Conformational Regulation of SNARE Assembly and Disassembly in Vivo*

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SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins function in intracellular trafficking by forming complexes that bridge vesicle and target membranes prior to fusion. Biochemical studies indicate that the entry of certain SNARE proteins into complexes is inhibited by intramolecular interactions that generate a closed conformation. For example, an essential N-terminal regulatory domain of the yeast plasma membrane SNARE Sso1p sequesters the C-terminal SNARE motif and prevents it from binding to its assembly partners Sec9p and Sncp. Here, we introduce mutations into Sso1p that cause it to remain constitutively open. These open mutants can functionally substitute for wild-type Sso1p protein in vivo, demonstrating that inhibition of SNARE assembly is not the essential function of the N-terminal regulatory domain. Furthermore, the open mutants suppress sec9–4, a mutation that causes a severe defect in SNARE assembly. Elevated levels of SNARE complexes are observed in cells expressing the open mutants. In the presence of sufficient Sec9p, these complexes accumulate to levels that cause severe growth defects. Similarly, overexpression of the open mutants in yeast carrying mutations in the SNARE disassembly machinery impairs growth. Our findings indicate that elevated levels of SNARE complexes can be toxic and that these levels are normally controlled by the SNARE disassembly machinery, by the limited availability of Sec9p, and by the closed conformation of Sso1p.

Intracellular trafficking in eukaryotic cells employs vesicular carriers that bud from one membrane and fuse with another. Central to this process are proteins anchored in the vesicle membrane called v-SNAREs1 and proteins anchored in the target membrane called t-SNAREs (1, 2). SNARE proteins contain one or two “SNARE motifs,” generally located adjacent to their C-terminal transmembrane anchors (3). The primary sequence of the SNARE motif is characterized by a repeating heptad pattern of hydrophobic amino acids. These SNARE motifs form helical bundles during the assembly of v- and t-SNAREs into “trans” (i.e. membrane bridging) complexes (4, 5). Trans-SNARE complexes hold the vesicle and target membranes in close apposition, leading directly (6) or indirectly (7, 8) to membrane fusion and delivery of vesicular cargo to its cellular destination.

Because SNARE complexes play essential roles in directing traffic among cellular compartments, substantial effort has been devoted to investigating the regulation of SNARE complex assembly. A number of proteins, especially those of the Rab and Sec1 families, have been implicated in the regulation of SNARE assembly (9). Recent reports have also shown that SNARE proteins themselves can adopt conformations that control their entry into intermolecular complexes (10–16). Structural studies of several SNAREs have identified so-called “closed” conformations, in which the SNARE motif, through intramolecular interactions with a regulatory domain, is rendered inaccessible (15, 16). Such regulatory domains impose a kinetic block on SNARE assembly in vitro (12, 15, 16). In the most dramatic case yet studied, an N-terminal regulatory domain of the yeast SNARE Sso1p slows its assembly into SNARE complexes by over 3 orders of magnitude (12). This regulatory domain might act in vivo to prevent the assembly of ectopic or promiscuous trans-SNARE complexes (12, 15).

The N-terminal regulatory domain of Sso1p might also act to limit the formation of “cis” SNARE complexes (i.e. complexes between v- and t-SNAREs localized to the same membrane). In vivo, these complexes are disassembled by a chaperone system consisting of the hexameric ATPase Sec18p/N-ethylmaleimide-sensitive factor and the accessory protein Sec17p/SNAP (17, 18). Active disassembly is necessary, because SNARE complexes are unusually stable. In some systems, Sec17p and Sec18p have been shown to act upstream of fusion, disassembling cis-SNARE complexes so that their constituent SNARE proteins can form membrane-bridging trans complexes (7). Sec17p and Sec18p also act downstream of membrane fusion, disassembling the resulting cis-SNARE complexes and allowing v-SNAREs to recycle (19). The N-terminal regulatory domain could prevent newly disassembled SNAREs from promptly reassembling.

The N-terminal regulatory domain of Sso1p is essential for viability (15). Nonetheless, this domain is not required for membrane fusion in vitro (20) or for protein stability or proper localization in vivo (15).2 Here, we ask whether the N-terminal domain regulates the entry of Sso1p into SNARE complexes in vivo and, if so, whether this regulatory activity is itself the essential function of this domain. By combining mutations known to destabilize the closed conformation, we have created constitutively “open” Sso1p proteins. These unregulated open mutants assemble rapidly into SNARE complexes in vitro.

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1 The abbreviations used are: v-SNARE, vesicle SNARE; SNARE, SNAP receptor; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; t-SNARE, target membrane SNARE; SNAP-25, synaptosome-associated protein of 25 kDa; GFP, green fluorescent protein.
2 M. Munson and F. M. Hughson, unpublished results.
Yeast expressing the open mutants exhibit substantially increased levels of Sso1p-containing SNARE complexes, confirming that the N-terminal domain inhibits SNARE assembly in vivo. This regulatory activity is not essential, since the open mutants can substitute functionally for wild-type Sso1p. Nonetheless, further analysis of the open mutants suggests that the closed conformation plays a major role in blocking formation of cis-SNARE complexes.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Bacterial expression plasmids encoding Sso1p mutants Sso1-Open1 (V84E,K95E,Y148A) and Sso1-Open2 (K95A,K99A,R119A,L123A,Y148A) were produced by PCR mutagenesis; the Sso1p coding region was sequenced to ensure the absence of unwanted mutations. The soluble cytoplasmic domains (residues 1–265) of Sso1p, Sso1p mutants, and the C-terminal SNAP-25-like domain (residues 416–651) of Sec9p were purified as described (12); this Sec9 domain is fully functional in yeast (21).

**Protein Expression**—Sso1, Sso1 mutants, and open mutants were cloned by two-step PCR schemes into pCu415CUP1 at the unique BamHI and PstI sites, creating the following plasmids: pMM268 (pCu-SSO1-Open1), or pMM266 (pCu-SSO1-Open2) were grown as described (24). Briefly, cells were pelleted and washed in ice-cold TAF buffer (20 mM Tris, pH 7.5, 20 mM sodium azide, and 20 mM sodium fluoride) to deplete cells of ATP and inhibit SNARE complex disassembly. Cells were resuspended in cold IP buffer (50 mM HEPES, pH 7.4, 150 mM potassium chloride, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and protease inhibitors (24)) and lysed using a Mini Bead Beater-8 (Biospec Products, Bartlesville, OK). Sso1p was immunoprecipitated from the precleared lysate using affinity-purified α-Ssoop antibodies (a generous gift from E. Grote, Yale University). Western blots were probed with biotinylated affinity-purified α-Ssoop, α-Snpp (both gifts from E. Grote), α-Sec9p (provided by P. Brennwald), or α-Sec1p antiserum (provided by C. Carr, Yale University) and developed using hors eradish peroxidase-conjugated avidin (α-Ssoop) or α-rabbit IgG (α-Ssoop, α-Sec9p, α-Sec1p), followed by chemiluminescent detection using ECL (Amersham Biosciences) and autoradiography.

**Fluorescence Microscopy**—Localization of Sec1p-GFP was performed as described (24). Briefly, cells containing either pCu415CUP1, pMM268 (pCu-SSO1), or pMM266 (pCu-SSO1-Open1) were grown overnight in SC −ura −leu. At early log phase, cells were pelleted, resuspended in YPD plus 100 μM copper sulfate, and grown for an additional 2 h. Cells were pelleted, washed in ice-cold TAF buffer, fixed in methanol at −20 °C for 10 min, and then washed once with −20 °C acetone and three times with ice-cold phosphate-buffered saline. Indirect immunofluorescence was carried out as described previously (25). Microscopy was carried out with the use of an Axiopt microscope equipped with a 1.3 numerical aperture ×100 FLUAR lens (Carl Zeiss, Thornwood, NY) or a 1.3 numerical aperture ×100 UplanFl iris lens (Olympus, Tokyo, Japan). Images were recorded with the use of a Hamamatsu SIT Video Camera 3200 (Hamamatsu, Hamamatsu City, Japan) with a Hamamatsu camera controller C2400. Images were initially processed with the use of an Omnix image-processing unit (Image, Princeton, NJ) and captured to computer disc with the use of a Scion image capture board (Las Vegas, NV).

**RESULTS**

**Creation of Constitutively Open Sso1p Mutants—In vitro**, and presumably in vivo, the formation of SNARE complexes at the yeast plasma membrane proceeds along an ordered assembly pathway (12, 13, 26). The initial step, which is slow, entails binding of Sso1p to its partner t-SNARE Sec9p. This binding reaction is inhibited by intramolecular interactions within Sso1p. Once binary Sso1p-Sec9p complexes are formed, they can bind the v-SNARE Sec8p in a reaction with a much faster in vitro rate constant.

The crystal structure of Sso1p reveals two hydrophobic cores that stabilize its native closed conformation (15). The conversion of this closed conformation to a more open one, with the concomitant release of the SNARE motif, appears to be required for binding to Sec9p (12, 15). As a consequence, single mutations within either hydrophobic core can generate substantial (up to 30-fold) increases in the rate of Sec9p binding (15). We wished to investigate the consequences of unregulated assembly in vivo. Previous biochemical studies showed that...
Fitted bimolecular rate constants are 2,400 (open blue line) and 2,800 (red dashed line) for Sso1p. A space-filling representation of Sso1p (left) and Sso1-Open2 (right). The N-terminal domain is shown in a space-filling representation, while the linker helices and the C-terminal SNARE motif are shown as a light blue coil. Red residues indicate those mutated to create open Sso1p mutants. This figure was produced using Molscript/Raster3D (37, 38).

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Fig. 1. Combining mutations creates conformationally open Sso1p. A, space-filling representation of Sso1-Open1 (left) and Sso1-Open2 (right). The N-terminal domain is shown in a space-filling representation, while the linker helices and the C-terminal SNARE motif helix are shown as a light blue coil. Red residues indicate those mutated to create open Sso1p mutants. This figure was produced using Molscript/Raster3D (37, 38). B, gel filtration chromatography of Sso1-Open1 (black dotted line), Sso1-Open2 (red dashed line), and wild-type Sso1p (solid blue line). C, kinetics of Sso1p-Sec9p complex formation. Fitted bimolecular rate constants are 2,400 ± 300 m⁻¹ s⁻¹ for Sso1-Open1 (blue) and 2,800 ± 100 m⁻¹ s⁻¹ for Sso1-Open2 (red). For comparison, the rate constant for wild-type Sso1p is 2.2 ± 0.2 m⁻¹ s⁻¹ (no change on the scale shown here), while the rate constant for Sso1ΔN2 (residues 146–265) is 6,300 ± 200 m⁻¹ s⁻¹ (12).

Deletion of the N-terminal domain of Sso1p increases its rate of binding to Sec9p by almost 3,000-fold (12). However, since the N-terminal domain is essential (15), we could not simply delete it for in vivo studies. Instead, we combined mutations in both hydrophobic cores to create two different "open" mutants (Fig. 1A). None of the mutations lie within the SNARE motif, where they might affect the structure or stability of SNARE complexes. Sso1-Open1 carries the mutations Val⁸⁴ → Glu, Lys⁹⁵ → Glu, and Tyr¹₄₈ → Ala. Sso1-Open2 carries alanine mutations at five positions: Lys⁹⁵, Lys⁹⁸, Arg¹₁₉, Leu¹₂₂, and Tyr¹₄₈.

Gel filtration analysis of the purified cytoplasmic domains of these proteins indicates that each has a significantly larger hydrodynamic radius than wild-type Sso1p, consistent with a more open conformation (Fig. 1B). Equilibrium analytical ultracentrifugation confirmed that this alteration in gel filtration behavior is not due to significant aggregation.³ To evaluate the in vitro assembly rate constants for Sso1-Open1 and Sso1-Open2, each protein was mixed with Sec9p. Formation of binary Sso1p-Sec9p complexes was monitored by circular dichroism, which registers the increase in α-helix content that accompanies assembly (12, 27). As anticipated, the rate of binary complex assembly is dramatically increased relative to wild-type Sso1p: 1,100- and 1,300-fold for Sso1-Open1 and Sso1-Open2, respectively (Fig. 1C). Within a factor of 3, these open mutants assemble as rapidly as proteins from which the N-terminal regulatory domain has been deleted.

Functionality of Open Sso1p Mutants in Vivo—The genes encoding Sso1-Open1 and Sso1-Open2 were cloned into a centromeric (CEN) plasmid and introduced into the yeast strain FYH102 (15). Wild-type yeast have two functionally redundant SSO genes (SSO1 and SSO2). Genomic copies of both genes have been deleted in FYH102; the essential SSO function in this strain is provided by an episomal copy of wild-type SSO1. This wild-type SSO1 plasmid can be functionally replaced by plasmids carrying SSO1-Open1 or SSO1-Open2 (Fig. 2). Therefore, the open Sso1p mutants, expressed at approximately wild-type levels (under the control of their own promoters on low copy CEN plasmids), can substitute for wild-type Sso1p. Furthermore, these yeast exhibit normal growth rates and cell morphology at various temperatures and in various media, and they secrete invertase and carboxypeptidase Y at levels similar to wild type (data not shown).

Increased SNARE Complex Assembly by Open Mutants in Vivo—The ability of the open Sso1p mutants to functionally replace wild-type Sso1p in vivo is subject to several possible interpretations. It is possible that assembly of the open Sso1p mutants into SNARE complexes is limited in vivo by some mechanism other than, or in addition to, the closed conformation. Alternatively, it could be that the open mutants assemble more readily in vivo, as they do in vitro, but that the resulting elevated levels of assembled SNARE complexes are not deleterious. Consistent with the latter possibility, coimmunoprecipitation experiments indicate that the levels of assembled exocytic SNARE complexes in cells expressing the open mutants are substantially increased (Fig. 3). An important caveat in this experiment, however, is the possibility that some fraction of the observed complexes assemble in the yeast extracts rather than in vivo. This possibility has been ruled out previously for wild-type Sso1p using mixing experiments (24); however, similar experiments using open Sso1p mutants show significant postlysyl assembly (data not shown). Therefore, we carried out additional experiments to confirm that the open mutants form higher levels of SNARE complexes in vivo.

The yeast Sec1 protein binds assembled exocytic SNARE complexes and, as a consequence, localizes to sites of secretion (24). In this respect, Sec1 differs from its neuronal homolog, which binds a closed conformation of the uncomplexed t-SNARE syntaxin (28–30). Therefore, GFP-tagged Sec1p can serve as an in vivo probe for the location and abundance of

³ M. Munson and R. Fairman, unpublished observations.
exocytic SNARE complexes. We used fluorescence microscopy to examine cells expressing Sec1p-GFP in conjunction with wild-type Sso1p or the open Sso1p mutants (Fig. 4 and data not shown). As expected, modest overexpression of wild-type Sso1p causes a modest increase in the level of localized Sec1p-GFP. Similar overexpression of Sso1-Open1 or Sso1-Open2, by contrast, causes a dramatic increase in the level of localized Sec1p-GFP. Increased levels of localized Sec1p are also observed in sec18-1 mutant cells, which are deficient in SNARE complex disassembly (24). In no case is mislocalization of Sec1p-GFP observed. Furthermore, total Sec1p-GFP levels were similar in all experiments (Fig. 4). These results demonstrate that open Sso1p mutants form increased levels of exocytic SNARE complexes at sites of exocytosis.

The level of SNARE complexes that can form in vivo is limited by the availability of Sec9p. This limitation is significant because the level of Sec9p in vivo is 5–10-fold lower than the levels of both Sso1p and Sncp (31). To relieve this limitation, we raised the levels of Sec9p by introducing a high copy 2μ-SEC9 plasmid into strains carrying the various SSO1 plasmids. The consequences were striking; the open mutants cause a strong growth defect when Sec9p is not limiting (Fig. 5). This growth defect was evident upon initial transformation of the open mutants with 2μ-SEC9; the resulting colonies were significantly smaller than with vector alone. The presence of overexpressed Sec9p during subsequent growth of the cells for several generations on −leu −ura plates and overnight in −leu −ura liquid media led to increased toxicity, presumably due to increased accumulation of SNARE complexes. The toxicity of the open mutants in the presence of overexpressed Sec9p prevented us from measuring the level of SNARE complexes in these cells using Sec1-GFP fluorescence. Nonetheless, it seems likely that sharply elevated levels of SNARE complexes are present and account for the observed growth phenotype.

**Genetic Interactions with Known Late Acting Secretory Mutants**—Overexpression of the open Sso1p mutants does not itself cause a significant growth defect (Table II). Growth defects are observed, however, when the open Sso1p mutants are overexpressed in a number of strains carrying temperature-sensitive mutations known to compromise Golgi-to-plasma membrane trafficking (Table II). These synthetic growth defects are observed at permissive or semipermissive temperatures. Synthetic toxicity is observed between open Sso1p mutants and mutations in exocyst subunits (sec3-2, sec5-24, sec6-4, sec8-9, sec10-2, and sec15-1), the relevant Sec1 and Rab family members (sec1-1, sec4-8), and general SNARE complex disassembly proteins (sec17-1 and sec18-1). Furthermore, growth defects are observed even for temperature-sensitive mutants, such as sec1-1, which are suppressed by overexpression of wild-type Sso1p (32). No effect was observed in the endoplasmic reticulum to Golgi SNARE mutant, sed5-1. These results indicate that the open Sso1p mutations impair Golgi to plasma membrane trafficking.

Several of these results were particularly striking. First, overexpression of open Sso1p mutants in sec17-1 and sec18-1 cells had a strong dominant negative effect on growth (Fig. 6). This effect was noticeable even at 28 °C, far below the restrictive temperature for either mutant. Sec17p binds to SNARE complexes and serves as an essential cofactor for their disassembly by the ATPase Sec18p. Presumably, even at permissive temperatures, these mutant cells have a SNARE disassembly defect that is exacerbated by the increased levels of SNARE complexes formed by the open mutants.

Of the mutations tested (Table II), only sec9-4 was suppressed by the constitutively open Sso1p mutant proteins (Fig.
The indicated yeast strains transformed with pRS425 (2μ-LEU2) (40), SSO1-2μ-LEU2, SSO1-Open1-2μ-LEU2, or SSO1-Open2-2μ-LEU2 were plated in serial dilutions on SC−leu and incubated at the indicated temperature. −−, no growth; +++, wild-type growth. Other combinations of + and − indicate intermediate growth defects.

| Strain  | Vector | SSO1  | SSO1-Open1 | SSO1-Open2 | Temperature |
|---------|--------|-------|------------|------------|------------|
| NY179   | +++++  | +++++  | +++++      | +++++      | All        |
| sec1–1  | +      | +++++  | −          | −          | 34         |
| sec3–2  | +      | +      | +          | +          | 37         |
| sec5–8  | +      | +      | +          | +          | 30         |
| sec6–24 | +      | +      | −          | −          | 34         |
| sec8–9  | +      | +      | +          | +          | 34         |
| sec9–4  | +      | +      | +          | +          | 34         |
| sec9–7  | +      | +      | +          | +          | 30         |
| sec10–2 | +      | +      | +          | +          | 35         |
| sec15–1 | +      | +      | +          | +          | 35         |
| sec17–1 | +      | +      | +          | +          | 23         |
| sec18–1 | +      | +      | +          | +          | 29         |
| sed5–1  | +      | +      | +          | +++        | 30         |

Fig. 6. Open mutants show dominant negative interactions with sec17-1 and sec18-1. 10-fold serial dilutions of sec17-1 or sec18-1 cells containing pRS425 (2μ-LEU2) (40), SSO1-2μ-LEU2, SSO1-Open1-2μ-LEU2, or SSO1-Open2-2μ-LEU2 were plated onto SC−leu and incubated at the indicated temperatures.

7). This robust, allele-specific suppression was striking compared with the relatively weak suppression conferred by wild-type Sso1p at this temperature. The sec9–4 allele is deficient in exocytic SNARE complex assembly (26). Therefore, the suppression of sec9–4 probably arises from the ability of the open Sso1p mutants to compensate for the assembly defect in sec9–4. These results provide further evidence that the open Sso1p mutants are enhanced in their ability to enter into productive membrane-bridging SNARE complexes.

**DISCUSSION**

Based on the x-ray structure of Sso1p in its closed conformation, we have generated constitutively open mutants that assemble into SNARE complexes much more rapidly than wild-type Sso1p (Fig. 1). Here, we establish that these open mutants form SNARE complexes more readily in vitro as well as in vivo (Figs. 3 and 4). Therefore, one function of the N-terminal domain of Sso1p is to limit, through the formation of a closed conformation, its entry into SNARE complexes. Despite forming elevated levels of SNARE complexes in vitro, the open mutants can functionally substitute for wild-type Sso1p in yeast (Fig. 2).

The deleterious consequences of the open Sso1p mutants appear to be masked in vivo by the limiting levels of its partner t-SNARE, Sec9p. Sec9p is normally present at levels 5–10-fold lower than those of Sso1p and Sec1p (31). Strikingly, we find that the open mutants cannot functionally substitute for wild-type Sso1p in yeast cells overexpressing Sec9p (Fig. 5). These results strongly suggest that high levels of cognate Sso1p-Sec9p-Sncp complexes are toxic and that unregulated SNARE assembly is deleterious.

The N-terminal domain appears to have an additional role beyond regulating SNARE assembly. An Sso1p deletion mutant that lacks the N-terminal domain altogether is expressed at wild-type levels (15) and correctly localized, yet unlike the open mutants it cannot substitute for full-length Sso1p in vivo (15). This mutant Sso1p is unlikely to be disabled for membrane fusion itself, since a corresponding deletion in syntaxin (the neuronal Sso1p homolog) enhances in vitro fusion (20). We favor the possibility that the N-terminal domain interacts with another factor essential for the efficient formation or functioning of trans-SNARE complexes. In this model, the open mutants retain the ability to interact with this as yet unidentified factor, but the deletion mutant does not.

Sec1p, which binds Sso1p-Sec9p-Sncp complexes in vitro and
in vivo (24), displays increased localization to sites of secretion in cells expressing open Sso1p (Fig. 4). Without a comparable in vivo probe for noncognate SNARE complexes, we cannot directly assess whether open Sso1p shows an increased level of promiscuous SNARE assembly. The ability of the open mutants to assemble in extracts also compromises our ability to use coimmunoprecipitation to evaluate in vivo binding to noncognate SNAREs. Nonetheless, the failure to observe growth or secretion defects in cells carrying only open Sso1p (in the absence of overexpressed Sec9p) argues against promiscuous formation of significant levels of SNARE complexes in vivo. Because Sso1p-Sec9p complexes are capable of interacting with noncognate vesicle SNAREs in vitro (33), our results imply that additional layers of regulation are needed to restrict promiscuous SNARE assembly in vivo.

If the open mutants have an enhanced capacity to enter into cognate SNARE complexes, why do they fail to grow in the presence of overexpressed Sec9p (Fig. 5)? The preponderance of evidence presented here suggests that this growth defect results from the accumulation of high levels of SNARE complexes. Further work, however, will be required in order to understand the molecular basis for this phenomenon. It may be that sequestered Snpc v-SNAREs fail to recycle properly (see below). Alternatively, the complexes may titrate out factors needed for fusion. It is possible, too, that the increased levels of SNARE complexes simply serve as substrates for, and ultimately overwhelm, the Sec17p/18p disassembly machinery. In support of this interpretation, in sec17-1 and sec18-1 mutant backgrounds, overexpression of open Sso1p causes synthetic toxicity well below the restrictive temperature (Fig. 6). In any case, our results are consistent with the conclusion that high levels of SNARE complexes are deleterious.

Does the overabundance of SNARE complexes formed by open Sso1p consist primarily of cis (v- and t-SNAREs anchored in the same membrane) or trans (membrane bridging) complexes? They seem unlikely to be trans for several reasons. First, the maximum level of trans-SNARE complexes is presumably limited by the availability of vesicles and the other components needed for vesicle transport, tethering, and docking. Furthermore, geometric considerations suggest that only a fraction of the v-SNAREs present on a trafficking vesicle can be involved in trans-SNARE complexes. Finally, the productive trans-SNARE complexes that do form are short lived, since they are converted to cis complexes by membrane fusion. Thus, we conclude that the excess cognate SNARE complexes formed by open Sso1p are likely to be predominantly cis complexes. Based on the localization of Sec1-GFP, which binds to ternary complexes, since the rate of this conversion is limited by the overall rate of secretion, which is unchanged in cells expressing the open mutants (data not shown). Instead, cis complexes may form on the plasma membrane, where all three constituent proteins are primarily localized (21, 34, 35), or on exocytic vesicles. Based on the arguments presented in the preceding paragraph, it seems likely that fusing secretory vesicles deposit large numbers of uncomplexed v-SNAREs into the plasma membrane. The uncomplexed v-SNAREs would normally be blocked from entering into nonproductive cis-SNARE complexes by the closed conformation of Sso1p and would be recycled via endocytosis (35, 36). If, on the other hand, the Sso1p molecules residing on the plasma membrane are open, assembly of cis-SNARE complexes could proceed unchecked. Furthermore, cis complexes disassembled by the Sec17p/18p chaperone system may readily reassemble when the functional “off switch” normally provided by the N-terminal domain is disabled (17). As a result, cis-SNARE complexes accumulate at sites of secretion.

Given the extremely slow bimolecular rate constant for binding of wild-type Sso1p to Sec9p (2.2 $\mu$m$^{-1}$ s$^{-1}$), the ability of exocytic SNARE complexes to assemble on a reasonable time scale in vivo seems likely to require additional factors. Such factors could either act directly, by relieving the inhibition caused by the Sso1p regulatory domain, or indirectly, by concentrating Sso1p and Sec9p within a localized region in order to overcome the intrinsically slow bimolecular rate constant. We tested for genetic interactions between open Sso1p and a number of late acting secretory mutants, in part to determine whether any of these other proteins have the characteristics expected of an “opener” protein. Specifically, mutations in an opener protein are expected to impair trafficking but should be suppressed by the constitutively open Sso1p mutants. Only one of the mutants tested, sec-4, has this property (Fig. 7). Thus, open Sso1p is a gain-of-function mutant with respect to the assembly of exocytic SNARE complexes. We have not ruled out the possibility that Sec9p acts as an opener protein; in addition, genetic screens aimed at identifying novel openers are in progress.

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