Genetic Evidence of a Role for Membrane Lipid Composition in the Regulation of Soluble NEM-Sensitive Factor Receptor Function in *Saccharomyces cerevisiae*

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ABSTRACT

SEC9 and *SPO20* encode SNARE proteins related to the mammalian SNAP-25 family. Sec9p associates with the SNAREs Sso1/2p and Snc1/2p to promote the fusion of vesicles with the plasma membrane. Spo20p functions with the same two partner SNAREs to mediate the fusion of vesicles with the prospore membrane during sporogenesis. A chimeric molecule, in which the helices of Sec9p that bind to Sso1/2p and Snc1/2p are replaced with the homologous regions of Spo20p, will not support vesicle fusion in vegetative cells. The phosphatidylinositol-4-phosphate-5-kinase *MSS4* was isolated as a high-copy suppressor that permits this chimera to rescue the temperature-sensitive growth of a *sec9-4* mutant. Suppression by *MSS4* is specific to molecules that contain the Spo20p helical domains. This suppression requires an intact copy of *SPO14*, encoding phospholipase D. Overexpression of *MSS4* leads to a recruitment of the Spo14 protein to the plasma membrane and this may be the basis for *MSS4* action. Consistent with this, deletion of *KES1*, a gene that behaves as a negative regulator of *SPO14*, also promotes the function of *SPO20* in vegetative cells. These results indicate that elevated levels of phosphatidic acid in the membrane may be required specifically for the function of SNARE complexes containing Spo20p.

THE endomembrane system of the eukaryotic cell consists of a series of distinct membrane-bound compartments. Lipids and proteins are shuttled between compartments by carrier vesicles. Maintenance of proper organization of the endomembrane system requires regulation of vesicle flow such that transport vesicles fuse only at the appropriate acceptor compartment. Soluble NEM-sensitive factor receptor (SNARE) complexes are thought to play a key role in maintaining the specificity of vesicle docking and may directly mediate vesicle fusion (Sollner *et al.* 1993; Rothman 1994; Rothman and Warren 1994; Pelham 1999; Parlati *et al.* 2002; Jahn *et al.* 2003).

SNAREs are a family of proteins that share a related 60-amino-acid (aa) helical region (Weimbs *et al.* 1997). Different SNAREs can interact to form oligomers at whose core is a bundle of four such helices (Sutton *et al.* 1998). In most cases, SNAREs are integral membrane proteins and the helical region of the SNARE protein is adjacent to a transmembrane domain. When SNAREs present in the membrane of an acceptor compartment (t-SNAREs) form complexes with SNAREs in the vesicle membrane (v-SNAREs) the transmembrane domains of the SNAREs in both membranes are brought into close proximity and this is proposed to lead, through an as yet undefined mechanism, to the fusion of the lipid bilayers (Weber *et al.* 1998). Specificity as to which combinations of SNAREs will form productive fusion complexes is proposed as the means by which fusion between vesicles and their appropriate target membranes is regulated (Sollner *et al.* 1993; Rothman and Warren 1994; Parlati *et al.* 2002). However, as isolated SNAREs show only limited binding specificity *in vitro* (Fasshauer *et al.* 1999), the molecular basis of *in vivo* SNARE specificity remains unknown.

One SNARE subfamily, the SNAP-25-related proteins, has an architecture different from that of other SNAREs in that these proteins lack a transmembrane domain but contain two SNARE helices (Hess *et al.* 1992; Weimbs *et al.* 1997). Complexes including these SNAREs are heterotrimers (Sutton *et al.* 1998). In the budding yeast *Saccharomyces cerevisiae*, the two SNAP-25 orthologs Sec9p and Spo20p interact with the t-SNAREs Sso1/2p and the v-SNAREs Snc1/2p to mediate the fusion of exocytic vesicles with the plasma membrane during vegetative growth or the prospore membrane during sporulation, respectively (Gerst *et al.* 1992; Protopopov *et al.* 1993; Brennwald *et al.* 1994; Couve and Gerst 1994; Rossi *et al.* 1997; Neiman 1998).

Sporulation is driven by an unusual cell division event in which daughter nuclei are enveloped within plasma membranes, termed prospore membranes, which form

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de novo within the cytoplasm of the mother cell. These prospore membranes are formed by the redirection of post-Golgi secretory vesicles away from the cell surface and the subsequent fusion of these vesicles within the cytoplasm (Neiman 1998). This fusion event requires much of the same machinery as fusion of vesicles with the plasma membrane, with the exception that Sec9p is largely replaced by Spo20p.

The Spo20 and Sec9 proteins share 40% sequence identity through their conserved SNAP-25 domain and both partner with Ssop and Sncp to mediate the fusion of post-Golgi vesicles with a target membrane (Neiman 1998; Neiman *et al.* 2000). However, they are specific to their respective compartments; Spo20p functions at only the prospore membrane and cannot function at the plasma membrane and the reverse is true for Sec9p. Thus, changing only one subunit of this SNARE complex, Sec9p or Spo20p, is sufficient to change the target membrane specificity of the complex. Sec9p and Spo20p therefore provide an excellent model system to investigate the basis for SNARE specificity *in vivo*.

In previous work, a collection of chimeric *SEC9/SPO20* genes was used to define domains important for the vegetative- or sporulation-specific activity of the two gene products (Neiman *et al.* 2000). Of particular relevance to the current study, an asymmetry was identified in the functionality of the helical regions that mediate SNARE assembly. Specifically, a chimeric protein in which the Spo20p helices have been replaced with those of Sec9p can rescue sporulation in a *spo20*Δ mutant; however, the reciprocal chimera, Sec9p with the Spo20p helices, cannot support growth of a *sec9-ts* mutant. In this report, we explore the basis for this inability of the Spo20p helices to support vesicle fusion at the plasma membrane. Our results suggest that the lipid composition of the membrane can affect the ability of specific SNARE complexes to mediate membrane fusion.

MATERIALS AND METHODS

Strains and media: Standard media and genetic methods were used (Rose and Fink 1990). Strain AN123-4A (*MAT***a** *ura3 leu2-2,113 trp1 lys2 sec9-4*) is a segregant of a cross of W303-1b and AN63-2C (Neiman 1998). AN123-4A derivatives expressing various SNARE chimeras under control of the *SEC9* promoter were constructed by transformation of AN123-4A with integrating plasmids expressing the appropriate chimera, as described previously (Neiman *et al.* 2000). Strain AN1076 (*MAT***a** *ura3 leu2-2,113 trp1 lys2 sec9-4 spo14::*Δ*URA3*) was constructed by transformation of AN123-4A with an *Xba*I-*Cla*I fragment from plasmid pKR466 (Rose *et al.* 1995). Strain AN1075 (*MAT***a** *ura3 leu2-2,113 trp1 lys2 sec9-4 kes1::*Δ*URA3*) was constructed by transformation of AN123-4A with an *Eco*RI-*Bam*HI fragment from pRE352 (Fang *et al.* 1996). Strain AC14 (*MAT***a** *ura3 leu2 arg4 lys2 ho*Δ*LYS2 kes1::*Δ*URA3 spo14::*Δ*URA3*) is a segregant of a cross of strain AN1075 with Y435 (Rose *et al.* 1995). Yeast strains were transformed by the lithium acetate procedure (Ito *et al.* 1983).

Identification of the suppressor gene: The suppressor plasmid pW18 contained an ~9-kb segment of chromosome *IV* carrying a portion of a TY element as well as open reading frames (ORFs) YDR210w, YDR209c, YDR208w (*MSS4*), and YDR207c (*UME6*). Deletion of ORFs YDR210w, YDR209c, and a portion of *MSS4* using internal *Spe*I sites led to a loss of suppressor activity, indicating that one of these three ORFs encodes the suppressor. Subcloning experiments further showed that suppressor activity was contained in a 3.9-kb *Bam*HI-*Xho*I fragment that contained only *MSS4* and a small portion of the *UME6* gene, thus identifying the suppressor as *MSS4*.

Plasmids: Integrating plasmids for expression of the various chimeric SNAREs have been described previously (Neiman *et al.* 2000). The pRS426-Sec9pr-SPO20 plasmid was constructed by cloning a 1.7-kb *Sac*I-*Kpn*I fragment carrying the *SPO20* coding region under control of the *SEC9* promoter from plasmid pRS306-Sec9pr-Spo20 (Neiman *et al.* 2000) into similarly digested pRS426 (Christianson *et al.* 1992). The YEp-GFP-Spo14 and GFP-Spo14^{K533E} plasmids (Sciorra *et al.* 2002) were provided by J. Engebrecht. To construct pRS425-MSS4, a 3.9-kb *Bam*HI-*Xho*I fragment carrying the *MSS4* ORF with ~1.2 kb of upstream and 400 bp of downstream sequence was subcloned from the suppressor plasmid pW18 into *Bam*HI-*Xho*I-digested pRS425 (Christianson *et al.* 1992).

Microscopy: For analysis of GFP-Spo14p, strains carrying pYEp-GFP-Spo14 were grown to midlog phase in YPD medium and examined on a Zeiss Axioplan 2 microscope. Images were acquired using a Zeiss Axiocam mRM and Axiovision 3.0.6 software.

RESULTS

Identification of a high-copy plasmid that promotes the function of a chimeric SNARE: In previous work (Neiman *et al.* 2000), we found that a *SEC9* gene in which the regions encoding the helical domains were replaced with the corresponding regions of *SPO20* (designated *SPSP*—each domain in a chimeric gene is designated "S" if the sequence comes from *SEC9* or "P" if the sequence comes from *SPO20*; letters indicate the origin, in order, of the amino-terminal domain, helix 1, the interhelical domain, and helix 2) could not rescue the temperature-sensitive growth defect of a *sec9-4* strain. By contrast, the reciprocal case in which the helical regions of *SPO20* were replaced with those of *SEC9* (*PSPS*) was competent to support the sporulation of a *spo20*Δ mutant. To investigate the reason for this difference, a screen was performed to identify genes that, when overexpressed, allow the *SPO20* helical regions to function during vegetative growth. A *sec9-4* strain was constructed that carried the *SPSP* SNARE integrated into the chromosome between a duplication of the *URA3* locus and expressed from the *SEC9* promoter. The expression level of the SPSP protein is comparable to native *SEC9* (Neiman *et al.* 2000).

This strain (AN123-4A-SPSP) was transformed with a high-copy genomic library (Yu and Hirsch 1995). From ~26,000 transformants, 60 clones capable of growth at 37° were isolated, of which 49 retested as displaying growth above background at 37°. To distinguish between plasmids that might directly suppress the *sec9-4* defect (*e.g.*, *SEC9*) and those that act by stimulating the

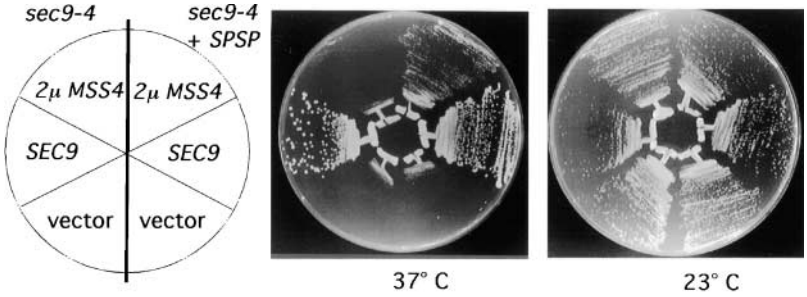

FIGURE 1.—*MSS4* rescues the growth of *sec9-ts* cells in the presence of the *SPSP* SNARE. Strains AN123-4A (*sec9-4*) and AN123-4A SPSP (*sec9-4 ura3::Sec9pr-SPSP::URA3*) were transformed with high-copy plasmids containing *MSS4* (pRS425-MSS4), *SEC9* (Yep352-SEC9), or an empty vector (pRS425). Cells were streaked out for single colonies and grown at the indicated temperature for 3 days.

activity of the *SPSP* chimera, the candidates were replica plated to medium containing 5-fluoroorotic acid to select for cells that had lost the *SPSP* expression construct. After loss of the *SPSP* gene, the strains were again tested for growth at 37°. Of the initial candidates, 10 reverted to temperature sensitivity after loss of the *SPSP* gene. Library plasmids were recovered from these 10 candidates and retransformed into strain AN123-4A-SPSP. Only one plasmid, pW18, reproducibly gave good growth at high temperatures (Figure 1). Sequencing of the ends of the genomic insert in this plasmid indicated that it carried a 9-kb segment of chromosome *IV* including several ORFs. Further deletion and subcloning of the suppressing activity (see MATERIALS AND METHODS) identified the suppressor as the *MSS4* gene.

MSS4 encodes phosphatidylinositol-4-phosphate-5-kinase (PI4P-5-kinase). *MSS4* is the only PI4P-5-kinase in *S. cerevisiae* and is an essential gene (DESRIVIERES *et al.* 1998; HOMMA *et al.* 1998). Although not previously implicated in secretory function, the protein is localized predominantly to the plasma membrane, as is the enzyme's product, phosphatidylinositol (4,5)-bisphosphate ($PI4,5P_2$) (DESRIVIERES *et al.* 1998; HOMMA *et al.* 1998; STEFAN *et al.* 2002). Importantly, it has been shown that expression of *MSS4* from a 2μ plasmid elevates intracellular $PI4,5P_2$ levels about twofold (DESRIVIERES *et al.* 1998). The identification of *MSS4* thus suggests that elevation of $PI4,5P_2$ levels might promote the function of the SPSP SNARE during vegetative growth.

Suppression by *MSS4* is specific to SNAREs containing the *SPO20* helices: In addition to the sporulation-specific function of the Spo20p helices, the Sec9/Spo20 chimera studies identified two distinct functional regions of the Spo20p amino terminus, a domain that is inhibitory to SNARE function and a domain specifically required for function at the prospore membrane (NEIMAN *et al.* 2000). To determine if suppression by *MSS4* was specific to the *SPO20* helices, the ability of *MSS4* to promote the rescue of *sec9-4* by other chimeras, as well as by native *SPO20*, was tested (Figure 2). To allow for a more quantitative assessment of the suppression, serial 10-fold dilutions of the indicated strains were spotted onto selective medium and incubated at either 37° or 25°.

In this assay, overexpression of *MSS4* alone gave a slight suppression of *sec9-4* (Figure 2, rows 2 and 3). Expression of *SPSP* alone showed no effect (Figure 2, row 5), but coexpression of both *MSS4* and *SPSP* (Figure 2, row 4) improved the plating efficiency by two to three orders of magnitude, comparable to expression of *SEC9* itself.

Overexpression of *MSS4* also allowed a *sec9-4* strain expressing only the conserved SNAP-25 domain of *SPO20* (*xPPP*), which contains the two SNARE helices, to grow well at 37° (Figure 2, row 6). By contrast, because it lacks the amino-terminal domain essential for function in sporulation, the SNAP-25 domain of Spo20p will not complement the *spo20*Δ sporulation defect (NEIMAN *et al.* 2000). Moreover, overexpression of *MSS4* does not allow *xPPP* to complement the *spo20* sporulation defect (data not shown). Thus, increased *MSS4* specifically allows the Spo20p SNARE domain to function in vesicle fusion at the plasma membrane.

Fusion of the *SPO20* amino-terminal domain to the *SEC9* SNAP-25 domain (*PSSS*) interferes with the ability of this region of *SEC9* to complement *sec9-4* (NEIMAN *et al.* 2000). To examine if *MSS4* can influence the inhibitory activity of the Spo20p amino terminus, the ability of *MSS4* to promote the function of *PSSS* was tested. Overexpression of *MSS4* does not allow rescue of *sec9-4* by *PSSS* (Figure 2, row 8). Thus, the ability of *MSS4* to promote Spo20p function appears to be specific to the Spo20p SNARE domain.

Consistent with the inability of *MSS4* to overcome inhibition by the Spo20p amino terminus, *MSS4* will not permit an integrated copy of *SPO20* to rescue *sec9-4* (Figure 2, row 10). However, when *SPO20*, expressed from the *SEC9* promoter, is introduced into *sec9-4* cells on a 2μ plasmid along with *MSS4*, then growth of the *sec9-4* strain is seen (Figure 2, row 12). As high-copy *MSS4* does not bypass the negative effect of the Spo20p amino terminus, this last result indicates that, in the presence of overexpressed *MSS4*, overexpression of *SPO20* itself overcomes the inhibitory property of the Spo20p amino terminus.

***SPO14* is required for suppression by *MSS4*:** The isolation of *MSS4* as a high-copy suppressor suggests that elevation of $PI4,5P_2$ levels promotes the function of the *SPO20* helices. This effect may be quite indirect; for example, elevation of $PI4,5P_2$ levels might promote the function of some $PI4,5P_2$ binding proteins. Indeed, a large number of $PI4,5P_2$ binding proteins with diverse intracellular functions have been identified (OSTRANDER

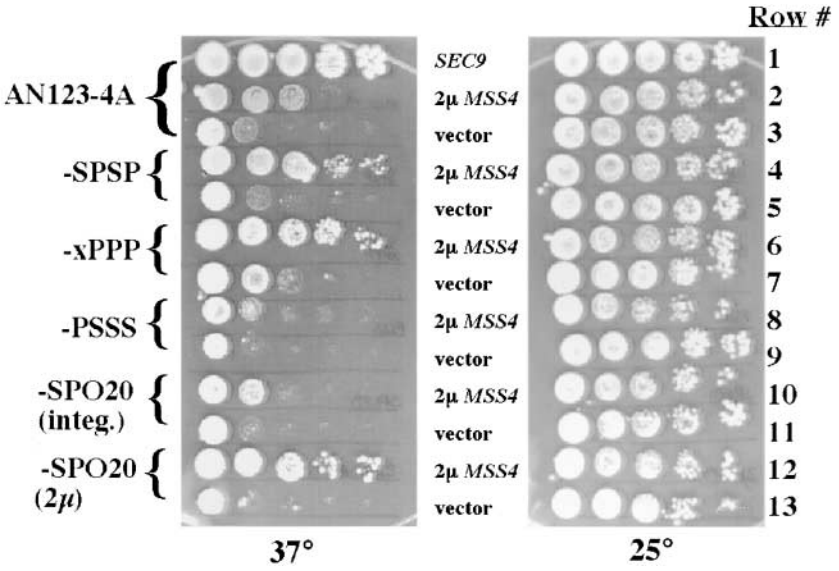

FIGURE 2.—*MSS4* specifically promotes the function of SNAREs containing the helical domains of Spo20p. Strain AN123-4A (*sec9-4*) and derivatives expressing the indicated SNARE were transformed with high-copy plasmids carrying *MSS4* (pRS425-MSS4), *SEC9* (Yep352-SEC9), or an empty vector (pRS425). Strains were grown to saturation in selective medium and 10-fold serial dilutions were plated. Plates were photographed after 3 days incubation at the indicated temperature.

et al. 1995; LEVINE and MUNRO 1998; OJALA *et al.* 2001; PALMGREN *et al.* 2001; HALLETT *et al.* 2002; SCIORRA *et al.* 2002). Of particular interest in this regard is the *SPO14* gene product. *SPO14* encodes a phosphatidylcholine-specific phospholipase D and the activity of the enzyme requires the presence of $PI4,5P_2$ (SCIORRA *et al.* 1999). Further, although *spo14* mutants have only minimal phenotypes in vegetative cells, they are defective in sporulation due to an inability to form the prospore membrane and, in fact, Spo14p is localized to the prospore membrane during sporulation (ROSE *et al.* 1995; RUDGE *et al.* 1998b).

Because *SPO14* encodes a $PI4,5P_2$-dependent enzyme required for formation of the same membrane at which Spo20p normally functions, we examined whether the action of *MSS4* on SNARE function required the presence of *SPO14*. The *SPO14* gene was deleted in our *sec9-4* strain and *MSS4* was introduced on a high-copy vector along with high-copy *SPO20*. Deletion of *SPO14* caused a slight decrease in the growth rate of the *sec9-4* strain (Figure 3, 25°). More importantly, in the absence of *SPO14*, overexpression of *MSS4* was unable to promote *SPO20* function (Figure 3, rows 4 and 5). Even the modest improvement of growth by overexpression of *MSS4* alone was lost (Figure 3, rows 3 and 6). Thus, the suppression activity of *MSS4* is dependent on *SPO14*. These data suggest that *MSS4* might promote *SPO20* function by altering the activity or localization of the *SPO14* gene product.

Localization of Spo14-GFP is altered by *MSS4* overexpression: To investigate the possible effect of the suppressor plasmids on *SPO14*, we first examined whether overexpression of *SPO14* itself could promote *SPO20* function. A high-copy *SPO14* plasmid was introduced

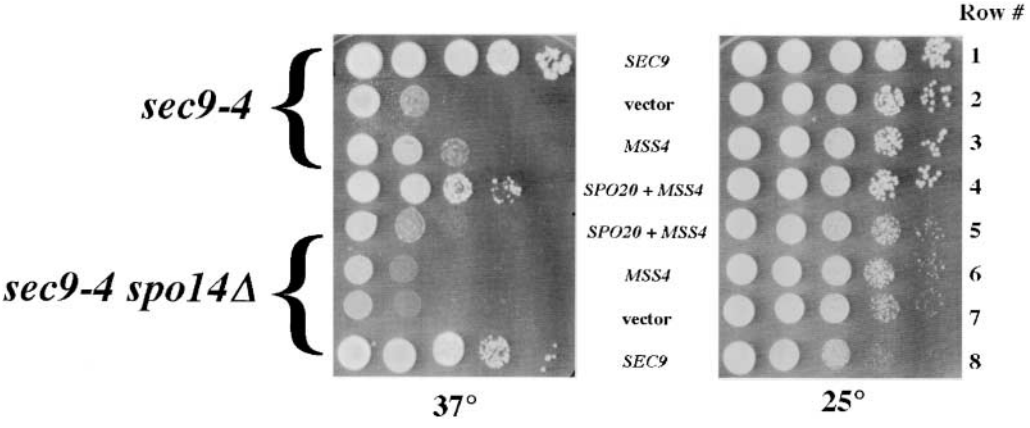

FIGURE 3.—Suppression by *MSS4* requires an intact *SPO14* gene. Strains AN123-4A (*sec9-4*) and AN1076 (*sec9-4 spo14Δ::URA3*) were transformed with high-copy plasmids carrying *MSS4* (pRS425-MSS4), *SEC9* (Yep-352-SEC9), an empty vector (pRS425), or two plasmids to introduce both *MSS4* (pRS425-MSS4) and *SPO20* (pRS426-Sec9pr-SPO20). Cultures were grown to saturation in selective medium and 10-fold serial dilutions were plated. Plates were photographed after 3 days incubation at the indicated temperature.

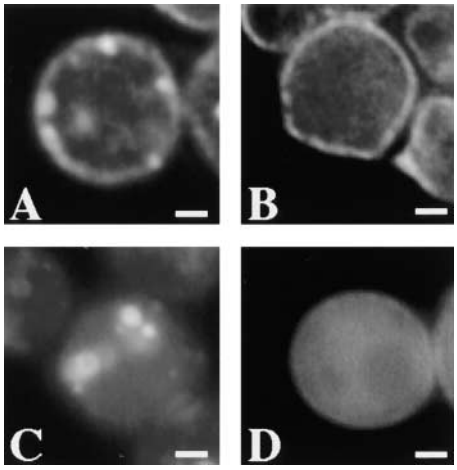

FIGURE 4.—Localization patterns of GFP-Spo14p in cells overexpressing *MSS4*. Strain AN123-4A was transformed with plasmids pRS425-MSS4 and pYEp-GFP-SPO14, and cells were grown to log phase at 30° and examined by fluorescence microscopy. Examples of each of the classes listed in Table 1 and described in the text are shown. (A) Dots underneath the plasma membrane. (B) Plasma membrane. (C) Intracellular rings and dots. (D) Diffuse cytosolic fluorescence. Bars: (A and D) 1 μm, (B and C) 1.5 μm.

into AN123-4A along with high-copy *SPO20*. Unlike *MSS4*, overexpression of *SPO14* was unable to promote rescue of *sec9-4* by *SPO20* (data not shown). This may indicate that the action of *MSS4* requires a change in the localization of *SPO14* rather than a simple increase in *SPO14* activity.

To examine the localization of Spo14p, a Spo14-green fluorescent protein (GFP) fusion was introduced into strain AN123-4A. Vegetatively growing cells expressing Spo14-GFP displayed a diffuse pattern throughout the cytosol (similar to Figure 4D) as has been reported previously (RUDGE *et al.* 1998b). A small but significant fraction of the cells (7.5%) displayed weak plasma membrane fluorescence. Introduction of high-copy *MSS4* produced a marked alteration in GFP-Spo14p localization. Four different patterns of localization were seen. Examples of each pattern are shown in Figure 4 and the distribution of cells displaying each pattern is given in Table 1. The most striking change was an increase in the fraction of cells (44%) showing plasma membrane localization of GFP-Spo14p (Figure 4B). Additionally, two minor classes showing distinct intracellular staining, either dots underneath the plasma membrane (Figure 4A) or larger intracellular rings and dots (Figure 4C), were identified. These patterns were very rare or absent in the control cells. The precise nature of these intracellular structures is not yet clear, although they may represent localization of Spo14-GFP to an endosomal compartment, as reported previously (LI *et al.* 2000). Nonetheless, these data indicate that overexpression of *MSS4* alters the intracellular localization of Spo14p, particularly by increasing its abundance on the plasma membrane. This relocalization of Spo14p may be the means by which *MSS4* promotes *SPO20* function.

Spo14p carries two PI4,5P2 binding domains. The first is a polybasic motif required for catalytic activity and the second is a pleckstrin homology (PH) domain by which $PI4,5P_2$ influences the localization of the protein (SCIORRA *et al.* 1999, 2002). To examine if elevated $PI4,5P_2$ levels generated by *MSS4* overexpression are responsible for the mislocalization of Spo14, the localization of $Spo14^{K533E}$ carrying a mutation that inactivates $PI4,5P_2$ binding by the PH domain was examined (Table 1). Unlike the wild-type protein, GFP-$Spo14^{K533E}$ was not relocalized by overexpression of *MSS4*. This result indicates that the elevation of $PI4,5P_2$ levels is responsible for plasma membrane recruitment of Spo14p.

Deletion of the *KES1* gene promotes *SPO20* function in vegetative cells: The results presented define a novel phenotype for *spo14* in vegetative cells. A known phenotype for *SPO14* in vegetative growth is its role in "bypass *sec14*" (SREENIVAS *et al.* 1998; XIE *et al.* 1998). The *SEC14* gene encodes an essential protein required for transport through the Golgi apparatus. A number of second-site suppressors that suppress the lethality of a *sec14* mutation have been identified (CLEVES *et al.* 1989, 1991; ALB *et al.* 1995; FANG *et al.* 1996). Although these mutations affect various aspects of lipid metabolism they share the common feature that *SPO14* activity is required for the bypass phenotype.

Mutation of the *KES1* gene produces a bypass *sec14*

TABLE 1

The effect of *MSS4* overexpression on the distribution of GFP-Spo14p

	Class A[a]	Class B	Class C	Class D
GFP-Spo14 + vector	0	7.5	0.5	92
GFP-Spo14 + *MSS4*	5	44	9	42
GFP-$Spo14^{K533E}$ + vector	0	3.5	0	96.5
GFP-$Spo14^{K533E}$ + MSS4	1	0	0.5	98.5

Strain AN123-4A was transformed with the indicated plasmids and examined in the fluorescence microscope and cells were grouped into classes on the basis of the observed distribution of GFP-Spo14p. The percentage of cells in each class is given. At least 400 cells were scored in each culture.

[a] Representative cells for each class are shown in Figure 4.

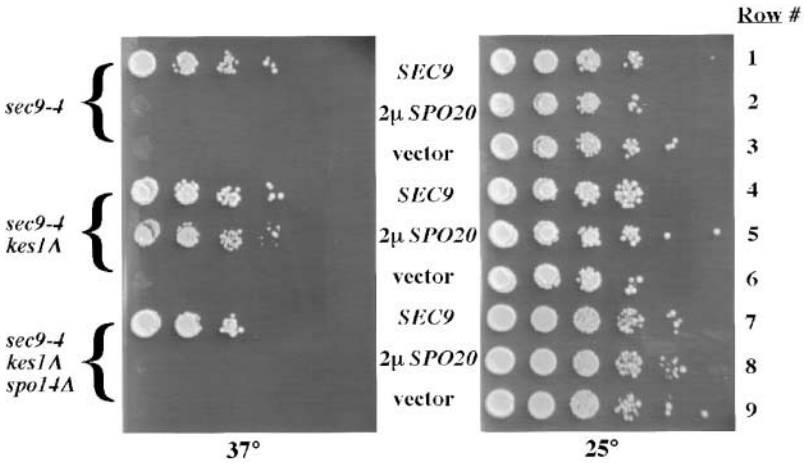

FIGURE 5.—Deletion of *KES1* allows rescue of *sec9-4* by *SPO20*. Strains AN123-4A (*sec9-4*), AN1075 (*sec9-4 kes1::ΔURA3*), or AC14 (*sec9-4 kes1::ΔURA3 spo14::ΔURA3*) were transformed with the indicated plasmid. Cultures were grown to saturation and 10-fold serial dilutions were plated and incubated at either 37° or 25°.

phenotype (FANG *et al.* 1996). To test whether the bypass *sec14* phenotype was related to *SPO14*-mediated promotion of *SPO20* function, the *KES1* gene was deleted from our *sec9-4* strain and the ability of 2μ *SPO20* to confer growth at 37° was examined. Remarkably, deletion of *KES1* was sufficient to allow high-copy *SPO20* to suppress *sec9-4* (Figure 5, row 5). As with overexpression of *MSS4*, the ability of the *kes1*Δ mutant to promote *SPO20* function required an intact copy of *SPO14* (Figure 5, row 8). These observations suggest that mutation of *KES1* might similarly cause a redistribution of Spo14p to the plasma membrane. In contrast to *MSS4* overexpression, however, the subcellular distribution of GFP-Spo14p in the *kes1*Δ mutant strain was not significantly different from that of wild-type cells (data not shown). Although the precise mechanism by which deletion of *kes1* leads to *SPO14*-dependent suppression remains unknown, these results provide further evidence that alterations in lipid metabolism enhance the ability of the Spo20p helices to promote vesicle fusion at the plasma membrane.

DISCUSSION

The results presented document a role for lipid-modifying enzymes in the function of a specific SNARE molecule. Overexpression of the PI4P-5-kinase *MSS4* allows SNAREs containing the Spo20p helical regions to support fusion of vesicles at the plasma membrane. However, this is true only if the phospholipase D encoded by *SPO14* is present. The exact relationship between *SPO14* and *MSS4* in this system is still unclear. The Spo14 protein has been shown to require binding to $PI4,5P_2$ for enzymatic activity and for localization (SCIORRA *et al.* 1999, 2002). Conversely, in higher cells, the activity of PI4P-5-kinase is promoted by phospholipase D activity (HONDA *et al.* 1999). Thus, overexpression of *MSS4* might promote Spo14p activity or *SPO14* might be necessary to support increased activity of Mss4p in the overexpressing cells. It should be noted, however, that stimulation of PI4P-5-kinase by phospholipase D in higher cells is mediated via the phospholipase D activator ARF (HAMMOND *et al.* 1995; HONDA *et al.* 1999). By contrast, Spo14p is not activated by ARF in yeast cells (RUDGE *et al.* 1998a). Moreover, recent studies suggest that *SPO14* acts downstream of *MSS4* in sporulating cells (J. ENGEBRECHT, personal communication). Given these results and our observation that high-copy *MSS4* leads to relocalization of Spo14p to the plasma membrane, the simplest interpretation of our results is that overexpression of *MSS4* elevates the levels of $PI4,5P_2$ in the plasma membrane. This elevation recruits Spo14p to the plasma membrane, leading to an elevation of the Spo14p product phosphatidic acid (PA) in the plasma membrane and, in turn, PA aids Spo20p in catalyzing membrane fusion.

Analysis of the lipids of the plasma membrane indicates that its composition is distinct from that of other intracellular membranes (SCHNEITER *et al.* 1999). Although no lipid analysis of the prospore membrane has been reported, studies of *SPO14* suggest that the prospore membrane may have a lipid composition different from that of the plasma membrane (RUDGE *et al.* 2001). *SPO14* is required for the formation of the prospore membrane and, further, Spo14p is localized to the prospore membrane during normal spore morphogenesis. These results suggest both that the production of PA by Spo14p is required for the coalescence of vesicles to form a prospore membrane and that the prospore membrane might be richer in PA than the plasma membrane. Thus, overexpression of *MSS4*, by recruiting Spo14p to the plasma membrane, may serve to make the plasma membrane more similar to the prospore membrane and thereby promote Spo20p function.

The proposition that alterations in lipid composition of the plasma membrane are promoting function of Spo20p is strengthened by our findings with *kes1*. *KES1* encodes a Golgi-resident protein that is a member of the oxysterol-binding protein family; however, the lipid ligand for Kes1p is not a sterol, but rather phosphatidyl-

inositol-4-phosphate (Li *et al.* 2002). Mutation of *KES1* bypasses the need for *SEC14* for Golgi function in a *SPO14*-dependent manner and, as with other *sec14*-bypass mutations, is thought to do so by elevating PA or diacylglycerol (DAG) levels in the Golgi. Further, overexpression of *KES1* phenocopies a deletion of the *SPO14* gene (Fang *et al.* 1996; Xie *et al.* 1998; Li *et al.* 2002). Thus, *KES1* behaves genetically as if it is antagonistic to *SPO14*, although no direct effect of *KES1* on Spo14p activity has been reported. Our observations strengthen the proposal that *KES1* acts in opposition to *SPO14* (Rivas *et al.* 1999).

One important unresolved issue is how PA acts to enhance Spo20p activity. For example, the critical lipid might be PA or some derivative thereof such as DAG or lyso-PA. More generally, it remains to be determined whether the effects we see are mediated through a protein intermediate or by the lipid directly. The two most straightforward possibilities are that (1) PA stimulates the activity of a PA-binding protein, which in turn aids in fusion of vesicles, or (2) the increased concentration of PA itself, by altering the lipid composition of the membrane, is directly responsible for rescuing vesicle fusion.

How might membrane composition promote or prohibit SNARE function? In current models of SNARE-mediated membrane fusion, the oligomerization of the v- and t-SNARE helices drives the two membranes together (Jahn *et al.* 2003). To drive the two membranes together, the energy provided by assembly must be sufficient to overcome the potential energy barrier to fusion. This barrier is produced by both repulsion of the negatively charged membrane surfaces and the stress of membrane bending in the fusion intermediates (Kuzmin *et al.* 2001; Kozlovsky and Kozlov 2002; Jahn *et al.* 2003). Perhaps the energy made available by assembly of SNARE complexes consisting of Spo20-Sso-Snc is insufficient to drive fusion at the yeast plasma membrane. Elevation of PA levels in the membrane, we suggest, might lower the potential energy barrier to fusion, permitting the Spo20p helices to work.

Previous chimera studies (Neiman *et al.* 2000) indicated that the inability of *SPO20* to function in vegetative cells is due to two factors: (1) inhibition of SNARE function by an amino-terminal domain and (2) the inability of the Spo20p helices to promote vesicle fusion at the plasma membrane. The results presented here suggest that this latter deficiency is related to the lipid composition of the plasma membrane. If the barrier to fusion at the plasma membrane is intrinsically higher than that at the prospore membrane, then the plasma membrane SNARE Sec9p might be expected to function well at the prospore membrane. Indeed, chimeras containing the Sec9p helices can promote fusion at the prospore membrane. However, intact Sec9p cannot. This failure is largely attributable to the fact that Spo20p contains, in its unique amino-terminal domain, a short region essential for SNARE activity at the prospore membrane (Neiman *et al.* 2000). This short region binds to PA *in vitro* (H. Nakanishi and A. M. Neiman, unpublished observations), suggesting that differences in lipid composition of the prospore membrane might also account for the inability of Sec9p to function at that compartment.

In a reconstituted liposome fusion assay, SNARE proteins display high selectivity in complex assembly (Parlati *et al.* 2002). However, additional factors appear to contribute to specificity *in vivo* (Jahn *et al.* 2003). Our results suggest that a specific lipid environment is required for the function of Spo20p. Some evidence suggests that other SNAREs also prefer specific lipid environments. In higher cells, the plasma membrane SNAREs have been shown to be present in lipid rafts or cholesterol-dependent clusters (Lafont *et al.* 1999; Chamberlain *et al.* 2001; Lang *et al.* 2001). Lipid rafts are enriched in cholesterol and, at synapses, this may enhance exocytosis by aiding in the recruitment of the cholesterol-binding SNARE cofactor synaptophysin (Mitter *et al.* 2003). However, plasma membrane SNAREs are also found in cholesterol-rich domains in nonneuronal cells that lack synaptophysin (Lafont *et al.* 1999; Chamberlain *et al.* 2001). In these cases, whether the lipids of the raft similarly serve to recruit necessary cofactors or, perhaps, directly influence the fusion of lipid bilayers remains to be determined.

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LITERATURE CITED

Alb, Jr., J. G., A. Gedvilaite, R. T. Cartee, H. B. Skinner and V. A. Bankaitis, 1995 Mutant rat phosphatidylinositol/phosphatidylcholine transfer proteins specifically defective in phosphatidylinositol transfer: implications for the regulation of phospholipid transfer activity. Proc. Natl. Acad. Sci. USA **92:** 8826–8830.

Brennwald, P., B. Kearns, K. Champion, S. Keranen, V. Bankaitis *et al.*, 1994 Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell **79:** 245–258.

Chamberlain, L. H., R. D. Burgoyne and G. W. Gould, 2001 SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. Proc. Natl. Acad. Sci. USA **98:** 5619–5624.

Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero and P. Hieter, 1992 Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.

Cleves, A. E., P. J. Novick and V. A. Bankaitis, 1989 Mutations in the *SAC1* gene suppress defects in yeast Golgi and yeast actin function. J. Cell Biol. **109:** 2939–2950.

Cleves, A. E., T. P. McGee, E. A. Whitters, K. M. Champion, J. R. Aitken *et al.*, 1991 Mutations in the CDP-choline pathway for phospholipid biosynthesis bypass the requirement for an essential phospholipid transfer protein. Cell **64:** 789–800.

Couve, A., and J. E. Gerst, 1994 Yeast Snc proteins complex with Sec9. Functional interactions between putative SNARE proteins. J. Biol. Chem. **269:** 23391–23394.

Desrivieres, S., F. T. Cooke, P. J. Parker and M. N. Hall, 1998

MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in Saccharomyces cerevisiae. J. Biol. Chem. **273:** 15787–15793.

Fang, M., B. G. Kearns, A. Gedvilaite, S. Kagiwada, M. Kearns *et al.*, 1996 Kes1p shares homology with human oxysterol binding protein and participates in a novel regulatory pathway for yeast Golgi-derived transport vesicle biogenesis. EMBO J. **15:** 6447–6459.

Fasshauer, D., W. Antonin, M. Margittai, S. Pabst and R. Jahn, 1999 Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. J. Biol. Chem. **274:** 15440–15446.

Gerst, J. E., L. Rodgers, M. Riggs and M. Wigler, 1992 *SNC1*, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the *RAS* and *CAP* genes. Proc. Natl. Acad. Sci. USA **89:** 4338–4342.

Hallett, M. A., H. S. Lo and A. Bender, 2002 Probing the importance and potential roles of the binding of the PH-domain protein Boi1 to acidic phospholipids. BMC Cell Biol. **3:** 16.

Hammond, S. M., Y. M. Altshuller, T. C. Sung, S. A. Rudge, K. Rose *et al.*, 1995 Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. J. Biol. Chem. **270:** 29640–29643.

Hess, D. T., T. M. Slater, M. C. Wilson and J. H. Skene, 1992 The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. J. Neurosci. **12:** 4634–4641.

Homma, K., S. Terui, M. Minemura, H. Qadota, Y. Anraku *et al.*, 1998 Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. J. Biol. Chem. **273:** 15779–15786.

Honda, A., M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura *et al.*, 1999 Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell **99:** 521–532.

Ito, H., Y. Fukuda, K. Murata and A. Kimura, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163–168.

Jahn, R., T. Lang and T. C. Sudhof, 2003 Membrane fusion. Cell **112:** 519–533.

Kozlovsky, Y., and M. M. Kozlov, 2002 Stalk model of membrane fusion: solution of energy crisis. Biophys. J. **82:** 882–895.

Kuzmin, P. I., J. Zimmerberg, Y. A. Chizmadzhev and F. S. Cohen, 2001 A quantitative model for membrane fusion based on low-energy intermediates. Proc. Natl. Acad. Sci. USA **98:** 7235–7240.

Lafont, F., P. Verkade, T. Galli, C. Wimmer, D. Louvard *et al.*, 1999 Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells. Proc. Natl. Acad. Sci. USA **96:** 3734–3738.

Lang, T., D. Bruns, D. Wenzel, D. Riedel, P. Holroyd *et al.*, 2001 SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. EMBO J. **20:** 2202–2213.

Levine, T. P., and S. Munro, 1998 The pleckstrin homology domain of oxysterol-binding protein recognises a determinant specific to Golgi membranes. Curr. Biol. **8:** 729–739.

Li, X., S. M. Routt, Z. Xie, X. Cui, M. Fang *et al.*, 2000 Identification of a novel family of nonclassic yeast phosphatidylinositol transfer proteins whose function modulates phospholipase D activity and Sec14p-independent cell growth. Mol. Biol. Cell **11:** 1989–2005.

Li, X., M. P. Rivas, M. Fang, J. Marchena, B. Mehrotra *et al.*, 2002 Analysis of oxysterol binding protein homologue Kes1p function in regulation of Sec14p-dependent protein transport from the yeast Golgi complex. J. Cell Biol. **157:** 63–77.

Mitter, D., C. Reisinger, B. Hinz, S. Hollmann, S. V. Yelamanchili *et al.*, 2003 The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content. J. Neurochem. **84:** 35–42.

Neiman, A. M., 1998 Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. J. Cell Biol. **140:** 29–37.

Neiman, A. M., L. Katz and P. J. Brennwald, 2000 Identification of domains required for developmentally regulated SNARE function in *Saccharomyces cerevisiae*. Genetics **155:** 1643–1655.

Ojala, P. J., V. Paavilainen and P. Lappalainen, 2001 Identification of yeast cofilin residues specific for actin monomer and PIP2 binding. Biochemistry **40:** 15562–15569.

Ostrander, D. B., J. A. Gorman and G. M. Carman, 1995 Regulation of profilin localization in Saccharomyces cerevisiae by phosphoinositide metabolism. J. Biol. Chem. **270:** 27045–27050.

Palmgren, S., P. J. Ojala, M. A. Wear, J. A. Cooper and P. Lappalainen, 2001 Interactions with PIP2, ADP-actin monomers, and capping protein regulate the activity and localization of yeast twinfilin. J. Cell Biol. **155:** 251–260.

Parlati, F., O. Varlamov, K. Paz, J. A. McNew, D. Hurtado *et al.*, 2002 Distinct SNARE complexes mediating membrane fusion in Golgi transport based on combinatorial specificity. Proc. Natl. Acad. Sci. USA **99:** 5424–5429.

Pelham, H. R., 1999 SNAREs and the secretory pathway-lessons from yeast. Exp. Cell Res. **247:** 1–8.

Protopopov, V., B. Govindan, P. Novick and J. E. Gerst, 1993 Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in S. cerevisiae. Cell **74:** 855–861.

Rivas, M. P., B. G. Kearns, Z. Xie, S. Guo, M. C. Sekar *et al.*, 1999 Pleiotropic alterations in lipid metabolism in yeast sac1 mutants: relationship to "bypass Sec14p" and inositol auxotrophy. Mol. Biol. Cell **10:** 2235–2250.

Rose, K., S. A. Rudge, M. A. Frohman, A. J. Morris and J. Engebrecht, 1995 Phospholipase D signaling is essential for meiosis. Proc. Natl. Acad. Sci. USA **92:** 12151–12155.

Rose, M. D., and G. R. Fink, 1990 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rossi, G., A. Salminen, L. M. Rice, A. T. Brunger and P. Brennwald, 1997 Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a novel function for the C terminus of the SNAP-25 homolog, Sec9. J. Biol. Chem. **272:** 16610–16617.

Rothman, J. E., 1994 Intracellular membrane fusion. Adv. Second Messenger Phosphoprotein Res. **29:** 81–96.

Rothman, J. E., and G. Warren, 1994 Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. Curr. Biol. **4:** 220–233.

Rudge, S. A., M. M. Cavenagh, R. Kamath, V. A. Sciorra, A. J. Morris *et al.*, 1998a ADP-ribosylation factors do not activate yeast phospholipase Ds but are required for sporulation. Mol. Biol. Cell **9:** 2025–2036.

Rudge, S. A., A. J. Morris and J. Engebrecht, 1998b Relocalization of phospholipase D activity mediates membrane formation during meiosis. J. Cell Biol. **140:** 81–90.

Rudge, S. A., T. R. Pettitt, C. Zhou, M. J. Wakelam and J. A. Engebrecht, 2001 SPO14 separation-of-function mutations define unique roles for phospholipase D in secretion and cellular differentiation in *Saccharomyces cerevisiae*. Genetics **158:** 1431–1444.

Schneiter, R., B. Brugger, R. Sandhoff, G. Zellnig, A. Leber *et al.*, 1999 Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acyl chain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. J. Cell Biol. **146:** 741–754.

Sciorra, V. A., S. A. Rudge, G. D. Prestwich, M. A. Frohman, J. Engebrecht *et al.*, 1999 Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. EMBO J. **18:** 5911–5921.

Sciorra, V. A., S. A. Rudge, J. Wang, S. McLaughlin, J. Engebrecht *et al.*, 2002 Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J. Cell Biol. **159:** 1039–1049.

Sollner, T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos *et al.*, 1993 SNAP receptors implicated in vesicle targeting and fusion. Nature **362:** 318–324.

Sreenivas, A., J. L. Patton-Vogt, V. Bruno, P. Griac and S. A. Henry, 1998 A role for phospholipase D (Pld1p) in growth, secretion, and regulation of membrane lipid synthesis in yeast. J. Biol. Chem. **273:** 16635–16638.

Stefan, C. J., A. Audhya and S. D. Emr, 2002 The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. Mol. Biol. Cell **13:** 542–557.

Sutton, R. B., D. Fasshauer, R. Jahn and A. T. Brunger, 1998 Crys-

tal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature **395:** 347–353.

Weber, T., B. V. Zemelman, J. A. McNew, B. Westermann, M. Gmachl *et al.*, 1998 SNAREpins: minimal machinery for membrane fusion. Cell **92:** 759–772.

Weimbs, T., S. H. Low, S. J. Chapin, K. E. Mostov, P. Bucher *et al.*, 1997 A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. Proc. Natl. Acad. Sci. USA **94:** 3046–3051.

Xie, Z., M. Fang, M. P. Rivas, A. J. Faulkner, P. C. Sternweis *et al.*, 1998 Phospholipase D activity is required for suppression of yeast phosphatidylinositol transfer protein defects. Proc. Natl. Acad. Sci. USA **95:** 12346–12351.

Yu, Y., and J. P. Hirsch, 1995 An essential gene pair in Saccharomyces cerevisiae with a potential role in mating. DNA Cell Biol. **14:** 411–418.

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