Binding interactions control SNARE specificity in vivo

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S. cerevisiae contains two SNAP25 paralogues, Sec9 and Spo20, which mediate vesicle fusion at the plasma membrane and the prospore membrane, respectively. Fusion at the prospore membrane is sensitive to perturbation of the central ionic layer of the SNARE complex. Mutation of the central glutamine of the t-SNARE Sso1 impaired sporulation, but does not affect vegetative growth. Suppression of the sporulation defect of an sso1 mutant requires expression of a chimeric form of Spo20 carrying the SNARE helices of Sec9. Mutation of two residues in one SNARE domain of Spo20 to match those in Sec9 created a form of Spo20 that restores sporulation in the presence of the sso1 mutant and can replace SEC9 in vegetative cells. This mutant form of Spo20 displayed enhanced activity in in vitro fusion assays, as well as tighter binding to Sso1 and Snc2. These results demonstrate that differences within the SNARE helices can discriminate between closely related SNAREs for function in vivo.

Introduction

Control of membrane fusion events is critical for the maintenance of an organized endomembrane system in eukaryotic cells. Fusion must be regulated so that carrier vesicles only fuse with the appropriate acceptor compartment. This control is exerted on several levels by a variety of regulatory proteins including SM proteins, Rab proteins, and tethering complexes (McNew, 2008). Additionally, specific interactions between SNARE proteins are an important factor in the specificity of vesicle fusion (Sollner et al., 1993; McNew et al., 2000).

SNARE proteins are the core machinery of intracellular membrane fusion (Weber et al., 1998). They are characterized by a ~60 amino acid domain (the SNARE domain) through which they form heterooligomers (Sutton et al., 1998; Weimbs et al., 1998). In addition, most SNARE proteins contain a C-terminal transmembrane domain adjacent to the SNARE domain. Interaction of a SNARE protein anchored in the vesicle membrane (a v-SNARE) with SNARE proteins in the target membrane (t-SNAREs) leads to the assembly of the SNARE domains into a parallel four-helix bundle (Poirier et al., 1998; Sutton et al., 1998). Bundle formation drives the transmembrane domains of the SNAREs into close proximity and is proposed to provide the potential energy necessary to allow mixing and fusion of the lipid bilayers (Weber et al., 1998; Jahn and Scheller, 2006).

Discrete SNARE complexes control fusion at every level of the secretory pathway (Pelham, 1999). This has led to the suggestion that assembly of cognate SNAREs into exclusive complexes could be a central mechanism for the control of vesicle fusion in the cell (Sollner et al., 1993; McNew et al., 2000). Though isolated SNARE domains show little or no binding specificity in vitro, when full-length SNAREs are reconstituted into synthetic liposomes, only specific combinations can mediate fusion of the artificial bilayers, suggesting that this could be the basis for in vivo control (Yang et al., 1999; McNew et al., 2000). However, many SNAREs have been found to participate in more than one fusion event in vivo and in vitro (Parlati et al., 2000, 2002; Paumet et al., 2001, 2004), again raising the question of how the participation of an individual SNARE in a particular fusion event is regulated.

The process of sporulation in the budding yeast S. cerevisiae provides a useful model in which to address the question of SNARE specificity. During sporulation, fusion of post-Golgi vesicles with the plasma membrane stops, and instead these vesicles are directed to specific sites in the cytoplasm.
where they fuse to form new membrane compartments termed prospore membranes (Neiman, 1998). One prospore membrane envelops each of the four nuclei produced by meiosis, packaging the nuclei into four daughter cells or spores (Neiman, 2005). In concert with this change in the target compartment of exocytic vesicles comes a change in one of the SNARE proteins required for their fusion (Neiman, 1998).

In vegetative cells, vesicles fusing with the plasma membrane use a SNARE complex that is composed of one of two redundant t-SNAREs Sso1 or Sso2, a second t-SNARE subunit, Sec9, and one of two redundant v-SNARE proteins Snc1 or Snc2 (Aalto et al., 1993; Protopopov et al., 1993; Brennwald et al., 1994). Sec9 is a member of the SNAP25 subfamily of SNAP proteins, which differ from other SNAP25 complexes in that they lack a transmembrane domain but contain two SNAP25 helices (Oyler et al., 1989; Brennwald et al., 1994; Weimbs et al., 1998). Thus, a SNARE complex acting at the plasma membrane contains one helix from Sso1 or Sso2, one helix from Snc1 or Snc2, and two helices from Sec9. During sporulation, exocytic vesicles fuse to generate a prospore membrane, the SNARE complex used is slightly different. Sso1 is required for this fusion, but Sso2 does not function in this process (Jantti et al., 2002). An sso1 single mutant, though normal for vegetative secretion, is completely blocked in fusion during sporulation. Direct evidence that Snc1 or Snc2 function at the prospore membrane has not been reported, but a role for these v-SNAREs has been inferred by their localization to the prospore membrane during sporulation (Neiman et al., 2000). Finally, the most notable difference is that the t-SNARE Sec9 is not required for fusion at the prospore membrane. Rather, it is replaced by a second sporulation-specific SNAP25 family member, the Spo20 protein (Neiman, 1998).

Sec9 and Spo20 are specialized for their sites of action. Ectopic expression of SPO20 in vegetative cells cannot rescue the growth defect of a sec9-4Δ mutant at 37°C, nor can overexpression of SEC9 during sporulation restore sporulation to a spo20 mutant (Neiman, 1998). Chimeras studies indicated that the basis of specificity is different for each protein. The ability of Spo20 to work at the prospore membrane requires a lipid-binding motif in its N-terminal domain that is not present in Sec9 (Neiman, 1998; Nakanishi et al., 2004). Targeting of Sec9 to the prospore membrane allows it to largely compensate for loss of SPO20 (Nakanishi et al., 2006). In contrast, the ability of Sec9 to function at the plasma membrane is a property of its SNARE domains. Forms of Spo20 in which the SNARE domains are substituted with those of Sec9 can replace SEC9 in vegetative cells (Neiman et al., 2000). Changes in genes involved in lipid metabolism were also found to promote the function of Spo20 in vegetative cells (Coluccio et al., 2004). Finally, in vitro experiments comparing Sso1/Sec9–Snc2 and Sso1/Spo20–Snc2 complexes demonstrated that the Sso20-containing assemblies are less potent fusogens and that the stability of the assembled complexes is lower (Liu et al., 2007). These results suggest that the inability of Spo20 to function at the plasma membrane may be a function of it forming complexes that provide insufficient binding energy to overcome a barrier to fusion at that compartment.

An assembled SNARE complex incorporates sixteen interfaces where the side chains from all four helices pack together (Sutton et al., 1998). The packing interactions are primarily hydrophobic contacts, except at the central or “zero layer” interface (Sutton et al., 1998). There, the interaction is mediated by polar binding between conserved glutamine and arginine side chains. Most SNARE complexes conform to a 3Q:1R rule, i.e., at the zero layer, three glutamine residues interact with one arginine (Fasshauer et al., 1998). In the yeast plasma membrane SNARE, Sso1/Sso2 and both helices from Sec9 contain a glutamine residue, and Snc1/Snc2 provides the arginine residue. If the central layer glutamine in Sso1 is mutated to arginine, this mutant form of Sso1 is not functional; however, function can be restored by coexpression of a form of Snc2 in which the arginine has been changed to glutamine (Katz and Brennwald, 2000). Such compensatory Q/R mutations also work in other SNARE complexes and have been used to demonstrate that specific pairs of SNARE proteins function together in vivo (Graf et al., 2005).

The interpretation of the Spo20 experiments described above assumes that the Snc1/Snc2 proteins function as the v-SNARE for fusion at the prospore membrane. To test this, we sought to use compensatory Q/R mutations in the SNARE domains of Sso1 and Snc2 to demonstrate a direct role of Snc2 during sporulation. We report here that strains carrying an sso1 Q224R mutant failed to sporulate, and that compensatory mutations in none of the S. cerevisiae R-SNAREs can rescue this sporulation defect. Sporulation is reduced by mutation of the central layer glutamine of Sso1 to any other residue, whereas vegetative growth is largely unaffected by these changes. The sensitivity of sporulation to changes in the Sso1 ion layer residue, we show, is due to the presence of Spo20 in the prospore membrane SNARE complex. Co-expression of a Spo20 chimera carrying the Sec9 SNARE helices with the Snc2 R52Q allele rescues the sporulation defect of the sso1 Q224R mutant. Mutation of two residues located at binding interfaces in the SNARE domain of Spo20 to the corresponding residue in Sec9 allows Spo20 to function in concert with Sso1 R224Q and Snc2 R52Q proteins. This mutant form of Spo20 also shows enhanced ability to rescue sec9-4Δ in vegetative cells. In vivo, the mutant Spo20 forms tighter complexes with Sso1 and Snc2 and is a more efficient fusogen than the wild-type protein. These results demonstrate that the intrinsic binding energy of the SNARE domains can help control the specificity of vesicle fusion in vivo.

Results

Compensatory mutations in Snc2 cannot rescue the sporulation defect of a mutation in the Sso1 central ionic layer

Prospore membrane formation requires the t-SNAREs Sso1p and Sso2p (Neiman, 1998; Jantti et al., 2002). Though the v-SNAREs Snc1p and Snc2p localize to the prospore membrane (Neiman et al., 2000), direct evidence of their involvement in prospore membrane assembly has not been reported. Compensatory Q/R mutations in the central ionic layer of a t-SNARE and a v-SNARE have been used to demonstrate specific SNARE interactions in vivo (Katz and Brennwald, 2000; Graf et al., 2005). To examine the possible role of the Snc proteins during sporulation, we introduced a plasmid carrying the sso1 Q224R allele into strain H13 (sso1Δ sso1) alone or in combination with a
relevant gene and expressed % asci ether test
sso1ΔSSO2 none < 0.2
sso1ΔSSO2 SSO1 40.4
sso1ΔSSO2 sso1Q224R < 0.2
sso1Δsso2Δ sso1Q224R snc2R52Q < 0.2

Figure 1. Compenatory mutation of SNP2 can rescue the sporulation defect of sso1Q224R. Strains H13 (sso1Δ/sso1Δ) or H175 (sso1Δ/sso1Δ sso2Δ/sso2Δ) were transformed with the CEN plasmids expressing the indicated genes and sporulated in liquid culture. Sporulation was assessed by observation in the light microscope and by spore test. To determine percentage of sporulation, at least 500 cells were counted for each strain. For H175, the plasmid carrying the wild-type SSO1 was lost by growth on 5-FOA before cells were assayed.

plasmid carrying the snc2R52Q allele. As expected, the sso1Q224R allele did not rescue the sporulation defect of the sso1Δ (Fig. 1). Neither SNC2 nor snc2R52Q were capable of restoring sporulation in this context (Fig. 1). This failure of snc2R52Q to rescue the sporulation defect raises the possibility that Snc2p does not participate in vesicle fusion at the prospore membrane. We therefore examined whether mutation of the central layer arginine to glutamine in any of the other S. cerevisiae R-SNAREs (Snc1, Ykt6, Sec22, Nyv1) could restore sporulation to the sso1Q224R strain. As with snc2R52Q, none of these mutant SNAREs could compensate for the sso1Q224R mutation (unpublished data).

To ensure that snc2R52Q was indeed capable of suppressing sso1Q224R in our strains, we constructed a strain homozygous for deletion of SSO1 and SSO2 and kept alive by the SSO1 gene on a centromeric plasmid (HI75). When the sso1Q224R mutation was introduced into this strain, the plasmid bearing the wild-type gene could be lost only when the snc2R52Q allele was also present. The resulting strain is viable because of the compensatory interaction between the two mutant SNAREs (Katz and Brennwald, 2000). However, as with the results in the sso1Δ/SSO1Δ strain, the presence of snc2R52Q does not rescue the sporulation defect associated with sso1Q224R (Fig. 1). Together, these results suggest either that no R-SNARE proteins are involved in fusion at the prospore membrane, and therefore they cannot compensate for the sso1Q224R mutation, or that fusion at the prospore membrane is particularly sensitive to the proper configuration of side chains at the central ionic layer in the SNARE complex.

Vegetative yeast cells are largely insensitive to mutation of the t-SNARE ionic layer

To further explore the role of the central ionic layer in Sso1 function, codon 224 was mutated and alleles bearing all possible amino acid replacements of the glutamine were constructed. Each of these sso1Q224R alleles was introduced on a plasmid into strain HI75 (sso1Δ/ssolΔ sso2Δ/ssol2Δ pSSO1) and the transformants were then transferred to plates containing 5-fluoroorotic acid (5-FOA) to select for loss of the wild-type SSO1-containing plasmid. Like sso1Q224K, sso1Q224P failed to grow on 5-FOA, indicating that a proline substitution at this position also interferes with Sso1 function. Though arginine cannot function, lysine is weakly tolerated at this position as cells containing sso1Q224R as their only source of Sso protein were viable, but slow growing at elevated temperature (Fig. 2). The phenotype of sso1Q224R may be due to the presence of the positively charged side chain because, as with sso1Q224R, coexpression of snc2R52Q suppressed the slow growth of sso1Q224R (unpublished data). However, other than these three mutations, all other amino acids at position 224 were well tolerated. As judged by colony size, strains carrying these sso1Q224X alleles as their sole form SSO grew as well as those carrying SSO1 even at low or high temperatures (Fig. 2; not depicted). Thus, despite the strong evolutionary conservation of ionic layer glutamine, the yeast plasma membrane SNARE can tolerate a wide variety of residues at this position.

Figure 2. Mutation of Sso1Q224 is well tolerated in vegetative growth. Strain HI75 (sso1Δ/ssolΔ sso2Δ/ssol2Δ) was transformed with CEN plasmids carrying all possible amino acid substitutions at position 224 of Sso1 and the plasmid carrying the wild-type SSO1 was lost by plasmid shuffle, leaving the mutant as the sole form of Sso protein in the cell. Transformants carrying substitutions that could support growth (all except arginine and proline) were streaked out on YPD plates and incubated at 37°C. Letters indicate the amino acid present at position 224 of Sso1 in each strain.
All of the mutations caused a reduction in sporulation efficiency, varying from a two- to fourfold to several hundredfold with small or polar amino acids better tolerated than large hydrophobic or positively charged side chains. Substitution of glutamine 224 to lysine, which causes slow vegetative growth, led to a complete loss of sporulation, as did the arginine and proline mutations (examined in the sso1 single mutant strain) that cannot support vegetative growth. Substitution to leucine or tryptophan had no effect on growth rate, yet these mutants displayed strong sporulation defects. Thus, mutations such as Sso1Q224X played strong sporulation defects. Moreover, in the context of the rearranged central layer, the partner SNARE for Spo20 to function with the altered Sso1/Snc2 complexes, suggesting that

The combination of Snc2R550 and Sec9 helices can rescue the sporulation defect of sso1Q224R

One possible explanation for the observation that snc2R550 can only rescue sso1Q224R during vegetative growth is that one or both of these mutant proteins is mislocalized during sporulation. However, examination of GFP-tagged forms of the proteins revealed that both display an SPB-associated fluorescence in sso1 mutant cells indistinguishable from wild-type Snc2 protein and consistent with localization to prospore membrane precursor vesicles (unpublished data).

An alternative possibility is the existence of a sporulation-specific protein whose interaction with Sso1 and/or Snc2 is sensitive to these mutations. As Sec9 works with these proteins in vegetative cells and Spo20 replaces it during sporulation, Spo20 would be a candidate for such a factor. To test the possibility that the switch to Spo20 during sporulation is the basis for the sso1Q224X phenotypes, we examined the ability of chimeras in which the helices of Spo20 are replaced with those of Sec9 (PSPS) to rescue the sso1Q224R sporulation defect. Strain HI3 (sso1Δ/sso1Δ spo20Δ/spo20Δ) carrying pso1Q224R was transformed with an empty vector, or one carrying snc2R550, as well as high copy plasmids expressing either wild-type SPO20 or the PSPS chimera. Expression of snc2R550 or SPO20 alone did not increase the frequency of sporulation and, similarly, co-overexpression of snc2R550 and SPO20 had no effect. Expression of PSPS alone resulted in some increase of sporulation; however, coexpression of both snc2R550 and the PSPS chimera resulted in sporulation at levels comparable to the same strain carrying SSO1 and SPO20 plasmids (Fig. 4). This result demonstrates that snc2R550 can contribute to suppression of sso1Q224R, indicating that Snc2 does participate in fusion at the prosopore membrane. Moreover, in the context of the rearranged central layer, the partner SNARE for Sso1/Snc2 must contain the Sec9 helical domains for sporulation to occur. Spo20 cannot support membrane assembly under these circumstances. Sensitivity of Spo20-containing complexes to perturbations of the ionic layer may also explain the sporulation-specific nature of other sso1I0224X mutations.

To more precisely define the differences between Sec9 and Spo20 in their ability to mediate fusion in the context of sso1Δ/sso1Δ(snc2R550), the ability of chimeras replacing only one of the two Spo20 helices with that of Sec9 was examined. A swap of the first Spo20 helix (PSPP) was as effective at rescuing sporulation as the PSPS chimera. A swap of only the second helix (PPPS) also increased sporulation, but to a lesser extent than the first helix (Fig. 4). These results suggest that differences in the first helical domains of Spo20 and Sec9 are largely responsible for their differing phenotypes in this assay.

Mutation of two interface residues allows Spo20 to function with the altered Sso1/Snc2

In vitro, Sso1/Spo20–Snc2 complexes have a lower melting temperature than Sso1/Sec9–Snc2 complexes, suggesting that...
Spo20 binds less tightly to these other SNAREs than does Sec9 (Liu et al., 2007). Packing interactions between side chains of amino acids located at interfaces on the SNARE helices are likely to determine how tightly the SNAREs in a given complex bind to each other. We aligned the interface residues of Spo20 and Sec9 to look for possible suboptimal residues in Spo20 (Fig. 5). As criteria to identify such residues, we looked for differences in the size and/or chemical properties of the side chains. In the first helix, only two positions looked significantly different, a cysteine at the +3 layer of Spo20 that is leucine in Sec9, and a serine at +5 that is an asparagine in Sec9. In the second helix, four differences of note were found; phenylalanines at the +2 and +1 layers that are threonine and leucine, respectively, in Sec9, an alanine in the +4 layer (leucine in Sec9) and a lysine residue at the +6 position (asparagine in Sec9). These six residues were mutated in pairs in the context of an otherwise wild-type SPO20 sequence. The resulting mutants, SPO20 C224L,S231N, SPO20 F357L,F361T, and SPO20 A378L,K385N were all capable of rescuing the sporulation defect of a spo20 mutant, indicating that the mutants encode functional proteins (unpublished data). They were then tested for their ability to rescue the sporulation defects of HJ3 (sso1Δ/sso1Δ spo20Δ/spo20Δ) expressing the sso1 Q224R and snc2 R52Q alleles (Fig. 5).

When sporulation was assessed on solid medium, the differences between the PSPP and PPSP chimeras were more pronounced than in liquid sporulation (Fig. 4). Consistent with the relative ability of the different chimeras to promote sporulation, the alterations in the second helix, SPO20 F357L,F361T and SPO20 A378L,K385N had little effect on suppression, though SPO20 C224L,S231N did display a reproducible, slight improvement in sporulation efficiency. In contrast, SPO20 C224L,S231N allowed sporulation at a level comparable to the PSPP chimera, indicating that these two residues are primarily responsible for the ability of this chimera to function in conjunction with sso1 Q224R and snc2 R52Q.

Ectopic expression of SPO20 cannot rescue the temperature-sensitive growth defect of a sec9-4 mutant, though a chimeric form of Spo20 carrying the Sec9 helical regions can rescue sec9-4 (Neiman et al., 2000). This suggests that the inability of Spo20 to function at the plasma membrane is tied directly to its SNARE domain. We examined whether the SPO20 C224L,S231N allele affects the ability of Spo20 to compensate for loss of SEC9. These experiments were performed with proteins lacking the inhibitory domain (amino acids 3–51) present in the N terminus of Spo20 (Neiman et al., 2000). As previously reported, Δ3-51SPO20 cannot rescue sec9-4, even when present on a high copy plasmid, though Δ3-51PSPS was capable of rescuing growth at high temperature. The Δ3-51SPO20 C224L,S231N allele also rescued growth of this strain at 37°C when present in high copy, though neither SPO20 C224L,S231N nor the PSPP chimera could rescue when
expressed from centromeric plasmids (Fig. 6). These results again suggest that the \textit{SPO20}\textsuperscript{G224L,S231N} mutations increase the strength of Spo20/Sso/Snc interactions, though not to the same degree as complete replacement with the Sec9 helices. To ensure the differences seen were not due to differential stability of the proteins, 3xHA-tagged versions of the different \textit{SPO20} and chimera genes were constructed. Western blotting with anti-HA antibodies indicated that all the \textit{SPO20} forms were present in comparable amounts (unpublished data). Therefore, the results reflect differences in the ability of the different forms to promote vesicle fusion, not differences in protein stability.

**Mutation of \textit{SPO20} increases association with Sso1 and Snc2 in vivo**

If alteration of the SNARE helices of Spo20 increases its affinity for its partner SNAREs, this should be reflected in increased binding of the protein to Sso1 and Snc2. To address this possibility, HA-tagged A50-Spo20, Spo20\textsuperscript{G224L,S231N}, or PSSP were expressed in combination with either wild-type Sso1 and Snc2 or Sso1\textsuperscript{G224R} and Snc2\textsuperscript{R530} in an \textit{sso1 sso2} strain. Lysates were made from each strain and the Spo20 proteins immunoprecipitated using anti-HA antibodies. Immunoprecipitates were then blotted and probed with anti-HA, anti-Sso1, or anti-Snc2 antibodies to examine association of the three SNARE proteins (Fig. 7). In the presence of both the wild-type and mutant Sso1 and Snc2 proteins the same pattern was seen; the PSSP chimera precipitated significantly more Sso1 and Snc2 than Spo20\textsuperscript{G224L,S231N}, which in turn brought down slightly more Sso1 and Snc2 than the wild-type Spo20. Though these immunoprecipitations do not provide a direct measure of affinity, the increased association of PSSP and Spo20\textsuperscript{G224L,S231N} with both forms of Sso1 and Snc2 are consistent with the idea that they bind more avidly to their partner SNAREs than wild-type Spo20. Interestingly, all three forms of Spo20 exhibited greater association with the Sso1\textsuperscript{G224R} and Snc2\textsuperscript{R530} proteins than with the wild-type SNAREs. Because the amount of SNAREs in complex reflects both the rates of assembly and of disassembly, we suggest that the general increase in the amount of SNARE complexes seen with Sso1\textsuperscript{G224R}/Snc2\textsuperscript{R530} might reflect a role for the central ionic layer in complex disassembly, as suggested previously (Scales et al., 2001).

**\textit{SPO20}\textsuperscript{G224L,S231N} improves function of the SNARE complex in vitro**

Studies of Sec9- and Spo20-containing SNARE complexes in an in vitro liposome fusion system indicate that, in a given lipid composition, Spo20-containing complexes are less fusogenic than Sec9 complexes (Liu et al., 2007). Moreover, this lesser activity correlates with decreased SNARE complex stability (measured as a lower melting temperature) of the Spo20 complexes compared with Sec9 complexes. The behavior of the \textit{SPO20}\textsuperscript{G224L,S231N} mutant in the genetic tests and immunoprecipitations described above suggests that these mutations might increase the binding energy of the Spo20-containing complexes. To test this directly, the recombinant SNARE domain (amino acids 147–397) of Spo20\textsuperscript{G224L,S231N} was purified from \textit{Escherichia coli} and tested with the Sso1 and Snc2 proteins in a liposome fusion assay. Using liposomes containing 85% POPC (palmitoyl oleoyl phosphatidylcholine) and 15% DOPS (di-oleoyl phosphatidylserine), Sso1/Sec9–Snc2 complexes promote liposome fusion at a greater rate than the Sso1/Spo20–Snc2 SNAREs (Fig. 8 A). In contrast, Sso1/Spo20\textsuperscript{G224L,S231N}–Snc2 mediates fusion at a rate comparable to the Sec9 complexes. Thus, parallel to the in vivo results, Spo20\textsuperscript{G224L,S231N} promotes fusion more efficiently than Spo20 in vitro.

To determine if the increased fusion activity was reflected in increased binding energy, the stability of the Spo20\textsuperscript{G224L,S231N} containing complexes was examined. We previously reported that Sec9-containing complexes are significantly more stable than Spo20 complexes during equilibrium unfolding reactions with chemical denaturants where the concentration of Guanidinium-HCL required to disrupt 50% of the ternary SNARE complex was reduced 2.1M for Sec9 vs. 0.9M for Spo20 (Liu et al., 2007). When the Spo20\textsuperscript{G224L,S231N}–containing SNAREs were examined, a concentration of 1.1M Guanidinium–HCL disrupted 50% of these ternary complexes, indicating that they were more stable than those with Spo20, though still well below the stability of Sec9 complexes (Fig. 8 B). This moderate improvement in stability of the Spo20\textsuperscript{G224L,S231N}–containing complexes is consistent with the slight increase in binding of Spo20\textsuperscript{G224L,S231N} to Sso1 and Snc2 seen in the IP experiments (Fig. 7). Together with the liposome fusion data, these results suggest that modest changes in affinity can have strong effects on the fusogenic properties of the SNAREs. For the neuronal SNARE SNAP-25 it has similarly been found that mutation of interface residues can result in large differences in function while only modestly altering stability of the SNARE complex (Sorensen et al., 2006).

![Figure 5. Mutation of two interface residues in the Spo20 SNARE helix allows it to function with sso1\textsuperscript{G224R}.](image-url)

(A) Alignment of the interface residues in the SNARE domains of Spo20 and Sec9. Residues chosen for mutation are in blocks. (B) Sporulation of sso1\textsuperscript{G224R} snc2\textsuperscript{R530} strains expressing different forms of SPO20. Strain HJ3 [sso1\textsuperscript{G224R} sso2 sso2 (sso23)] was transformed with plasmids carrying sso1\textsuperscript{G224R} and snc2\textsuperscript{R530} as well as the indicated form of SPO20. The sso1\textsuperscript{G224R} allele was expressed from a CEN plasmid; snc2\textsuperscript{R530} and the SPO20 alleles were expressed from high copy plasmids. These strains were sporulated and sporulation efficiency measured in the light microscope. At least 500 cells were scored for each strain. Results are the average of three experiments. Error bars indicate one standard deviation.
Discussion

The use of compensatory mutations in the central ionic layer of the SNARE domain has proven to be an effective means to demonstrate the participation of different SNARE proteins in the same complex in vivo (Graf et al., 2005). Here, we attempted to use this technique to demonstrate a role for the Snc1/2 proteins in fusion at the prospore membrane. A compensatory mutation in SNC2 could only rescue the sporulation defect of sso1Q224R when expressed in concert with forms of Spo20 carrying the Sec9 SNARE helices. Similar results were obtained using a compensatory mutation in SNC1 (unpublished data). These results demonstrate, first, that the Snc1 and Snc2 proteins indeed function as the R-SNARE subunit of the prospore membrane SNARE complex and, second, that placement of the central layer arginine in different helices is not functionally equivalent. In this instance, swapping the glutamine and arginine between the Sso1 and Snc2 helices creates a SNARE bundle that is more sensitive to the composition of other interface layers in the complex. When Spo20 is the partner, the binding energies at other interfaces are insufficient to overcome the weaker central layer interactions.

During the course of this work, a crystal structure of the SNARE complex containing the Sso1, Snc2, and Sec9 helical domains was published (Strop et al., 2008). When this structure is used to model in the Spo20 cysteine and serine side chains at the +3 and +5 interface layers, the Spo20 residues result in an apparent loss of packing interactions between the side chains (unpublished data), consistent with our results indicating that the Sec9 residues at these positions improve stability of the SNARE complex. Mutational analysis of interface residues in SNAP-25 revealed that interactions in the N-terminal half of the SNARE domain are important for promoting priming or docking of the vesicle, whereas interactions in the C-terminal half of the SNARE helix are critical to drive membrane fusion (Sorensen et al., 2006). In this regard, it is noteworthy that the critical interfaces differentiating the ability of Spo20 and Sec9 to promote fusion at the plasma membrane lie in the C-terminal domain, suggesting that fusion and not docking is the affected step.
would introduce positive charges that clash with the arginine on the Snc2 helix, reduced or eliminated function. However, any other amino acid at this position was well tolerated. This is quite surprising in light of the strong conservation of this glutamine in all syntaxin-family SNARE proteins (Bock et al., 2001). Our results with Spo20 suggest one possible explanation for this apparent paradox. The sensitivity of Spo20-containing complexes to alteration of glutamine 224 would provide selective pressure for its maintenance in Sso1. It may be that other SNARE complexes more closely resemble Spo20- than Sec9-containing SNAREs and are sensitive to perturbation of the central ionic layer.

The ability of mutant forms of Sso1 to function well raises the question of the conservation not just of the glutamine residue, but also of the ionic layer. The ionic layer has been shown to be important for efficient disassembly of the neuronal SNARE complex in vitro (Scales et al., 2001), and our immunoprecipitation data are consistent with this idea. However, the lack of growth phenotype of the \( SSO1 \) \( Q224 \) mutations suggests that disassembly must still occur with reasonable efficiency in the mutants. Another suggested explanation is that the ionic layer allows the multiple helices to assemble in the appropriate register (Fasshauer et al., 1998). Examination of the interface residues in defined SNARE complexes reveals that interfaces with one or two polar...
residues are not uncommon. However a charged residue or more than two polar residues is quite rare (unpublished data). In our experiments, three of the four helices still contain polar or charged residues (two glutamates and an arginine). Therefore, this may still provide sufficient information to assemble the complex in register. Though mutation of all the central ionic layers to hydrophobic residues did not disrupt assembly of the neuronal SNARE complex in vitro (Scales et al., 2001), it would be interesting to determine if combining additional ionic layer changes with SSO1Q224 changes in the yeast SNARE would result in a much more severe fusion defect.

Control of SNARE specificity in vivo

The switch from Sec9- to Spo20-dependent fusion during sporulation provides an excellent system to explore the mechanisms by which a change in a single SNARE subunit can alter the target specificity of a particular class of vesicle. Our results here, along with those previously reported, allow us to answer this question. The specificity of Sec9 and Spo20 for their respective membranes is reinforced in three ways. First, transcriptional control, in wild-type cells SPO20 is transcribed only during sporulation and so cannot function in constitutive secretion (Neiman, 1998). The second mechanism is control of intracellular localization. Efficient targeting of Sec9 to the prospore membrane, either by fusing it to the Spo20 lipid binding motif or to an integral membrane protein, allows Sec9 to restore some degree of sporulation to spo20 cells (Neiman et al., 2000; Nakaniishi et al., 2006). Finally, as we show here, SNARE specificity can be controlled by the strength of the binding interactions between the SNAREs themselves. As the binding energy required for a given fusion event will depend on the potential energy barrier to fusion of the two membranes involved, this form of regulation is linked to the lipid composition of the membranes.

Control of localization and strength of binding are likely to be general mechanisms contributing to SNARE specificity. In liposome binding experiments, the R-SNARE Sec22 is capable of mediating fusion in concert with Sso1 and Sec9 (McNew et al., 2000). This result has been suggested to indicate the existence of a direct ER-to-plasma membrane secretion step in yeast, as found in mammalian cells (Becker et al., 2005). Alternatively, it may be that, though Sec22 is capable of forming productive complexes with Sso1 and Sec9, it does not do so because its localization as a v-SNARE is limited to the cis Golgi- and ER-directed vesicles that do not dock with the plasma membrane in vivo. Consistent with this idea, overexpression of a Sec22R157Q mutant cannot rescue sporulation of the Sso1Q224R mutant even in the presence of the PSPS chimera (unpublished data), suggesting that Sec22 cannot participate in prospore membrane fusion events in vivo.

A recent study revealed that a suboptimal interface at the +7 layer in the neuronal syx-1A gene is important for allowing calcium-mediated regulation of secretion (Lagow et al., 2007). Mutation of the threonine residue at this position in syx-1A to the corresponding isoleucine residue in syx-2 led to constitutive fusion. Thus, as with Spo20 and Sec9, in the neuronal SNARE complex tuning of the strength of binding interactions is important for allowing proper regulation of vesicle transport.

Finally, Spo20 and Sec9 provide a useful model for the evolution of novel SNARE complexes. During the evolution of Saccharomyces, a whole genome duplication occurred that ultimately gave rise to many related gene pairs in the S. cerevisiae genome (Wolfe and Shields, 1997). Sec9 and Spo20 arose from this duplication event. In yeasts that diverged from the S. cerevisiae lineage before the duplication, such as Schizosaccharomyces pombe, a single Sec9/Spo20 related gene participates in fusion at both the plasma membrane and the prospore membrane (Nakamura et al., 2005). Thus, in the S. cerevisiae lineage, the duplication event allowed the two paralogues to become specialized for action at distinct compartments where the ancestral protein functioned at both membranes. Similar patterns are likely at work in the expansions of particular SNARE families seen in plant and mammalian genomes (Sanderfoot et al., 2000; Bock et al., 2001).

Materials and methods

Yeast strains and genetics methods

Unless otherwise noted, standard media and genetic methods were used (Rose and Fink, 1990). The strains used in this study are listed in Table I. Strain HI3 was constructed by PCR-mediated replacement (Longtine et al., 1998) of the SSO1 gene in the haploid strains AN117-4B andAN117-16D (Neiman et al., 2000) and mating of the resulting haploids. Strain HI75 was constructed by mating the sso1Δhis5 derivative of AN117-4B to an sso1Δkan strain from the S. cerevisiae knockout collection (Winzeler et al., 1999). The resulting diploid was transformed with pRS316-SSO1 and then sporulated. Segregants lacking both SSO1 and SSO2 were then mated to generate HI75. To construct strain HI3, a strain from the S. cerevisiae knockout collection carrying the sso1Δhis5 allele was first mated to AN117-4B. A haploid segregant from this cross was mated to strain AN1052 (Neiman et al., 2000), and this diploid was dissected and double mutant sso1Δ spo20Δ haploids were mated.

Plasmids

Plasmids used in this study are listed in Table II. Plasmids pRS314-SSO1 and pRS314-sso1Q224 were constructed by digesting pRS316-SSO1 and pRS316-sso1Q224 (Katz and Brennwald, 2000) with PvuII. These fragments were cotransformed into yeast with KpnI–SacI-digested pRS314 (Sikorski and Hieter, 1989), and the reconstituted plasmids were recovered from yeast. To construct the other glutamine 224 substitutions, the sso1Q224 gene...
was first cloned as a BamHI–HindIII fragment from pRS314-ssO1 into similarly digested pUC119. Site-directed mutagenesis was then performed using oligonucleotides ANO377 and ANO378, which contain randomized nucleotides at codon 224. Sequencing of individual clones from the mutagenesis identified particular substitutions. All substitutions except lysine, glutamine, and aspartate were obtained in this way. For lysine, mutagenesis was performed using oligos HNO961 and HNO962, which contain randomized nucleotides at codon 224. Sequencing of individual clones from the mutagenesis identified particular substitutions. All substitutions except lysine, glutamine, and histidine, the randomized oligos HNO991 and HNO992, were used. For aspartate, mutagenesis was performed using oligos HJO31 and HJO32, HJO33 and HJO34, and HJO35 and HJO36, respectively (Sikorski and Hieter, 1989; Christianson et al., 1992). To construct chimera were isolated from the corresponding integrating plasmids (Neiman et al., 2000) and cloned into similarly digested pRS426 or pRS316, respectively (Sikorski and Hieter, 1989; Christianson et al., 1992). To construct SPO20 C224L;S231N, pRS426-SPO20pr-SEC9 was first cloned as a BamHI–HindIII fragment from pRS306-SEC9pr-3xHA and HNO991, and cloned into similarly digested pRS426-SPO20pr-SEC9, respectively (Sikorski and Hieter, 1989; Christianson et al., 1992). To construct SPO20 F357T;F361L, pRS426-SPO20pr-SEC9 was first cloned as a BamHI – HindIII fragment from pRS304-SEC9pr-3xHA and HNO991, and cloned into similarly digested pRS426-SPO20pr-SEC9, respectively (Sikorski and Hieter, 1989; Christianson et al., 1992). To construct SPO20 A378L,K385N, and HNO992, after specific mutations were identified by sequencing, the plasmid pRS426-SPO20pr-3xHA was swapped into pRS314-SEC9pr-3xHA as an NcoI–SalI fragment. To construct the 3xHA tagged versions of the different SPO20 mutants, two complimentary oligos (HJO72 and HJO73) were synthesized that encoded 3 HA epitopes and anneal to XhoI-compatible ends. The oligos were phosphorylated with T4 polynucleotide kinase (Invitrogen) at 37°C for 10 min, mixed, and then allowed to anneal. The annealed oligos were then ligated with XhoI-digested pRS306-SEC9pr-SEC9 promoter control, KpnI–SacI fragments containing SEC9 promoter with indicated genes were cloned from the integrating and CEN plasmids into KpnI and SacI sites of pRS426.

To construct 3xHA tagged versions of the different SPO20 mutants, two complimentary oligos (HJO72 and HJO73) were synthesized that encode 3 HA epitopes and anneal to XhoI-compatible ends. The oligos were phosphorylated with T4 polynucleotide kinase (Invitrogen) at 37°C for 10 min, mixed, and then allowed to anneal. The annealed oligos were then ligated with XhoI-digested pRS306-SEC9pr-SEC9. To construct SPO20pr-SEC9chimaeras under SEC9 promoter control, KpnI–SacI fragments containing SEC9 promoter with indicated genes were cloned from the integrating and CEN plasmids into KpnI and SacI sites of pRS426.

Table II. Plasmids used in this study

| Name | Source |
|------|--------|
| pRS314 | Sikorski et al., 1989 |
| pRS314-SSO1 | This study |
| pRS314-ssO1 | This study |
| pRS425 | Christianson et al., 1992 |
| pRS425-SSO2 | This study |
| pRS426 | Christianson et al., 1992 |
| pRS426-PPPP | This study |
| pRS426-SPO20 | This study |
| pRS426-PSPP | This study |
| pRS426-PPSS | This study |
| pRS426-SPO20C224L,S231N | This study |
| pRS426-SPO20F357T,F361L | This study |
| pRS426-SPO20A378L,K385N | This study |
| pRS306-SEC9pr-SEC9 | This study |
| pRS306-SEC9pr-3x-SI SPO20 | This study |
| pRS306-SEC9pr-3x-SI PSSP | This study |
| pRS306-SEC9pr-3x-SI SPO20C224L,S231N | This study |
| pRS316-SEC9pr-3x-SI PSSP | This study |
| pRS316-SEC9pr-3x-SI PPPS | This study |
| pRS426-SEC9pr-SEC9 | This study |
| pRS426-SEC9pr-3x-SI PSSP | This study |
| pRS426-SEC9pr-3x-SI PPPS | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |
| pRS426-SEC9pr-3xHA | This study |

Sporulation assays

Sporulation assays were performed as described previously (Neiman et al., 2000). For tests on solid medium, the strains to be tested were grown overnight on selective media, and then replica plated to sporulation medium. After 24 h, spore formation was quantified by direct observation in the light microscope.
For liquid sporulation and ether tests, 1.5 ml of overnight-cultured cells were pelleted, washed once in 1 ml 2% potassium acetate, and resuspended in 10 ml 2% potassium acetate. After 2 d of incubation at 30°C, the sporulation frequency was determined by observation under the light microscope; meanwhile, 5 µl of the culture was spotted onto a YPD plate. The plate was inverted over a paper filter soaked with 2 ml of ethyl ether for 30 min. After 30 min the paper filter was removed, and the plate was incubated at 30°C overnight.

Growth assays
To assay the growth defect of the spo20A sec9::Δ4 mutant, cells were first cultured overnight at 25°C in YPD. Thereafter, 10-fold serial-diluted cell culture was spotted onto two identical plates selective for the plasmid. One plate was placed at 25°C, and the other at 37°C to monitor the growth rate of the spo20A sec9::Δ4 mutant.

Immunoprecipitations
The immunoprecipitation assays were modified from Carr et al. (1999). Strain H75 was transformed with CEN plasmids expressing the different SSO1 genes and high copy plasmids expressing the different SNC2 and SPO20 alleles. 5 ml of overnight culture was diluted into 100 ml of selective medium and grown to mid-log phase. Cells were harvested and resuspended in 1 ml of ice-cold wash buffer (20 mM Tris, pH 7.5, 20 mM NaN3, and 20 mM EDTA). Washed cells were pelleted at 4°C, resuspended in 1 ml ice-cold IP buffer (50 mM Hepes, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.5% NP-40), and treated with zymolyase (100 µg/ml) for 10 min. Cells were pelleted and resuspended in 500 µl ice-cold IP buffer with protease inhibitors. Cells were lysed by shaking with glass beads (0.5 mm) at 4°C for 10 min. Lysed cells were pelleted for 10 min at 13,000 g. The mixtures were rocked for 30 min at 4°C and then centrifuged for 15 min at 13,000 g at 4°C to pellet the beads, debris, and non-specifically bound products. To precipitate the HA-tagged proteins, anti-HA antibodies (Aves Laboratories) were added. Sso1 and Snc2 were detected by rabbit anti-Sso1 and rabbit anti-Snc2 (Sogaard et al., 1994), respectively. Peroxidase-conjugated secondary antibodies (anti-chicken or anti-rabbit) were used. The band intensities were determined using ImageJ and the ratios of Sso1 or Snc2 to the precipitated 3HA-Δ15-Spo20 protein were calculated to compare coprecipitation of Sso1 and Snc2 with the different 3HA-Δ15-Spo20 species.

Protein expression and purification
Sso1, Snc1, Sec9, and Spo20 were expressed and purified as previously described in detail (Liu et al., 2007). Spo20C224L,S231N was expressed and purified from pET24a (+)-based plasmid pJM557 as done previously for Spo20. The Spo20C224L,S231N (147–397) fragment for pJM557 was amplified from pRS30 6SEC9pr::5OSpo20C224L,S231N using primers #303 (CGGGATCCGAGATATCCACAGGG) and #304 (CCACCGCATCTGCACTAATCCTTTCGCCG). The authors wish to thank Pat Brennwald and Reinhard Jahn for plasmids. This work was supported by National Institutes of Health grants GM62184 (to A.M. Neiman) and GM071832 (to J.A. McNiew).

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