Fus1p Interacts With Components of the Hog1p Mitogen-Activated Protein Kinase and Cdc42p Morphogenesis Signaling Pathways to Control Cell Fusion During Yeast Mating

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ABSTRACT

Cell fusion in the budding yeast *Saccharomyces cerevisiae* is a temporally and spatially regulated process that involves degradation of the septum, which is composed of cell wall material, and occurs between conjugating cells within a prezygote, followed by plasma membrane fusion. The plasma membrane protein Fus1p is known to be required for septum degradation during cell fusion, yet its role at the molecular level is not understood. We identified Sho1p, an osmosensor for the HOG MAPK pathway, as a binding partner for Fus1 in a two-hybrid screen. The Sho1p-Fus1p interaction occurs directly and is mediated through the Sho1p-SH3 domain and a proline-rich peptide ligand on the Fus1p COOH-terminal cytoplasmic region. The cell fusion defect associated with *fus1*Δ mutants is suppressed by a *sho1*Δ deletion allele, suggesting that Fus1p negatively regulates Sho1p signaling to ensure efficient cell fusion. A two-hybrid matrix containing fusion proteins and pheromone response pathway signaling molecules reveals that Fus1p may participate in a complex network of interactions. In particular, the Fus1p cytoplasmic domain interacts with Chs5p, a protein required for secretion of specialized Chs3p-containing vesicles during bud development, and *chs5*Δ mutants were defective in cell surface localization of Fus1p. The Fus1p cytoplasmic domain also interacts with the activated GTP-bound form of Cdc42p and the Fus1p-SH3 domain interacts with Bni1p, a yeast formin that participates in cell fusion and controls the assembly of actin cables to polarize secretion in response to Cdc42p signaling. Taken together, our results suggest that Fus1p acts as a scaffold for the assembly of a cell surface complex involved in polarized secretion of septum-degrading enzymes and inhibition of HOG pathway signaling to promote cell fusion.

MATING yeast cells achieve cytoplasmic continuity through a combination of degradation of the prezygote septum and fusion of the plasma membrane in a process termed cell fusion (Marsh and Rose 1997). The mating reaction proceeds as an ordered set of events (Sprague and Thorner 1992; Elion 2000). First, mating partners, which are arrested in the G_1 phase of the cell cycle due to mating-pheromone-induced signaling, make contact and remodel their cell surface to form a prezygote, in which two cells are joined by a continuous extracellular matrix while their plasma membranes remain separated by an intervening septum. Second, localized degradation of the septum facilitates membrane fusion, which requires the membrane-spanning protein Prm1p (Heiman and Walter 2000) and leads to cytoplasmic mixing. Finally, nuclear migration and nuclear fusion leads to the formation of a diploid zygote that resumes vegetative growth.

Several signaling pathways have been implicated in the regulation of cell fusion. Strains with reduced pheromone production accumulate prezygotes during mating, indicating that cell fusion is dependent upon a critical pheromone level (Brizzio *et al.* 1996; Dorer *et al.* 1997). The pheromone response pathway may also directly control cell fusion since mutations in *FUS3*, encoding the pheromone response mitogen-activated protein kinase (MAPK), lead to a fusion defect (Elion *et al.* 1990; Fujimura 1990). Activated alleles of *PKC1* inhibit cell fusion, implicating the PKC pathway in negative regulation of cell fusion (Philips and Herskowitz 1997). Finally, high levels of internal glycerol relative to the external medium inhibit cell fusion (Philips and Herskowitz 1997), which suggests that the HOG MAPK pathway, which increases production of glycerol in response to high-osmotic conditions (Posas and Saito 1997), may also influence cell fusion.

The HOG pathway contains two major branches, each with its own surface-localized sensors, that feed into the MAPK kinase, Pbs2p, which activates the Hog1p MAPK (O'Rourke *et al.* 2002). Sho1p, an osmosensor for one branch of the pathway, contains four putative transmem-

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brane domains and a COOH-terminal SH3 domain (Maeda *et al.* 1995). SH3 domain peptide recognition modules often occur within signaling molecules and bind to proline-rich peptide ligands on specific target proteins (Tong *et al.* 2002). The COOH-terminal SH3 domain of Sho1p binds the proline-rich ligand Pbs2p (Maeda *et al.* 1995), which facilitates its cell surface localization and activation of the Hog1p MAPK (Raitt *et al.* 2000).

These various signaling pathways may control the activity of the fusion-specific proteins Fus1p and Fus2p, whose expression is pheromone induced (McCaffrey *et al.* 1987; Trueheart *et al.* 1987). Fus1p is a plasma membrane protein with an external NH2-terminal *O*-glycosylated region, a single transmembrane domain, and a larger cytoplasmic region containing a COOH-terminal SH3 domain (Trueheart and Fink 1989). Fus1p localizes to the growing tip of mating projections and the cortical septum region of the prezygote. Fus2p is an intracellular protein that binds Rvs161p, a homolog of mammalian amphiphysin, and, like Fus1p, both Fus2p and Rvs161p localize to the shmoo tip and are required for cell fusion (Elion *et al.* 1995; Brizzio *et al.* 1998).

Electron microscopy has revealed some clues to the roles of fusion proteins; vesicles cluster along the zone of cell fusion in wild-type cells where cell wall thinning occurs (Gammie *et al.* 1998). Fusion mutants appear to fall into two classes: mutants unable to deliver vesicles and mutants blocked at some later stage. *fus1*Δ mutants show a striking absence of vesicles at the zone of cell fusion and appear to fall into the first class of mutants, whereas Rvs161p and Fus2p are not required for vesicle delivery but are blocked with vesicles at the sites of cell fusion (Gammie *et al.* 1998). Because amphiphysin is able to bind lipid bilayers and remodel membranes, the Rvs161p-Fus2p pair may perform a similar role during cell fusion (Takei *et al.* 1999).

Bni1p, Pea2p, and Spa2p are also required for efficient cell fusion (Dorer *et al.* 1997). These proteins form a complex that regulates polarized cell growth in response to signals involving the Cdc42p Rho-type GTPase (Chenevert *et al.* 1994; Amberg *et al.* 1997; Evangelista *et al.* 1997; Sheu *et al.* 1998). Bni1p and its paralog Bnr1p are members of the highly conserved formin family of actin assembly proteins and are required for the assembly of tropomyosin-stabilized actin cables (Evangelista *et al.* 2002; Sagot *et al.* 2002). Actin cables probably act as tracks for type V myosin motors, which direct the trafficking of secretory vesicles. Consistent with the role of these proteins in polarized secretion, *spa2*Δ mutants contain vesicles that fail to cluster in a polarized manner (Gammie *et al.* 1998).

Chs5p plays a role in cell fusion that, by analogy to its role in Chs3p trafficking and localization during budding, may involve a cell-fusion-specific secretory pathway (Dorer *et al.* 1997; Santos *et al.* 1997). Chs3p is an enzyme involved in the assembly of the cell wall at the mother-bud junction and is maintained intracellularly by a Chs5p-dependent cycle of transport between the *trans*-Golgi network and early endosomes (Santos and Snyder 1997; Ziman *et al.* 1998). Thus, Chs5p is dedicated to a specialized secretory pathway during budding, and components of this pathway may be recruited to a fusion-specific pathway required for zygote formation during mating.

Taken together, these studies provide a general model for regulation of prezygote septum degradation during cell fusion. A cell fusion signaling pathway, presumably originating with a stimulus generated within the cell-cell contact region of the prezygote and transmitted or modulated by components of the pheromone response, PKC, and HOG MAPK pathways, activates Fus1p and Fus2p-Rvs161p to control actin-based polarization machinery that directs secretion of specialized vesicles containing a cargo of septum-degrading enzymes. To substantiate this model at the molecular level, we examined the role of a protein-protein interaction between Fus1p and the HOG MAPK pathway osmosensor Sho1p and we generated a network of protein-protein interactions involving Fus1p, a number of other proteins implicated in cell fusion, and the components of the yeast pheromone response MAPK pathway.

MATERIALS AND METHODS

Strain construction: The yeast strains used in this study are all derivatives of W3031A (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*) and W3031B (*MAT*α *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*) except for Y704 (*MAT***a** *LexA-LEU2 ura3::URA3-lexAop-LacZ sst1 his3 trp1 ura3-52 leu2*; Evangelista *et al.* 1997) and Y1356 (*MAT***a** *ura3::URA3-lexAop-LacZ ste12*Δ*::kanR leu2 his3 trp1 ade2 lys2 gal80 GAL4*), which were used in two-hybrid experiments. We constructed W303 derivatives Y2106 (*MAT***a** *ssk1*Δ*::HIS3MX6 far1-f3 sst1::LEU2 fus1*Δ*::URA3*) and Y2108 (*MAT***a** *ssk1*Δ*::HIS3::MX6 far1-f3 sst1::LEU2*) through crosses between Y23 (*MAT*α *far1-f3 sst1::LEU2*), Y427 (*MAT***a** *fus1*Δ*::URA3*), and Y1668 (*MAT***a** *ssk1*Δ*::HIS3MX6*). Y23 (*MAT*α *far1-f3 sst1::LEU2*) was constructed by transforming SY2624 (*MAT*α *far1-f3*) with *Hin*dIII-*Bam*HI-digested pZV77 (*sst1::LEU2*). SY2624 (*MAT*α *far1-f3*) was constructed by transformation of a W3031B-derived strain with *Eco*RI-digested pSL2287 (*far1-f3*); transformants were then streaked onto 5-fluoroorotic acid (5-FOA)-containing medium and screened for pheromone response cell-cycle arrest defect. Y427 (*MAT***a** *fus1*Δ*::URA3*) was constructed by transformation of a W3031A-derived strain with *Eco*RI-*Bgl*II-digested p307(*fus1*Δ*::URA3*). Y1668 (*MAT***a** *ssk1*Δ*::HIS3MX6*) was constructed by replacing the *SSK1* protein-coding sequence with a *HIS3MX6* cassette (Longtine *et al.* 1998). To construct Y579 (*MAT***a** *fus1*Δ*::LEU2*), *Sma*I-digested p1288 [*URA3* to *LEU2* switcher plasmid (Cross 1997)] was transformed into Y427, which was then backcrossed to a W3031B derivative to create Y2816 (*MAT*α *fus1*Δ*::LEU2*). Y1657 (*MAT*α *sho1*Δ*::TRP1MX6*) was constructed by replacing the *SHO1* protein-coding sequence with *TRP1MX6* and the resultant strain was backcrossed to generate Y2653 (*MAT***a** *sho1*Δ*::TRP1MX6*). Y2601 (*MAT***a** *ssk1*Δ*::HIS3MX6 sho1*Δ*::TRP1 MX6*) and Y2602 (*MAT*α *ssk1*Δ*::HIS3MX6 sho1*Δ*::TRP1MX6*) were constructed by crossing Y1657 (*MAT*α *sho1*Δ*::TRP1MX6*)

and Y1668 (*MAT***a** *ssk1*Δ*::HIS3MX6*). Y1690 (*MAT***a** *sho1*Δ*::TRP1 MX6 fus1*Δ*::LEU2*) and Y1691 (*MAT*α *sho1*Δ*::TRP1MX6 fus1*Δ*::URA3*) were constructed by crossing Y448 (*MAT***a** *fus1*Δ*::LEU2*) and Y1657 (*MAT*α *sho1*Δ*::TRP1MX6*). To construct Y1005 (*MAT***a** *chs5*Δ*::TRP1 sst1*Δ), SY2625 (*MAT***a** *sst1*Δ) was transformed with *Xho*I-*Sst*I-digested p220 (*chs5*Δ*::URA3*) to produce Y374 (*MAT***a** *chs5*Δ*::URA3 sst1*Δ), which was then transformed with *Sma*I-digested p1289 [*URA3* to *TRP1* switcher plasmid (Cross 1997)]. Y2843 (*MAT***a** *spa2*Δ*::HIS3 sst1::LEU2*) was created by transformation of *Sal*I-*Hin*dIII-digested p219 (*spa2*Δ*::URA3*) into a W3031A derivative to produce Y485 (*MAT***a** *spa2*Δ*::URA3*), which was then transformed with *Bam*HI-*Hin*dIII-digested pZV77 (*sst1::LEU2*) to create Y586 (*MAT***a** *spa2*Δ*::URA3 sst1::LEU2*), which was transformed with p1287 [*URA3* to *HIS3* switcher plasmid (Cross 1997)]. To create Y334 (*MAT***a** *fus1*Δ *sst1*Δ), SY2625 (*MAT***a** *sst1*Δ) was transformed with *Bgl*II-digested pSL1475 (*fus1*Δ); transformants were then streaked onto FOA medium and screened for a fusion defect. To create Y2813 (*MAT***a** *fus1*Δ *sst1*Δ *spa2*Δ*::LEU2*), Y334 was transformed with *Sal*I-*Hin*dIII-digested p219 (*spa2*Δ*::URA3*) to produce Y2657 (*MAT***a** *fus1*Δ *sst1*Δ *spa2*Δ*::URA3*), which was transformed with *Sma*I-digested p1288 [*URA3* to *LEU2* switcher plasmid (Cross 1997)].

Plasmid construction: The following plasmids were used: p307, which encodes a *fus1*Δ*::URA3* plasmid; pSL1475, which encodes a *fus1*Δ URA3-based yeast integrating plasmid (YIP); pSL1851, which encodes a *ste4*Δ*::URA3* plasmid; pSL2068, which encodes a *far1*Δ*::URA3* plasmid; and pSL2287, which encodes a *far1-f3::URA3* YIP plasmid, provided by George Sprague. pZV77, a *sst1::LEU2* plasmid, was provided by Vivian Mackay. pMA106 (*RAS2-GFP*), a CEN *TRP1* vector, derived from YCplac22, carrying a *RAS2-GFP* fusion gene, was provided by Jennifer Whistler and Jasper Rine. p1287, a *URA3* to *HIS3* switcher plasmid, p1288, a *URA3* to *LEU2* switcher plasmid, and p1289, a *URA3* to *TRP1* switcher plasmid, were provided by Fred Cross (Cross 1997). To create p220 (*chs5*Δ*::URA3*), an *Xho*I-*Bgl*II fragment from p181 (*CHS5* in pRS316, Sikorski and Hieter 1989) was cloned into KS+ (Stratagene, La Jolla, CA) and a *URA3* fragment was inserted into the *Bam*HI sites within *CHS5*; this deleted the *CHS5* sequence encoding amino acids 98–181 and left the downstream *CHS5* sequence out of frame. To create p219 (*spa2*Δ*::URA3*), a *Sal*I-*Hin*dIII fragment from p185 (*spa2*Δ*::URA3* in YCp50, provided by Nicole Valtz) was cloned into KS+ (Stratagene). p2226, carrying *ADH1-BNI1-GFP*, was created in two steps. First, p532 (Evangelista *et al.* 1997), carrying full-length *BNI1* with a *Bam*HI site immediately 5′ to the start ATG, was cut with *Bam*HI-*Not*I and ligated into a pRS316-based vector carrying the *ADH1* promoter to produce p2224. Second, a 500-bp *Nhe*I to *Not*I fragment of p1912 (Evangelista *et al.* 2002), carrying *BNI1-GFP* behind its own promoter, was ligated into p2224 to produce p2226.

Two-hybrid constructs were based on pEG202 (Gyuris *et al.* 1993) and pBTM116 (Hollenberg *et al.* 1995) encoding the LexA-DNA binding domain (DBD), and pJG4-5 (Gyuris *et al.* 1993), pACT (Durfee *et al.* 1993), and pGAD-C (James *et al.* 1996) encoding a transcriptional-activation (AD) domain. DNA fragments of various genes were amplified by the polymerase chain reaction (PCR) with primers that incorporated 5′-*Bam*HI and 3′-*Not*I (unless otherwise stated) restriction sites for insertion into the vectors. DBD plasmids were pEG202 unless otherwise stated: p1002 encodes Fus1p (97–513) and incorporates a 3′-*Xho*I site; p2100 encodes Fus1p (401–513); p1450 encodes full-length Fus2p; p1795 encodes full-length Fus3p; p810 contains a Bni1p (1–1214) *Bam*HI-*Not*I fragment cut from p717 (Evangelista *et al.* 1997); p813 encodes Bni1p (1414–1953); p890 encodes Bni1p (1227–1397); p2086 encodes full-length Pea2p; p1272 encodes full-length Rvs161p in pBTM116; p2276 encodes Chs5p (1–261) and incorporates a 5′-*Eco*RI site and 3′-*Xho*I site; p458 encodes Cdc42p that incorporates a C188S substitution, preventing prenylation, and a G12V mutation, which locks Cdc42p into the GTP-bound state (Stevenson *et al.* 1995); p701 encodes Cdc42p that incorporates a C188S substitution, preventing prenylation, and a D188A mutation locking Cdc42p into the GDP-bound state (Stevenson *et al.* 1995); p3479 encodes Fus1p (401–513) with a P422A mutation, Fus1p(P422A)-SH3, and was created in a two-step PCR procedure (primers available upon request); p3475 encodes Fus1p (401–513) with a W473S mutation, Fus1p-SH3(W473S), and was created in a two-step PCR procedure (primers available upon request).

AD plasmids were pJG4-5 unless otherwise stated: p1111 is the empty vector; p3503 encodes Sho1p (281–368); p1481 encodes full-length Fus2p; p2101 encodes Fus1p (401–513); p717 encodes Bni1p (1–1214); p558 encodes Bni1p (1215–1953); p913 encodes Bni1p (1227–1397); p929 encodes Bni1p (1414–1953); p2155 encodes full-length Rvs161p; p2273 encodes Chs5p (1–261) and incorporates a 5′-*Eco*RI site and a 3′-*Xho*I site; p464 encodes an AD-Cdc42p fusion that incorporates a C188S substitution, preventing prenylation, and a G12V mutation, which locks Cdc42p in a GTP-bound state (Stevenson *et al.* 1995); p461 encodes Rga1p (provided by John Pringle); p993 encodes full-length Far1p (Butty *et al.* 1998); p2106 encodes Bnr1p (1–753) and incorporates a 5′-*Sal*I; p2098 encodes Bnr1p (754–1376) and incorporates a 3′-*Sal*I site; p2099 encodes Bnr1p (856–1376) and incorporates a 5′-*Sal*I; p3503 encodes Sho1p-SH3 (280–368); p3492 encodes Sho1p-SH3 (281–368) with a P352A mutation, Sho1p-SH3 (P352A), and was created in a two-step PCR procedure (primers available upon request). The following pACT-derived AD plasmids (Durfee *et al.* 1993) were all obtained from G. Sprague (Printen and Sprague 1994): p1422 encodes Ste4p, p1423 encodes Ste11p, p1426 encodes Kss1p, p1428 encodes Ste7p, p1432 encodes Ste5p, p1435 encodes Fus3p, p1438 encodes Ste20p, and p1487 encodes Ste12p. p1517 encodes Gpa1p(R297A) in pGAD3F, and the R297A mutation should lock Gpa1p in a GTP-bound state. p628 encodes Fus1p (97–513) in pACT-derived plasmids (Amberg *et al.* 1997). p2073 encodes Spa2p (1–1466), p2074 encodes Spa2p (743–1466), and p2075 encodes Spa2p (512–1118), all in pACT-derived plasmids obtained from M. Snyder (Sheu *et al.* 1998). p500 encodes an AD-Cdc24p fusion in a pACT-derived plasmid (a gift from Alan Bender); p831 encodes Bud6p (274–789) in a pACT-derived plasmid (Evangelista *et al.* 1997); p1124 encodes Act1p (Evangelista *et al.* 1997); and p1586 encodes full-length Pea2p in pGAD-C.

To create p3998 encoding GST-Sho1p (281–368) with a P352A mutation, the insert from p3492 was ligated in frame with the GST sequences of pGEX-3X (Pharmacia). To create p3999 encoding Sho1p (281–368), the insert from p3503 was ligated in frame with the GST sequences of pGEX-3X (Pharmacia). p4040, encoding MBP-Fus1p (96–513), was created by ligation of a *FUS1* PCR fragment in frame with the maltose binding protein (MBP) sequences of pMAL-c2 (New England Biolabs, Beverly, MA).

p4263 is a pRS316 (Cen *URA3*)-based plasmid (Sikorski and Hieter 1989) carrying a gene encoding Fus1p(P422A), driven by 800 bp of the *FUS1* promoter, and was created in a two-step PCR procedure (primers available upon request). p4597 is a pRS314 (Cen *TRP1*)-based plasmid (Sikorski and Hieter 1989) encoding Fus1p-SH3(W473S), driven by 800 bp of the *FUS1* promoter, and was created in a two-step PCR procedure (primers available upon request). p4598 is a pRS314 (Cen *TRP1*)-based plasmid encoding Fus1p (P422A)-SH3(W473S), driven by 800 bp of the *FUS1* promoter, and was created in a two-step PCR procedure. Similarly, we created a set of pRS316 (Cen *URA3*) plasmids encoding full-length

TABLE 1

Two-hybrid interactions between Fus1p and Sho1p and Bni1p

	Fus1p-SH3(401–513)	Fus1p-SH3(W473S)	Fus1p(P422A)-SH3
Vector	0.1 ± 0.01	0.01 ± 0.001	0.05 ± 0.06
Sho1p-SH3	302 ± 32	114 ± 33	0.07 ± 0.01
Sho1p-SH3(P352A)	0.02 ± 0.01	0.03 ± 0.002	0.1 ± 0.2
Bni1p	122 ± 13	3 ± 0.4	119 ± 10

Assays were done as described (see MATERIALS AND METHODS); the plasmids used were p2100[Fus1p-SH3(401–513)], p3475[Fus1p-SH3(W473S)], p3479[Fus1p(P422A)-SH3], p3505(Sho1p-SH3), p3492[Sho1p-SH3(P352A)], and p717[Bni1p (1–1214)]; pJG4-5 was the vector control. Two-hybrid reporter *lexAop-lacZ* expression was measured as β-galactosidase activity (in Miller units), and a mean and standard deviation were calculated from three independent samples.

FUS1-GFP gene fusion derivatives, under control of the *FUS1* promoter: p1491 encodes Fus1p-GFP; p4269 encodes Fus1p (P422A)-GFP; p4580 encodes Fus1p-SH3(W473S)-GFP; and p4667 encodes Fus1p(P422A)-SH3(W473S)-GFP.

GST-binding experiments: BL21 cells expressing MBP-Fus1p (96–513; p4040) were lysed and mixed (45 min) with GST fusion proteins, GST-Sho1p (281–368; p3999) or GST-Sho1p (P352A) (281–368; p3998), derived from BL21 cells and purified on glutathione-Sepharose beads. The proteins associated with the glutathione-Sepharose beads were processed for Western immunoblot analysis using monoclonal MBP antibody (New England Biolabs) and monoclonal GST antibody (Santa Cruz), as described previously (EVANGELISTA *et al.* 1997).

Fusion assays: *MAT***a** strains were transformed with pMA106 (Cen *TRP1*) or p2664 (Cen *LEU2*) carrying a gene fusion encoding Ras2p-GFP. Strains were grown in synthetic medium lacking the appropriate amino acids for plasmid selection to mid-logarithmic phase. A total of 100 μl of cells of each mating type were added to 500 μl of synthetic medium and then concentrated on 0.45-μm filters that were placed on solid synthetic medium containing all amino acids. Cells were allowed to mate for the designated time until wild type (wt) × wt controls reached 70–80% fusion (~2.5 hr). Prezygotes were considered fused upon entry of Ras2p-GFP in the *MAT*α cell as monitored under fluorescence microscopy.

Two-hybrid analysis: For the yeast two-hybrid experiments (PHIZICKY and FIELDS 1995), two-hybrid strains (Y704 and Y1356) were cotransformed with DBD and AD plasmids, and cells were grown in liquid culture to mid-log phase and then assayed for expression of *lexAop-lacZ* as described (HAGEN *et al.* 1991), with a mean and standard deviation calculated from three independent samples. To preclude complications associated with activation of the yeast pheromone response pathway, Y1356, which carries a *ste12*Δ mutation, was used for interactions involving Gpa1p, Ste4p, Ste5p, Ste7p, Ste11p, Ste20p, Ste12p, Kss1p, and Fus3p. A table of the results of *lexAop-lacZ* expression assays in Figure 6 is available on request.

RESULTS

Sho1p-SH3 domain binds a peptide ligand in Fus1p: In a previous study, we identified the SH3 domain of the HOG MAPK pathway osmosensor Sho1p as a binding partner for the cytoplasmic COOH-tail of the cell fusion protein Fus1p in a two-hybrid screen (TONG *et al.* 2002). By phage display, we found that Sho1p-SH3 binds a K/RxLPxxP consensus ligand (TONG *et al.* 2002). A peptide matching this consensus (KPLPPLP) occurs within Pbs2p MAPK kinase (MAEDA *et al.* 1995) and binds Sho1p-SH3, thereby localizing the Pbs2p MAPK kinase to the cell surface and enabling Hog1p MAPK activation (RAITT *et al.* 2000). A global scan for Sho1p-SH3 consensus ligands (TONG *et al.* 2002) identified another putative binding site (KPLPLTP) within the COOH-terminal region of Fus1p, just NH2-terminal to the Fus1p-SH3 domain. To test if the two-hybrid interaction between Fus1p and Sho1p requires this sequence, we generated a mutation that leads to a single amino acid substitution of one of the proline residues, Fus1p(P422A), which should be critical for SH3 domain binding (altering KPLPLTP to KPLALTP). We also generated amino acid substitutions of conserved residues of the Fus1p-SH3 domain [Fus1p-SH3(W473S)] and the Sho1p-SH3 domain [Sho1p-SH3(P352A)]. In two-hybrid assays, a Fus1p-SH3 (401–513) fragment, containing the putative Sho1p-SH3 ligand and the Fus1p-SH3 domain, was competent for interaction with Sho1p-SH3 (Table 1). The Fus1p-SH3(W473S) version of this fragment interacted with Sho1p-SH3, whereas the Fus1p-(P422A)-SH3 version did not. Sho1p-SH3(P352A) failed to interact with any of the Fus1p fragments (Table 1). These results suggest that binding between Fus1p and Sho1p is mediated through the Sho1p-SH3 domain and its consensus ligand (KPLPLTP) in Fus1p. This binding is likely direct, because Fus1 tagged with the MBP in bacterial extracts, Fus1p(96–513)-MBP, bound to Sho1p-SH3-GST, but not Sho1p-SH3(P352A)-GST (Figure 1). Because both Sho1p and Fus1p localize to the tip of the mating projection, this interaction may occur *in vivo* during pheromone-induced polarized morphogenesis (RAITT *et al.* 2000), suggesting that Fus1p may compete with Pbs2p for Sho1p binding and thereby prevent Pbs2p activation.

Fus1p sequesters Sho1p during pheromone response: The HOG pathway contains two distinct arms, both of which activate the MAPK kinase, Pbs2p, in response to hyperosmotic shock. Removal of either arm of the pathway still allows cells to respond to high-osmotic conditions; removal of both arms renders cells defective

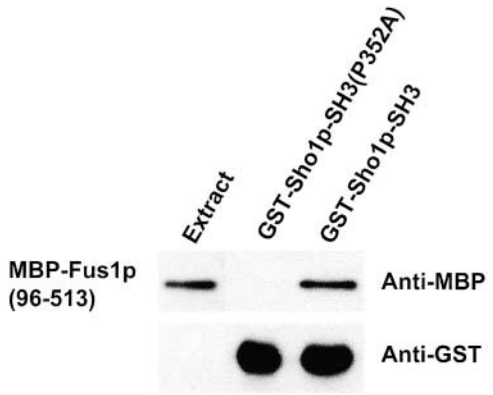

FIGURE 1.—Direct association of Fus1p with Sho1p. GST-Sho1p or GST-Sho1p(P352A) was purified from *Escherichia coli* extracts, bound to glutathione-Sepharose beads, and then mixed with *E. coli* extracts containing MBP-Fus1p (96–513). Bound proteins were detected by immunoblot analysis with antibodies to MBP (top) or GST (bottom).

for growth in high osmolarity. To test whether Sho1p bound Fus1p *in vivo* during pheromone response, we first constructed a strain missing *SSK1* to force all high-osmotic response to initiate from the Sho1p branch of the HOG pathway and, containing a *far1-f3* mutation (PETER *et al.* 1993), to render the cells defective for pheromone-induced G_1 arrest. When *ssk1*Δ *far1-f3* cells are transferred to a medium of high osmolarity, Sho1p-Pbs2p interaction is required for cells to respond to the high osmolarity and grow, but if Fus1p sequesters Sho1p away from Pbs2p, Pbs2p activation will be prevented, resulting in a halo of growth inhibition caused by a defect in Hog pathway signaling (Figure 2). Indeed, within a pheromone diffusion halo, *MAT***a** *ssk1*Δ *far1-f3* failed to grow on high-osmolarity medium. In contrast, cells outside the halo grew normally due to a lack of pheromone-induced Fus1p expression (Figure 3). This pheromone-induced growth defect was dependent on Fus1p expression, because *MAT***a** *ssk1*Δ *far1-f3 fus1*Δ cells continued to divide, adhere to one another, and invade into the agar in the presence of pheromone in a process known as pheromone-induced invasion (ROBERTS *et al.* 2000). These results suggest that Fus1p sequesters Sho1p away from Pbs2p in pheromone-responding cells.

The Sho1p-Fus1p interaction is required for efficient cell fusion and the Fus1p-SH3 domain contributes to cell fusion: To determine if disruption of the Sho1p-Fus1p interaction affects cell fusion, we introduced a plasmid that encodes a full-length consensus ligand mutant Fus1p(P422A) into a *fus1*Δ deletion mutant strain and scored cell fusion efficiency in mating assays. Relative to a *FUS1* wt × wt control, cells expressing Fus1p (P422A) were compromised for cell fusion in both unilateral (wt × mutant) and bilateral (mutant × mutant) mating assays (77.1 ± 1.8% of mating pairs were fused in the control and 51.6 ± 10.1% and 26.3 ± 6.1% in unilateral and bilateral fusion assays, respectively; Figure 4A). This fusion defect was not caused by protein instability or mislocalization because Fus1p(P422A)-GFP was expressed at normal levels and concentrated in the growing shmoo tip (Figure 5), as observed for a Fus1p-GFP fusion that appears to be fully functional in mating assays (data not shown). These findings indicate that the Sho1p-Fus1p complex promotes cell fusion.

The reduced fusion efficiency associated with Fus1p (P422A) cells was less than the reduction seen for cells lacking Fus1p altogether (*fus1*Δ; Figure 4A), suggesting that other domains of Fus1p are functionally important. To test if the Fus1p-SH3 domain contributes to efficient cell fusion, a construct encoding full-length Fus1p-SH3(W473S) was introduced into *fus1*Δ cells and fusion efficiency was monitored in mating assays. Fus1p-SH3(W473S) was also expressed at normal levels and localized to the growing shmoo tip (Figure 5). Fus1p-SH3(W473S) cells were compromised for cell fusion in both unilateral and bilateral mating assays (72.4 ± 3.2% were fused in the control and 54.3 ± 3.1% and 35.0 ± 1.6% in unilateral and bilateral assays, respectively; Figure 4A). Thus, the Fus1p-SH3 domain also mediates the formation of a complex important for cell fusion.

A version of Fus1p containing both the P422A and the W473S mutations, Fus1p(P422A)-SH3(W473S), was expressed and localized normally (Figure 5), but was associated with a more pronounced fusion defect than was either single mutant alone (72.4 ± 3.2% were fused

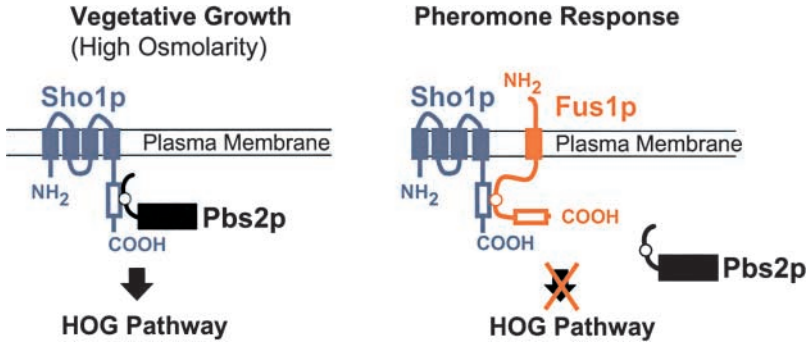

FIGURE 2.—A model for Fus1p regulation of Sho1p. In the presence of pheromone, Fus1p is expressed and binds Sho1p, thereby sequestering Sho1p away from Pbs2p and rendering cells unable to respond to high osmolarity through the Sho1p arm of the HOG pathway.

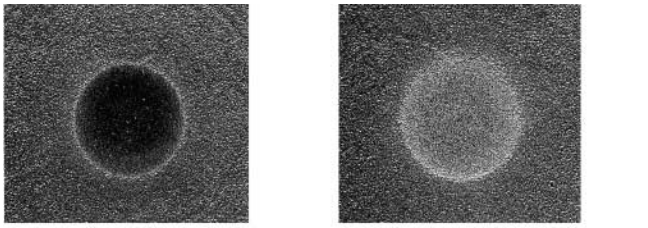

FIGURE 3.—Fus1p sequesters Sho1p away from Pbs2p. Survival of *ssk1*Δ strains exposed to pheromone and high osmolarity is Fus1p dependent; lawns of the noted strains were plated onto rich media containing 1 M sorbitol and 1 μl of 1 mM α-factor was spotted on the surface of the plate. Strains lacking Fus1p were able to grow in the presence of pheromone and 1 M sorbitol. Strains used were *ssk1*Δ *far1-f3 sst1* (Y2108) and *ssk1*Δ *far1-f3 sst1 fus1*Δ (Y2106).

in the control and 26.8 ± 1.0% and 15.6 ± 1.1% in unilateral and bilateral assays, respectively; Figure 4A). Because this fusion defect was not as severe as that associated with the *fus1*Δ deletion mutant (81.5 ± 3.3% were fused in the control and 27.9 ± 7.2% and 0.7 ± 0.6% in unilateral and bilateral assays, respectively), additional Fus1p domains may contribute to its function.

An inhibitory role for Sho1p in cell fusion: If Sho1p is required to promote cell fusion, then *sho1*Δ cells should display a fusion defect. However, we found that *sho1*Δ cells fused normally, if not slightly more efficiently than wild-type cells in bilateral assays (74.5 ± 4.8% were fused in the control and 73.3 ± 6.5% and 83.3 ± 8.3% in unilateral and bilateral mating assays, respectively; Figure 4B). We conclude that Sho1p is not required for cell fusion.

To assay components of the other arm of the HOG pathway for a role in cell fusion, we examined a *sho1*Δ *ssk1*Δ double mutant in mating assays. The *sho1*Δ*ssk1*Δ double mutant also appeared to fuse slightly more efficiently than wild-type cells (71.4 ± 0.6% were fused in the control and 87.4 ± 0.8% and 82.5 ± 1.2% in unilateral and bilateral mating assays, respectively; Figure 4B). Thus, disruption of HOG pathway signaling at the level of its cell-surface sensors may accentuate the efficiency of cell fusion.

If Sho1p negatively regulates cell fusion and Fus1p normally binds and inhibits Sho1p, then a *sho1*Δ mutation should at least partially alleviate the fusion defect in *fus1*Δ cells. Indeed, *sho1*Δ suppressed the fusion defect of *fus1*Δ cells (fusion efficiency was 27.9 ± 7.2% for *fus1*Δ cells and 50.0 ± 4.6% for *fus1*Δ*sho1*Δ cells in unilateral assays; 0.7 ± 0.6% for *fus1*Δ cells and 8.8 ± 1.6% for *fus1*Δ*sho1*Δ cells in bilateral assays; Figure 4B). Furthermore, the fusion defect of Fus1p(P422A) was completely suppressed in *sho1*Δ cells, thereby demonstrating the dependence of Sho1p binding during fusion (data not shown). Thus, Fus1p sequestration repre-

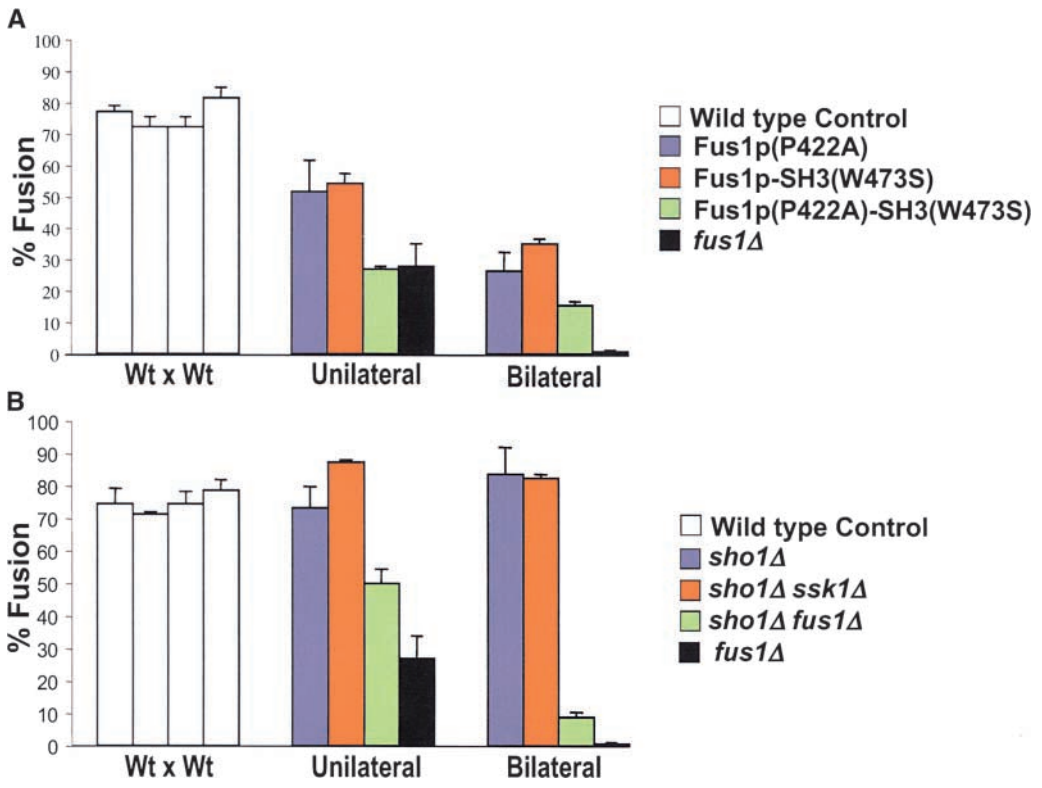

FIGURE 4.—Fusion assays on various strains. (A) Fusion efficiency was measured in *fus1*Δ strains carrying a vector control or a plasmid encoding various versions of Fus1p, Fus1p (P422A), Fus1p-SH3 (W473S), or Fus1p(P422A)-SH3(W473S). For each experiment, a wt × wt control assay, a unilateral or mutant × wt assay, and a bilateral or mutant × mutant fusion assay was performed simultaneously. The wt × wt control processed with Fus1p(P422A) unilateral and bilateral fusion assays is the leftmost bar, the wt × wt control processed with the Fus1p-SH3(W473S) assays is the second bar from the left, the wt × wt control processed with Fus1p(P422A)-SH3(W473S) assays is the third bar from the left, and the wt × wt control processed with *fus1*Δ assays is the rightmost bar. The strains used were wild type (W3031A, W3031B) and *fus1*Δ (Y579, Y2816). (B) Effect of Sho1p on fusion efficiency. The strains used in the fusion assays were wild type (W3031A, W3031B), *fus1*Δ (Y579, Y2816), *sho1*Δ (Y2653, Y1657), *sho1*Δ *ssk1*Δ (Y2601, Y2602), and *sho1*Δ *fus1*Δ (Y1690, Y1691).

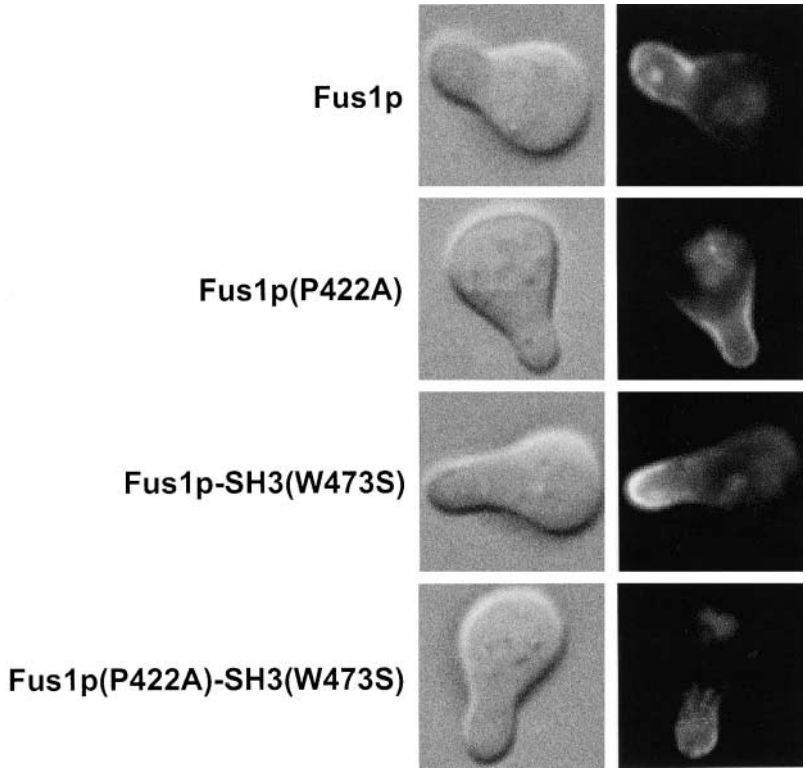

FIGURE 5.—Localization of Fus1p-GFP. SY2625 cells carrying p1491 (Fus1p-GFP), p4269 [Fus1p (P422A)-GFP], p4580 [Fus1p-SH3(W473S)-GFP], or p4667 [Fus1p(P422A)-SH3W473S)-GFP] were exposed to 500 nM α-factor for 2 hr and visualized by Nomarski (left) or fluorescent (right) microscopy.

sents a negative regulation of Sho1p, which appears to inhibit cell fusion. The finding that *sho1*Δ did not fully suppress the *fus1*Δ fusion defect suggests that Fus1p has an additional role(s) during fusion, perhaps one involving a Fus1p-SH3-mediated complex.

Two-hybrid matrix reveals a network of fusion protein interactions: To identify additional Fus1p-binding partners, we constructed a matrix of pairwise two-hybrid tests (UETZ *et al.* 2000) in which the fusion proteins were tested for interactions with components of the pheromone response pathway, polarity proteins, and each other. The positive interactions, most of which have not been observed previously, were quantified on the basis of expression of a *lacZ* two-hybrid reporter and represented as fold induction with respect to a control lacking the activation domain fusion (Figure 6A). Visualization of these interactions as a network (Figure 6B) revealed that Fus1p showed an interaction with Fus2p and that both Fus1p and Fus2p showed interactions with Fus3p, Bni1p, Pea2p, and the activated GTP-bound form of Cdc42p. Because all of these proteins localize to the growing shmoo tip, Fus1p and Fus2p may function as scaffolds for assembly of signaling and polarity proteins that control cell fusion. Two-hybrid tests comparing interactions with Fus1p-SH3 with Fus1p-SH3(W473S) indicated that interactions with Bni1p were SH3 dependent (Table 1). Phage display analysis identified a Fus1p-SH3 consensus ligand (RxxRs/ts/tSl; TONG *et al.* 2002), but none of these potential Fus1p-SH3 targets contained a perfect match to this consensus ligand; therefore, either the SH3-dependent interactions are indirect or a different ligand mediates the protein-protein interactions *in vivo*. Nevertheless, these findings suggest that the Fus1p-SH3 domain may mediate a complex with Bni1p to control actin cable assembly and regulate polarized secretion during cell fusion.

CHS5 controls Fus1p localization: Chs5p is required for cell fusion in addition to the specialized secretion of Chs3p vesicles during bud development (DORER *et al.* 1997; SANTOS *et al.* 1997; SANTOS and SNYDER 1997). Since Chs5p showed a two-hybrid interaction with Fus1p (Figure 6A), we tested Chs5p for a role in targeting Fus1p-GFP to the tip of shmooing cells. In wild-type cells, Fus1p-GFP, which functions normally in cell fusion assays (data not shown), was found concentrated in shmoo tips in 99.7 ± 0.4% of cells (Figure 7). In *chs5*Δ cells, the Fus1p-GFP signal appeared fainter and concentrated at shmoo tips in only 44.7 ± 3.6% of the cells examined. Western blot analysis revealed that the same amount of Fus1p-GFP protein was made in both wt and *chs5*Δ cells (data not shown). We infer that the fainter Fus1p-GFP signal in *chs5*Δ cells may reflect the lack of a concentrated localization of this protein in *chs5*Δ cells.

Evidence for the formation of a functional Fus1p-Bni1p complex: Bni1p localizes to the sites of surface growth during budding and mating (EVANGELISTA *et al.* 1997). In budding cells, a Spa2p-Bni1p interaction appears to contribute to Bni1p localization, because a substantial fraction of *spa2*Δ deletion mutant cells mislocalize Bni1p (FUJIWARA *et al.* 1998). In shmooing

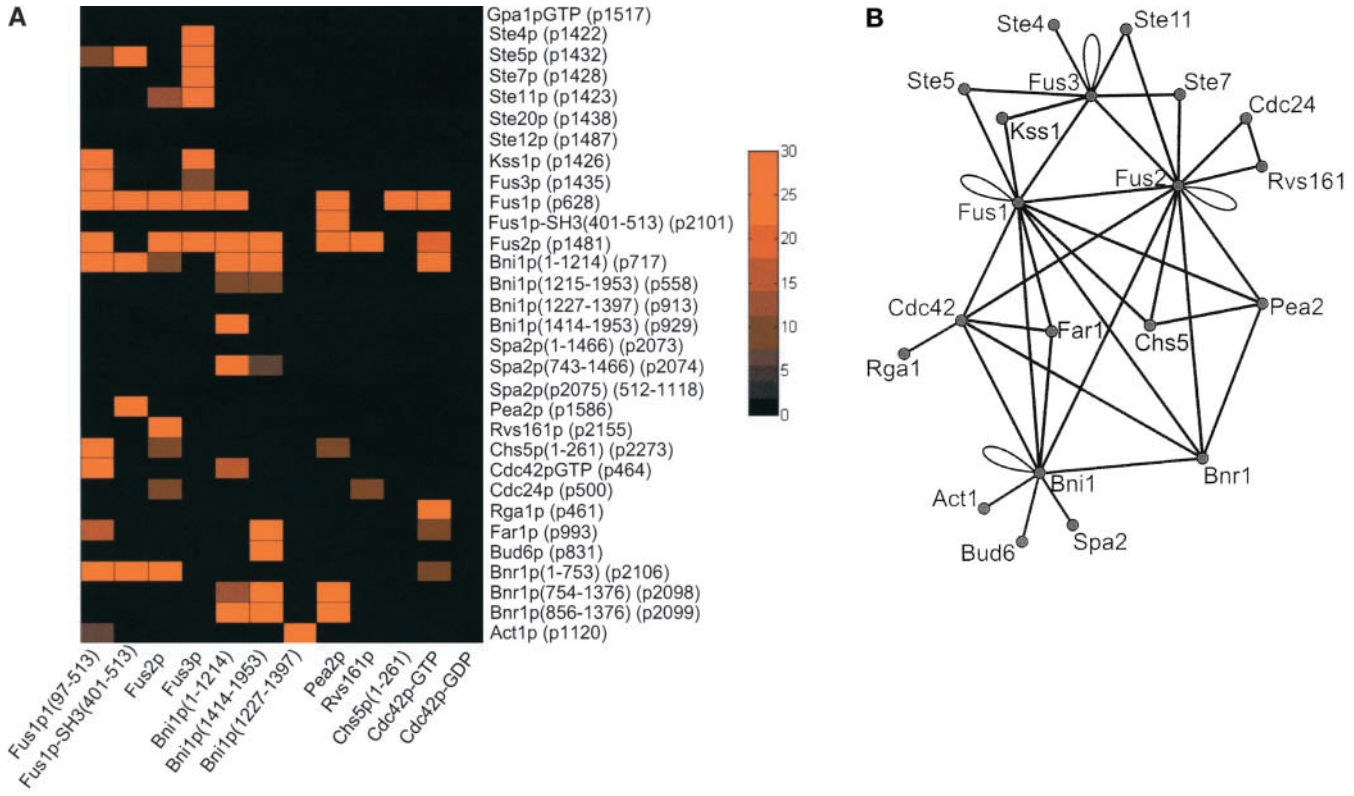

FIGURE 6.—Two-hybrid interaction network. (A) DBD fusions are listed along the horizontal axis and AD fusions are listed along the vertical axis. Two-hybrid reporter *lexAop-lacZ* expression was measured as β-galactosidase activity (Miller units) with a mean and standard deviation calculated from three independent samples. Two-hybrid reporter expression levels are presented as fold-induced above that observed for control cells, which expressed the DBD fusion only, and are represented by a color scale, with stronger interactions denoted by brighter colors. Only those interactions that were at least fivefold above the control are shown. (B) Schematic representation of the two-hybrid interactions as network. Loops indicate a positive two-hybrid interaction when a protein interacted with itself.

cells, however, an additional protein-protein interaction may contribute to the localization of Bni1p because almost all *spa2*Δ cells scored (97.1 ± 1.4%) showed normal localization of Bni1p-GFP, which is comparable to that observed for wt cells (99.4 ± 0.8%; Figure 8, A and B). Since we observed a Fus1p-SH3-dependent two-hybrid interaction between Fus1p and Bni1p, we tested whether pheromone-induced Fus1p contributed to Bni1p localization. We examined Bni1p-GFP localization in *fus1*Δ cells and *fus1*Δ *spa2*Δ double-mutant cells and found that Bni1p-GFP localized normally to the growing tip of most *fus1*Δ shmoos scored (96.9 ± 0.5%). However, Bni1p-GFP localized correctly only in a subset of the *fus1*Δ *spa2*Δ cells scored (53.5 ± 4.7%; Figure 8, A and B). Thus, both Fus1p and Spa2p appear to contribute to Bni1p localization during pheromone response.

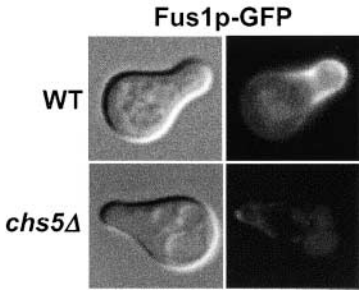

FIGURE 7.—Chs5p is required for Fus1p localization. SY2625 or Y1005 (*chs5*Δ) cells carrying p1491 (Fus1p-GFP) were exposed to 500 nM α-factor for 2 hr before visualization by Nomarski (left) or fluorescent (right) microscopy.

DISCUSSION

Our results suggest that Fus1p binds a number of different signaling, fusion, and polarity proteins and may act as a scaffold protein to coordinate multiple aspects of the cell fusion process. We found that a peptide in the Fus1p cytoplasmic domain binds to the SH3 domain of Sho1p, an osmosensor for the HOG MAPK pathway (MAEDA *et al.* 1995), which controls glycerol production and the osmotic state of the cell. Previous work established that the osmotic balance can regulate cell fusion (PHILIPS and HERSKOWITZ 1997); in particular, differential osmolarity between prezygotic partners can inhibit cell fusion. Our results suggest a molecular

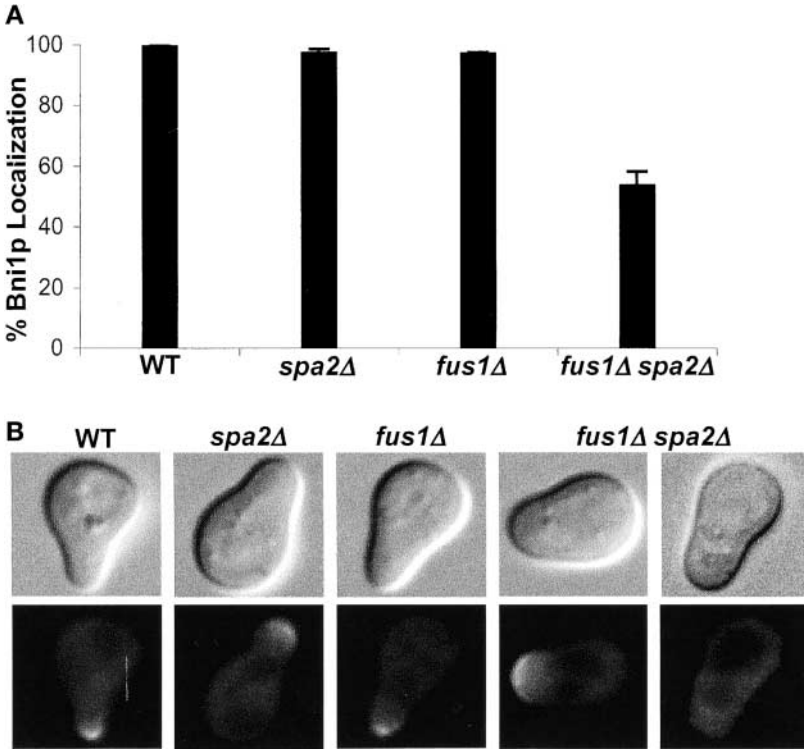

FIGURE 8.—Fus1p and Spa2p collaborate to control Bni1p localization in shmoos. (A) Quantification of Bni1p localization. Cells carrying p2226 (Bni1p-GFP) were exposed to 500 nM α-factor for 2 hr before visualization. (B) Nomarski (top) of cells and fluorescent (bottom) microscopy of Bni1p-GFP in wild-type (SY2625), *spa2*Δ (Y2843), *fus1*Δ (Y334), and *spa2*Δ *fus1*Δ (Y2813) cells. For the *fus1*Δ *spa2*Δ cells, an example of a cell that localized (left) Bni1p-GFP and one that mislocalized (right) Bni1p-GFP is shown.

model for negative regulation of cell fusion by osmosensor signaling. Specifically, Sho1p appears to block cell fusion in the absence of Fus1p, and pheromone-induced expression of Fus1p prevents Sho1p from signaling HOG MAPK-dependent growth on high-osmolarity medium. Thus, Fus1p appears to compete with the HOG MAPKK Pbs2p for binding to Sho1p (Figure 2). Downregulation of Sho1p signaling by Fus1p may enable cells to lower their internal osmolarity, which would reduce the chance of cell lysis and death during cell fusion. The HOG pathway contains at least two other osmosensors in addition to Sho1p: Sln1p, which controls the Ssk1p branch (O'ROURKE *et al.* 2002), and Msb2p, which appears to be partially redundant with Sho1p (O'ROURKE and HERSKOWITZ 2002). Therefore, additional modes of pheromone-induced negative regulation of HOG pathway signaling may also occur. An analysis of Hog1p activation or intracellular glycerol levels during pheromone response and during zygote formation may further substantiate our model.

The PKC pathway is activated during projection formation and likely remains so until cell contact has been achieved (ZARZOV *et al.* 1996; BUEHRER and ERREDE 1997; ROBERTS *et al.* 2000). Activated alleles of *PKC1* inhibit cell fusion, suggesting that the PKC pathway must be downregulated before cell fusion can proceed (PHILIPS and HERSKOWITZ 1997). Because a two-hybrid screen with a Fus1p bait identified interactions with both Sho1p and Pkc1p (TONG *et al.* 2002), there is the potential for coordinate regulation of multiple MAP kinase pathways through Fus1p complexes.

A two-hybrid matrix analysis of cell fusion and signaling proteins showed that Fus1p interacts with several fusion and polarity proteins. In particular, we identified a Fus1p-Chs5p interaction and found that Chs5p controlled Fus1p localization to the growing mating projection, suggesting that Fus1p may be localized by a specialized secretory pathway in a manner similar to Chs3p localization. Recently, Santos and Snyder showed that the role of Chs5p in cell fusion is specific to Fus1p, as Chs5p is not required for localization of other fusion proteins, such as Fus2p and Spa2p (SANTOS and SNYDER 2003). Our two-hybrid matrix revealed that the Fus1p-SH3 domain binds to the NH2-terminal regulatory domain of Bni1p, a formin protein that controls nucleation of actin cables, which determine polarized secretion and morphogenesis (EVANGELISTA *et al.* 2002; SAGOT *et al.* 2002). The regulatory domain of formins is known to be involved in their localization and negative regulation of their actin nucleation activity (EVANGELISTA *et al.* 2003). The Fus1p-Bni1p interaction is important for the localization of Bni1p, as Fus1p appears to collaborate with Spa2p to concentrate Bni1p at the cortical shmoo tip. Fus1p also interacts with the pheromone response MAPK Fus3p, which is required for cell fusion and thought to regulate the process (ELION *et al.* 1990; FUJIMURA 1990), suggesting that Fus1p may be regulated by Fus3p phosphorylation. The activated GTP-bound form of Cdc42p interacted with Fus1p and subsequent testing of *cdc42-6*, an allele defective in exocytosis (ADAMO *et al.* 2001), showed a requirement for Cdc42p during cell fusion (data not shown). Finally, Fus1p

showed that a two-hybrid interacted with Fus2p, another cortically localized protein required for cell fusion (Elion *et al.* 1995; Brizzio *et al.* 1998), and Fus2p showed many of the same interactions as Fus1p, including Fus2p-Bni1p, Fus2p-Fus3p, and Fus2p-Cdc42-GTP. Taken together, the results of our two-hybrid matrix analysis support a model in which Fus1p and Fus2p collaborate to assemble a cortical fusion complex in which Cdc42p regulates actin-based polarization machinery, perhaps to direct secretion of specialized vesicles containing a cargo of septum-degrading enzymes.

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