globular protein used as a standard, sedimented to a peak in the second fraction of an identical gradient (data not shown), the complexes in the top fraction of the gradient must be associated with a buoyant component such as a detergent-insoluble lipid raft.

If the assembly of SNARE trimers into higher-order multimers requires hydrophobic interactions mediated by transmembrane domains (Laage et al., 2000), ggSNAREs might be excluded from the multimeric SNARE complexes or might limit their assembly. Therefore, assembly of higher-order SNARE multimers was examined in cells expressing ggSNAREs. To streamline the assay for higher-order SNARE complex assembly, we tested for coinmunoprecipitation of Sso1p with HA-Sso2p (Fig. 4 c). As shown above, this interaction occurs primarily within the rapidly sedimenting complexes. To further validate the assay, we compared HA-Sso2p immunoprecipitates from sec18-1 cells that were either maintained at the permissive

**Higher-Order SNARE Complex Assembly**

SNARE proteins isolated from rat brains are found in 7S heterotrimeric VAMP/syntaxin/SNAP-25 complexes. 20S SNARE complexes are formed upon the addition of recombinantly expressed α-SNAP and NSF (Sollner et al., 1993a). To examine the sedimentation rate of yeast exocytic SNAREs, a detergent-solubilized yeast lysate was sedim-
Lipid-anchored SNAREs Inhibit Fusion

Since Sncp and Ssop are implicated in the final stage of secretion, we tested the effect of high level Snc-CIIL and Sso-CIIL expression on secretion. 6 h after Snc-CIIL induction, cells were metabolically labeled with a 5-min pulse of [35S]methionine/cysteine and then chased for various periods of time. Proteins secreted into the media were concentrated by TCA precipitation and then separated on a polyacrylamide gel. The pattern of bands secreted by wild-type and SNC-CIIL cells was similar, but the rate of secretion was significantly slower in the SNC-CIIL cells (Fig. 5). No reduction in protein synthesis was observed. Similar results were observed for SSO-CIIL as shown below. Thus, the growth inhibitory effects of the ggSNAREs are coincident with an inhibition of secretion.

Because Sncp binds to several syntaxin-like t-SNAREs in addition to Ssop (Abeliovich et al., 1998; Holthuis et al., 1998; Grote and Novick, 1999), we tested whether transport to the vacuole is also inhibited by Snc-CIIL expression. Newly synthesized carboxypeptidase Y was targeted to vacuoles and processed to its mature form at the same rate in wild-type and SNC-CIIL cells after 4 h growth in galactose medium (data not shown). Similarly, transport of the fluorescent dye FM4-64 from the plasma membrane to vacuoles was normal in SNC-CIIL cells after 4 h growth in galactose (data not shown). Because secretion is inhibited at this time point by 50% in SNC-CIIL cells, we conclude that exocytosis is the first transport step to be inhibited by Snc-CIIL expression. However, after 10 h growth in galactose medium, FM4-64 was internalized, but was not transported to a single large vacuole (data not shown). This endocytic defect may be either a direct consequence of Snc-CIIL binding to endocytic t-SNAREs (Grote and Novick, 1999) or an indirect effect of the block in secretion.

To determine which stage of the secretory pathway is inhibited by ggSNARE expression, we began with testing for vesicle targeting by immunofluorescent staining of the vesicle-associated Rab GTPase Sec4p. Vesicle targeting defects in mutants that affect the actin cytoskeleton or prevent nucleotide exchange on Sec4p result in a loss of polarized Sec4p immunofluorescent staining (Walch-Solimena et al., 1997). In SNC-CIIL and SSO-CIIL cells, Sec4p fluorescence is concentrated in bud tips and mother–daughter necks as it is in SEC+ yeast (Fig. 6). However, Sec4p staining is more intense and fills a larger area of the cytosol in the mutant cells. Thus, ggSNAREs

Figure 5. SNC-CIIL inhibits secretion. Snc-CIIL expression was induced for 6 h in SC galactose medium without methionine at 25°C. Cells were labeled for 5 min with [35S]-ProMix, and chased with excess methionine and cysteine. Aliquots were removed at the indicated time points and separated into cell pellet and media fractions by centrifugation. (a) Media proteins collected by TCA precipitation and (b) cellular proteins released by glass bead lysis were run on SDS-PAGE gels. Autoradiographs were exposed to film for 4 d (secreted proteins) and 2 h (2% of lysate). (c) Secretion of the 185-kD protein (arrow in a) was quantified using a Storm™ PhosphorImaging system.

temperature of 25°C or shifted to 37°C for 10 min. As expected, the amount of Sso1p, Sncp, and Sec1p bound to HA-Sso2p increased when the mutant Sec18-1 protein was inactivated. When HA-Sso2p was immunoprecipitated from SNC-CIIL cells, both Sncp and Snc-CIIL coprecipitated, as shown in Fig. 2. The amount of Sso1p bound to HA-Sso2p also increased in Snc-CIIL–expressing cells. Therefore, cells expressing Snc-CIIL have more higher-order SNARE multimers in addition to more SNARE trimers. Consistent with the GFP-Sec1p fluorescence results (Fig. 3), the amount of Sec1p bound to HA-Sso2 also increased in the Snc-CIIL–expressing cells. Unexpectedly, blocking SNARE complex disassembly by shifting sec18-1 SNC-CIIL cells to 37°C before lysis resulted in only a small increase in the amount of Sec1p, Sso1p, and Sncp bound to HA-Sso2p. Since SNARE complex assembly depends on flux through the secretory pathway (Grote and Novick, 1999), one interpretation of this result is that an exocytosis block resulting from Snc-CIIL expression prevents additional SNARE complex assembly.

We also examined sedimentation of SNARE complexes from SNC-CIIL and SSO-CIIL cells into glycerol gradients. ggSNARE expression did not affect the sedimentation behavior of the wild-type SNAREs, and the behavior of the ggSNAREs and wild-type SNAREs was identical. In particular, <2% of the Snc-CIIL and Sso-CIIL sedimented at ≥20S, but the pools of both Snc-CIIL and Sso-CIIL in SNC-CIIL complexes were highly enriched in the rapidly sedimenting fractions (data not shown). We conclude that assembly of Sncp and Sso1p into multimeric SNARE complexes is unaffected by replacement of the transmembrane domain with a lipid anchor.
do not inhibit vesicle targeting but may cause an accumulation of vesicles that occupy a large fraction of the bud.

To test for secretory vesicle accumulation more directly, OsO$_4$/UAc-stained ultrathin sections were observed by transmission electron microscopy. There was a significant accumulation of 100-nm secretory vesicles in cells expressing Snc-CIIL or Sso-CIIL. We observed the time course of vesicle accumulation after induction of Sso-CIIL expression. 1 h after shifting to galactose medium, the small number (5–30) of vesicles observed were often located adjacent to the plasma membrane in the bud. After 2 h induction, hundreds of vesicles filled the entire bud and spilled over into the mother cell. At later time points, other abnormal membrane structures including enlarged secretory vesicles and Berkeley Bodies, swollen derivatives of the yeast Golgi, were observed (data not shown).

To look at higher resolution for evidence of vesicle docking, freeze-substituted samples were prepared for electron microscopy (Fig. 7). On these specimens, vesicles were often surrounded by a regularly spaced coat structure that excluded densely staining ribosomes. A small number of vesicles (five or less per cell cross-section) were located within 15 nm of the plasma membrane. These vesicles often bulged out towards the plasma membrane, suggesting a strong physical attachment. It is difficult to trace membranes at sites of contact between vesicles and the plasma membrane because ultrathin sections are 60-nm thick, whereas only 10 nm separates the two hydrophilic layers of phospholipid bilayer membranes. In some instances, distinct secretory and plasma membrane bilayers can be observed, but these may have been sectioned above or below the point of contact. In other images, the outer leaflet of the secretory vesicle and the inner leaflet of the plasma membrane appear to merge at the intersection point and in rare cases vanish. These images may represent the initiation of membrane fusion. No contacts were found where the outer leaflet of the plasma membrane and the inner leaflet of the vesicles coalesced to form a single extended bilayer. Thus, if exocytosis is arrested at a hemifusion intermediate in cells expressing ggSNAREs, the intermediate must either be short-lived or have a diameter less than the thickness of the sections. In addition, a similar number of vesicles closely apposed to the plasma membrane were observed in cells expressing the dominant-negative mutant sec4-N34 (data not shown). GTP-Sec4p is known to be required for vesicle transport to bud tips (Walch-Solimena et al., 1997) and to bind to Sec15p, a component of the exocyst tethering complex (Guo et al., 1999). Furthermore, Sec4-N34 expression inhibits SNARE complex assembly (Groje and Novick, 1999). It is therefore possible that some of the vesicles that appear to be docked in ggSNARE cells have randomly localized adjacent to the plasma membrane.

**LPC Rescues Secretion in SNC-CIIL and SSO-CIIL Cells**

The major difference between the lipid anchors of gg-SNAREs and the transmembrane domains of wild-type SNAREs is that a geranylgeranyl group cannot interact with the hydrophilic portion of the distal leaflet of a phospholipid bilayer, whereas the transmembrane domain must interact with the distal leaflet because it spans the entire membrane and is followed by a short hydrophilic sequence. We reasoned that an agent that increases the fusion potential of the distal leaflet might rescue secretion from cells expressing ggSNAREs. 10:0 LPC is an inverted cone–shaped lipid with a bulky, positively charged choline head group and a single, 10-carbon saturated aliphatic chain. The choline head group prevents spontaneous transport of LPC across lipid bilayers, and there is no enzymatic LPC flip-flop across the yeast plasma membrane (Tang et al., 1996). Thus, LPC has the potential to induce positive curvature in the outer leaflet of the plasma membrane (Sheetz and Singer, 1974).

To assay for LPC-mediated suppression of the exocytic block, we pulse labeled cells with [35S]methionine/cysteine and measured secretion of the labeled 185-kD protein identified in Fig. 5 in the presence or absence of LPC. An
advantage of this assay is that effects on exocytosis can be observed immediately after adding LPC, thereby minimizing the possibility of being misled by indirect effects. Secretion from both SNC-CIIIL (P > 0.90) and SSO-CIIIL (P > 0.99) cells was stimulated upon addition of 0.3 mM 10:0 LPC (Fig. 8 a). Interestingly, LPC stimulated more 35S-p185 secretion from SSO-CIIIL cells than from SNC-CIIIL cells. This difference may be related to the fact that the geranylgeranyl group of Sso-CIIIL is embedded in the plasma membrane where LPC acts.

However, in addition to stimulating secretion from gg-SNARE-expressing cells, LPC can also inhibit secretion. Higher concentrations of LPC (>0.6 mM) inhibited secretion from ggSNARE-expressing cells. This inhibition excludes the possibility that LPC stimulates secretion from ggSNARE-expressing cells by lysing membranes. Furthermore, the possibility that LPC stimulates exocytosis from ggSNARE-expressing cells via nonspecific detergent artifacts can be excluded because 0.5% Triton X-100 and Tween 20 have no effect on p185 release (data not shown).

If a large pool of hemifused secretory vesicles accumulates in Sso-CIIIL-expressing cells, addition of LPC might result in a burst of secretion. To test for this effect, the amount of 35S-p185 release in SSO-CIIIL cells was quantified at three time points after LPC addition (Fig. 8 b). Instead of a burst of 35S-p185 release, there was a sustained increase in the secretion rate. These data suggest that only a small fraction of the secretory vesicles accumulated in

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**Figure 7.** Ultrastructure of SNC-CIIIL and SSO-CIIIL cells. SNC-CIIIL (a–e) and SSO-CIIIL (f–j) cells were grown for 2 h in YP galactose and then prepared for electron microscopy by freeze-substitution. Low magnification images of a bud (a) and the septum forming between dividing mother cells (f) demonstrate an accumulation of 100-nm secretory vesicles, several of which are closely associated with the plasma membrane (see arrows). Higher magnification images (b–e, g–j) of vesicles adjacent to the plasma membrane are also shown. The vesicles in d and h evaginate towards the plasma membrane. Possible merger of the outer leaflet of the secretory vesicle with the inner leaflet of the plasma membrane can be seen in e. Bars, 100 nm.
CIIL mM LPC. Secretion of 35S-p185 was quantified (error bars indicate SEM, n = 5). Secretion from wild-type cells not treated with LPC was defined as 100%. (b) Kinetics of secretion from LPC-stimulated cells. SSO-CIIL cells were pulse labeled for 5 min with [35S]-ProMix, chased for 5 min with excess methionine, and then stimulated with 0.3 mM LPC. Secretion of 35S-p185 was quantified (error bars indicate SEM, n = 3). Secretion from wild-type cells not treated with LPC was defined as 100%. (b) Kinetics of secretion from LPC-stimulated cells. SSO-CIIL cells were pulse labeled for 5 min with [35S]-ProMix, chased for 5 min, and then LPC was added for the indicated times. Secretion of p185 was quantified.

Figure 8. LPC-stimulated secretion. (a) SNC-CIIL and SSO-CIIL cells were pulse labeled for 5 min with [35S]-ProMix, chased for 5 min with excess methionine, and then stimulated with 0.3 mM LPC. Secretion of 35S-p185 was quantified (error bars indicate SEM, n = 3). Secretion from wild-type cells not treated with LPC was defined as 100%. (b) Kinetics of secretion from LPC-stimulated cells. SSO-CIIL cells were pulse labeled for 5 min with [35S]-ProMix, chased for 5 min, and then LPC was added for the indicated times. Secretion of p185 was quantified.

Discussion

The SNC-CIIL and SSO-CIIL mutations were designed by analogy to the GPI-HA mutant of the influenza HA fusion protein to test the hypothesis that SNARE transmembrane domains have a critical function in membrane fusion. High expression of Snc-CIIL or Sso-CIIL inhibited exocytosis, but did not inhibit transport of secretory vesicles to the bud or docking of vesicles to the plasma membrane. Instead, exocytosis was arrested at a late, LPC-sensitive stage. These observations suggest that an interaction between the transmembrane domain of SNARE proteins and the distal leaflets of apposed membranes is essential for fusion.

We propose that a functional SNARE complex must include at least two transmembrane domains, one for the vesicle membrane and one for the target membrane. Similarly, viral fusion requires both a transmembrane domain and a fusion peptide for fusion activity, and mutations in either domain can result in hemifusion (Kemble et al., 1993; Qiao et al., 1999). Several SNARE proteins are naturally modified with lipids (Hess et al., 1992; Couve et al., 1995; McNew et al., 1997; Vogel and Roche, 1999). For example, Snc1p is palmitylated on Cys95, the same site that is geranylglyceroylated in Snc-CIIL (Couve et al., 1995). Nevertheless, neither overexpression of palmitylated Snc1p nor a snc1-Cys95Ala mutation that prevents palmitylation has a deleterious effect on secretion (Protopopov et al., 1993; Couve et al., 1995). Thus, Snc-CIIL toxicity correlates with the lack of a transmembrane domain rather than the presence of a lipid anchor. Another interesting example is Ykt6p, the Sed5p-associated v-SNARE implicated in ER to Golgi complex transport and vacuole fusion (Sogaard et al., 1994; Lupashin et al., 1997; McNew et al., 1997; Ungermann et al., 1998a). Ykt6p is attached to vesicles via a COOH-terminal farnesyl group (Sogaard et al., 1994). Since Ykt6p does not have a transmembrane domain, we suggest that Ykt6p incorporates into SNARE complexes that have a second v-SNARE protein with a transmembrane domain such as Vt1p.

Snc-CIIL and Sso-CIIL both assemble into SNARE complexes indistinguishable by several criteria from complexes containing wild-type SNAREs. These complexes can assemble into higher-order multimers, recruit GFP-Sec1p to bud tips, and be disassembled by Sec18p. Nevertheless, ggSNAREs do not interfere with the assembly of wild-type SNARE proteins into complexes. Therefore, there is the presence of the mutant SNARE complexes rather than the absence of wild-type complexes that is responsible for the inhibition of exocytosis. Assembly of Sso-CIIL into SNARE complexes indicates that vesicles have docked to the plasma membrane because the temperature-sensitive sec5 mutation inhibited assembly of SNARE complexes containing Sso-CIIL as well as native Ssop. Sec5p is a component of the exocyst complex that is thought to tether secretory vesicles to the plasma membrane before SNARE complex assembly (Guo et al., 1999; Grote et al., 2000). Because only a short span of amino acids separates the transmembrane domains of Sncp and Ssop from their SNARE complex forming α-helical domains, the completion of SNARE complex assembly should bring the two membranes into close proximity and may also distort the membranes at the point of contact leading to the initiation of fusion (Sutton et al., 1998).

One mechanism for ggSNAREs to inhibit fusion without affecting the assembly of normal SNARE complexes is by poisoning the function of a higher-order SNARE complex. In support of this model, we found that the majority of the exocytic SNARE complexes in a yeast lysate sediment at >20S. These rapidly sedimenting complexes must contain at least two copies of Ssop because untagged Sso1p coimmunoprecipitates with HA-Ssop2p from fractions near the bottom of the gradient. Furthermore, these higher-order SNARE complexes are likely to have assembled in vivo rather than in the lysate because if different populations of cells expressing myc-Ssop and HA-Sncp are mixed before lysis, the myc-Ssop and HA-Sncp do not bind to each other in vitro (Carr et al., 1999). Because Snc-CIIL toxicity requires a high ratio of Snc-CIIL to wild-type Sncp expression, higher-order SNARE complexes...
may retain partial function if only a minority of the Snc proteins have lipid anchors. Similarly, viral fusion involves cooperative interactions between multiple HA trimers (Blumenthal et al., 1996; Daniël et al., 1996), but based on theoretical calculations, less than half of these HA trimers must undergo a conformational shift for fusion to occur (Bentz, 2000).

To test the hypothesis that ggSNAREs and GPI-HA are defective at a similar stage of fusion, we tested whether LPC would stimulate exocytosis. LPC, which induces positive curvature in the outer leaflet of the plasma membrane, stimulated secretion from ggSNARE-expressing cells. Similarly, chlorpromazine, which induces positive curvature in the inner leaflet of the plasma membrane, stimulates fusion of GPI-HA-expressing fibroblasts with red blood cells (Melikyan et al., 1997; Chernomordik et al., 1999). In the absence of chlorpromazine, GPI-HA mediates hemifusion, the fusion of the proximal leaflets of two membranes but not their distal leaflets. Thus, our data support the model that ggSNAREs are defective at the final stage of exocytic fusion—the merging of the inner leaflet of the secretory vesicle with the outer leaflet of the plasma membrane (Fig. 9). However, because it is not possible to selectively label the outer leaflet of secretory vesicles with a fluorescent marker in living cells, there is no definitive evidence that ggSNAREs induce hemifusion. Also, in contrast to the consistent observation that LPC stimulates secretion from ggSNARE-expressing cells, in wild-type cells LPC slightly inhibited secretion in some experiments and stimulated secretion in others. These disparate results suggest that the transition from hemifusion to complete fusion may be rate limiting in wild-type cells under appropriate conditions.

We considered several alternative explanations for the mechanism of LPC stimulation. LPC might interact with proteins rather than lipids (Gunther-Ausborn et al., 1995). Since LPC inhibits several enzymes including adenylyl cyclase in yeast (Resnick and Tomaska, 1994), it is possible that LPC stimulates fusion by interacting with a protein. However, LPC does not cross the yeast plasma membrane (Tang et al., 1996), so it would not have access to cytoplasmic proteins including those involved in membrane fusion. Although LPC might exert effects on the cytoplasmic side of the plasma membrane via a cell surface receptor, there is no evidence for LPC receptors or ligand-stimulated exocytosis in yeast.

LPC stimulated release of 58% of the accumulated 35S-p185 in SSO-CIIIL cells and 16% in SNC-CIIIL cells. Perhaps, the effectiveness of LPC is limited by our experimental system. First, LPC has direct access to only one leaflet of the putative hemifusion diaphragm. LPC might enter the inner leaflet of secretory vesicles after recycling from the plasma membrane to the Golgi by endocytosis, but this LPC cannot stimulate 35S-p185 release in our experiments because secretory vesicles are loaded with 35S-p185 before the addition of LPC. Second, there is a massive buildup of secretory vesicles in the cells before the 35S–amino acid pulse. These vesicles are likely to be sequestering some of the components involved in vesicle targeting and fusion. For comparison, addition of 0.4 mM chlorpromazine to a GPI-HA fusion assay resulted in full fusion of only 36% of the cells (Melikyan et al., 1997). Thus, we consider the partial rescue of secretion by LPC to be highly significant.

One unresolved question is why a large number of SNARE complexes, docked secretory vesicles, and perhaps hemifusion intermediates, did not accumulate in the ggSNARE-expressing cells if secretion is blocked at a stage after vesicle docking. One possibility is that docking and hemifusion are reversible. Interestingly, a reversible hemifusion intermediate has been detected for hemifusion catalyzed by HA (Leikina and Chernomordik, 2000). However, there is some evidence that SNARE complex assembly is not reversible. If sec5-24 SSO-CIIIL cells are grown for 11 h in YP galactose to completely inhibit secretion, there is no reduction in SNARE complex levels upon shifting to 37°C (our unpublished observation). Since Sec5p is required for assembly of exocytic SNARE complexes, and SNARE complex levels do not decrease in this situation, there must also be no SNARE complex disassembly. The implication of this result is that there is a small, stable population of docked secretory vesicles rather than a continuous cycle of vesicle docking and undocking. An alternative explanation for the failure of docked vesicles to accumulate is that there are a limited number of secretory vesicle docking sites on the plasma...
membrane. Since there are large un assembled pools of all of the known secretory proteins (SNAREs, Sec1p, the exocyst, and Sec4p), availability of these proteins is not likely to be limiting for vesicle docking. Thus, we suggest that the number of docking sites is limited either by an unknown component or by a sensor of assembled SNARE complexes that negatively regulates vesicle docking.

The hypothesis that SNAREs catalyze membrane fusion has been challenged by two groups who have reported that SNARE complexes can be disassembled before membrane fusion (Coorssen et al., 1998; Tahara et al., 1998; Ungermann et al., 1998b). They have proposed that gSNAREs assemble into SNARE complexes, dock secretory vesicles to plasma membrane, and recruit GFP-Sec1p to exocytic sites, yet still block fusion. To conform to the alternative hypothesis, the transmembrane domains of both Sncp and Ssop must be essential to recruit the alternative fusion protein. Thus, our data are more consistent with the model that SNARE complexes directly catalyze fusion.

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