Actin Recovery and Bud Emergence in Osmotically Stressed Cells Requires the Conserved Actin Interacting Mitogen-activated Protein Kinase Kinase Kinase Ssk2p/MTK1 and the Scaffold Protein Spa2p

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Osmotic stress causes actin cytoskeleton disassembly, a cell cycle arrest, and activation of the high osmolarity growth mitogen-activated protein kinase pathway. A previous study showed that Ssk2p, a mitogen-activated protein kinase kinase kinase of the high osmolarity growth pathway, promotes actin cytoskeleton recovery to the neck of late cell cycle, osmotically stressed yeast cells. Data presented herein examined the role of Ssk2p in actin recovery early in the cell cycle. We found that actin recovery at all stages of the cell cycle is not controlled by Ssk1p, the known activator of Ssk2p, but required a polarized distribution of Ssk2p as well as its actin-interacting and kinase activity. Stress-induced localization of Ssk2p to the neck required the septin Shs1p, whereas localization to the bud cortex depended on the polarity scaffold protein Spa2p. *spa2Δ* cells, like *ssk2Δ* cells, were defective for actin recovery from osmotic stress. These *spa2Δ* defects could be suppressed by overexpression of catalytically active Ssk2p. Furthermore, Spa2p could be precipitated by GST-Ssk2p from extracts of osmotically stressed cells. The Ssk2p mediated actin recovery pathway seems to be conserved; MTK1, a human mitogen-activated protein kinase kinase kinase of the p38 stress response pathway and Ssk2p homolog, was also able to localize at polarized growth sites, form a complex with actin and Spa2p, and complement actin recovery defects in osmotically stressed *ssk2Δ* and *spa2Δ* yeast cells. We hypothesize that osmotic stress-induced actin disassembly leads to the formation of an Ssk2p–actin complex and the polarized localization of Ssk2p. Polarized Ssk2p associates with the scaffold protein Spa2p in the bud and Shs1p in the neck, allowing Ssk2p to regulate substrates involved in polarized actin assembly.

INTRODUCTION

Growth and survival of eukaryotic cells requires they be able to rapidly sense and adapt to changing environmental conditions. This is typically achieved through the action of sensor proteins found in the plasma membrane that are coupled to mitogen-activated protein (MAP) kinase, signaling pathways. In the case of osmolarity, a conserved MAP kinase cascade (the high osmolarity growth [HOG] pathway in *Saccharomyces cerevisiae* and the c-Jun NH₂-terminal kinase/p38 pathway in human cells) functions to sense increases in extracellular osmolarity and to initiate an adaptive response. The yeast HOG pathway has been an effective

model for studying how eukaryotic cells sense and respond to osmotic stress.

Three plasma membrane proteins Sho1p, Msb2 and Sln1 have been shown to be involved in activation of the HOG pathway (Maeda *et al.*, 1995; Posas and Saito, 1997; O'Rourke and Herskowitz, 2002) (Figure 1). Signal transmission through the Sho1p branch of the HOG pathway requires the yeast PAK homolog Ste20p in complex with the small GTPase Cdc42p leading to phosphorylation of the MAPKKK Ste11p, which in turn phosphorylates the MAPKK Pbs2p (Raitt *et al.*, 2000; Reiser *et al.*, 2000). Msb2p, a recently discovered plasma membrane protein, is partially redundant with Sho1p for activation of the HOG pathway (O'Rourke and Herskowitz, 2002). Signal transmission through the Sln1p branch of the HOG pathway involves inhibition of Sln1p histidine kinase activity and a resulting accumulation of unphosphorylated Ssk1p (Maeda *et al.*, 1994; Posas *et al.*, 1996). Unphosphorylated Ssk1p binds to the N terminus of Ssk2p and activates Ssk2p to autophospho-

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Figure 1. The *S. cerevisiae* HOG MAP kinase pathway.

rylate on Thr¹⁴⁶⁰ (Posas and Saito, 1998). Activated Ssk2p, and its close homolog Ssk22p, phosphorylate Pbs2p (Maeda *et al.*, 1995; Posas and Saito, 1998), which in turn phosphorylates and activates the MAPK Hog1p (Brewster *et al.*, 1993; Maeda *et al.*, 1995; Posas and Saito, 1997). Phosphorylated Hog1p accumulates in the nucleus and alters gene expression most notably increasing the expression of genes involved in glycerol synthesis (Gustin *et al.*, 1998; Hohmann, 2002). A human homologue of Ssk2p and Ssk22p, MTK1, was initially cloned by functional complementation of the osmosensitivity of a yeast *ssk2Δ ssk22Δ sho1Δ* strain and has been shown to mediate activation the JNK and the p38 stress response pathways (Takekawa *et al.*, 1997). Activation of MTK1 in mammalian cells is controlled by GADD45-like proteins that are believed to regulate MTK1 kinase activity by disrupting autoinhibition of the C-terminal kinase domain, by the N-terminal regulatory domain (Takekawa, 1997; Mita *et al.*, 2002; Takekawa *et al.*, 2002). Recently, a *Drosophila* homologue of MTK1, D-MEKK1, has been identified, and its importance for viability of *Drosophila* embryos under high osmotic condition has been reported previously (Inoue *et al.*, 2001).

It has long been known that the actin cytoskeleton of yeast is rapidly induced to disassemble upon application of osmotic stress and that the cell will not return to the cell cycle until osmotic balance is restored and a polarized actin cytoskeleton is reassembled (Chowdhury *et al.*, 1992). Actin polarization in yeast is controlled by small GTPases, such as Cdc42 that recruit and activate proteins involved in actin assembly (Pruyne and Bretscher, 2000). A key effector of

these activated GTPases is the formin Bni1p, which in concert with Aip3p/Bud6p drives polarized actin cable formation (Evangelista *et al.*, 2002; Sagot *et al.*, 2002b). The scaffold protein Spa2p displays two-hybrid interactions with both Bni1p (Fujiwara *et al.*, 1998) and Aip3p/Bud6p and is believed to be in complex with these proteins (Fujiwara *et al.*, 1998; Sheu *et al.*, 1998). Polarized actin cables serve as the main conduit for polarized secretion and polarized cell growth (Pruyne and Bretscher, 2000).

Although, the response of the HOG MAP kinase pathway is very well understood, little is known about the mechanisms underlying actin regulation during osmotic stress. Given its very prominent role in adaptation to osmotic stress, the HOG pathway would be expected to be involved. This was recently confirmed by a previous report from our laboratory (Yuzyuk *et al.*, 2002). We found that the MEK kinase Ssk2p of the HOG pathway, at the end of the cell cycle, facilitates efficient reassembly of the actin cytoskeleton at the neck of osmotically stressed cells. Actin recovery required localization of active Ssk2p kinase to the neck region in a septin-dependent manner. The septins Cdc3p, Cdc10p, Cdc12p, Cdc11p, and Shs1p are members of a conserved family of GTP-binding proteins that have been shown to form filaments that are required for cytokinesis and that function as a scaffold for the assembly of signaling complexes involved in cell cycle coordination (Field and Kellogg, 1999). Additionally, we found that Ssk2p forms a 1:1 complex with actin minutes after the application of osmotic stress and that a mutant of *ssk2Δ* (*ssk2ΔLD*), unable to interact with actin, cannot localize to the neck or mediate actin recovery. These observations, in conjunction with our finding that complete disassembly of the actin cytoskeleton with latrunculin A in the absence of osmotic stress induces translocation of Ssk2p to the neck, suggests that Ssk2p can sense damage to the actin cytoskeleton. Interestingly, Ssk2p localization at the neck and Ssk2p-mediated actin recovery did not require the activation of Ssk2p by HOG pathway components.

In this report, we have extended our findings by investigating the role of Ssk2p in bud emergence after osmotic stress. At early stages of the cell cycle Ssk2p localizes at the incipient bud site and the bud tips and necks of small and medium budded osmotically stressed cells. The kinase activity of Ssk2p is required for polarized actin assembly to the bud, thus facilitating bud formation in osmotically stressed cells. Efficient localization of Ssk2p at the bud tip largely depends on the scaffold protein Spa2p, whereas Ssk2p localization at the neck requires an intact septin cytoskeleton and, in particular the septin Shs1p. *spa2Δ* cells, like *ssk2Δ* cells, are delayed for actin recovery and bud emergence but this defect can be suppressed by overexpression of catalytically active Ssk2p. These data suggest that the Ssk2p kinase functions with Spa2p to facilitate actin recovery in osmotically stressed cells, perhaps by regulating SpaII associated proteins involved in actin polarization. Moreover, Ssk2p's functions in actin cytoskeleton recovery are likely to be conserved among all eukaryotes. Its human homologue, MTK1, was able to localize at sites of polarized growth, interact with actin and Spa2p and complement the actin recovery defects of osmotically stressed *ssk2Δ* and *spa2Δ* cells.

Table 1. Yeast strains

Strain	Genotype	Source
FY86	α <i>ura3-52 leu2 his3</i> 200	F. Winston, personal communication
FY23	a <i>trp1</i> 63 <i>ura3-52 leu2</i>	F. Winston, personal communication
TTYD6B	a <i>ssk2::URA3 his3</i> 200	Yuzyuk <i>et al.</i> , 2002
TTY7Da	a <i>ssk1::URA3 his3</i> 200	Yuzyuk <i>et al.</i> , 2002
TTY3Da	a <i>ssk22::URA3 trp1</i> 63	Yuzyuk <i>et al.</i> , 2002
TTY1Ba	a <i>pbs2::URA3 his3</i> 200	Yuzyuk <i>et al.</i> , 2002
TTY11	a <i>ssk1::URA3 sln1::URA3 sho1::URA3 trp1</i> 63	Yuzyuk <i>et al.</i> , 2002
TTY146	a <i>ssk1::URA3 sln1::URA3 sho1::URA3 msb2::KAN leu2 trp1</i> 63 <i>his3</i> 200	This study
TTY150	α <i>shs1::KAN leu2 ura3-52 his3</i> 200	This study
TTY157	a <i>shs1::KAN spa2::KAN leu2 ura3-52 his3</i> 200	This study
TTY47	a <i>ssk2</i> LD (a.a. 426-466) <i>trp1</i> 63 <i>ura3-52 leu2</i>	This study
38F1	a <i>his3</i> 1 <i>leu2 met15 ura3 shs1::KAN</i>	Research Genetics
1HI	a <i>his3</i> 1 <i>leu2 met15 ura3 spa2::KAN</i>	Research Genetics
49G5	a <i>his3</i> 1 <i>leu2 met15 ura3 pea2::KAN</i>	Research Genetics
24F4	a <i>his3</i> 1 <i>leu2 met15 ura3 msb2::KAN</i>	Research Genetics

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods

Strains used in this study are listed in Table 1. The haploid *msb2Δ sho1Δ ssk1Δ sln1Δ* (TTY146) strain was obtained by mating the *sho1Δ sln1Δ ssk1Δ* (TTY11) and *msb2Δ* (24F4) haploid strains followed by tetrad dissection. The haploid *shs1Δ* strain (TTY150) was obtained by mating the *shs1Δ* strain (38F1) to FY86 stain followed by tetrad dissection. The haploid *shs1Δ spa2Δ* strain (TTY157) was obtained by mating *shs1Δ* (TTY150) and *spa2Δ* (1HI) strains followed by tetrad dissection. The *ssk2ΔLD* allele was integrated at the *SSK2* locus by a two-step gene replacement to create strain TTY47. Plasmid pTY21 was digested with *SpeI* and transformed into strain FY23. Integrants were plated on 5-fluoroorotic acid medium and recombinants were screened by polymerase chain reaction (PCR) to confirm proper replacement of *SSK2* with the *ssk2ΔLD* allele. Standard yeast media and genetic procedures were as described previously (Burke *et al.*, 2000). To induce osmotic shock, cells were grown in YPD medium for 6 h at 25°C and an equal volume of 1.8M NaCl

in YPD was added to a final concentration of 0.9 M NaCl. In each case, ≥ 100 cells were counted. Data from three or more independent experiments were used to calculate statistical errors.

Plasmid Constructions and DNA Manipulations

Plasmids used in this study are listed in Table 2. General cloning methods were as described previously (Sambrook *et al.*, 1989). For the green fluorescent protein (GFP)-Shs1p expression construct (pTY20), the *SHS1* coding region was PCR amplified from *S. cerevisiae* genomic DNA with primers TY-Shs1-*HindIII* (5'-CCCA-GAAGCTTTAATGAGCACTGCTTCAACACCG-3') and TYO-Shs1-*XbaI* (5'-CCTAGTCTAGAATCTCTACCCGATGCAATAGA-3'). The PCR fragment was digested with *HindIII* and *XbaI* and cloned as a fusion to the N terminus of the GFP-coding region in plasmid pRB2214 (Doyle and Botstein, 1996), which had been digested with *HindIII* and *XbaI*. Plasmid pTY21, carrying a fragment of the *ssk2ΔLD* allele (coding for aa 1-566, 426-466Δ), was generated by double fusion PCR with primers TYO-ΔLD-*HindIII* (5'-CCCGTA-

Table 2. Plasmids

Plasmid	Description	Source
pTY111L	GFP-SSK2 LEU2 CEN	Yuzyuk <i>et al.</i> , 2002
pTY112L	GFP-ssk2(T1460A) LEU2	Yuzyuk <i>et al.</i> , 2002
pTY113L	GFP-ssk2(K1295N) LEU2	Yuzyuk <i>et al.</i> , 2002
pTY119L	GFP-ssk2 LD (a.a. 426-466) LEU2	Yuzyuk <i>et al.</i> , 2002
pTY20*	SHS1-GFP URA3	This study
pTY21**	ssk2 (aa 1-566, 426-466) URA3	This study
pRB2214	URA3 CEN ACT1p-GFP-ACT1t	Doyle <i>et al.</i> , 1996
YIplac211	URA3	Gietz, 1988
pRS315	LEU2 CEN GFP-CDC3	B. Haarer, personal communication
pEG(kl)	URA3 CEN GAL10p-ACT1t	Mitchell <i>et al.</i> , 1993
pTY118	GST-SSK2 URA3 CEN GAL10p-ACT1t	Yuzyuk <i>et al.</i> , 2002
pTY131	GFP-MTK1 (aa 22-1607) LEU2 CEN	This study
pTY132	GST-MTK1 (aa 22-1607) URA3 CEN GAL10p-ACT1t	This study

* based on plasmid pRB2214

** based on plasmid YIplac211

AGCTTGCTAAGAACGGGTGTTTTCAA-3') and TYO-NoFrag1-2 (5'-CTCGTCAGCGCTCATATTATCGTCATCTGAAACTGAGTATTGAA-3'), TYO-ΔLD-*Bam*HI (5'-CGCGGGATCCATTAGTGCGGAAAACGGCTGG-3') and TYO-NoFrag1-3 (5'-AATACTCAGTTTTTCAGATGACGATAATATGAGCGCTGACGAGGCT-3'). The PCR fusion product was digested with *Hind*III and *Bam*HI and ligated into *Hind*III/*Bam*HI digested plasmid YIplac211 (Gietz and Sugino, 1988). For the GFP-MTK1 expression construct (pTY131) the *MTK1* coding region (aa 22–1607) was PCR amplified from plasmid pcDNA1-MTK1 (kindly provided by Haruo Saito, Harvard Medical School, Boston, MA) with primers TYO-MTK1-M22BgIII (5'-CGCAGATCT ATGGAGGAGCCGCC CCACCG-3') and TYO-MTK1-*Nhe*I (5'-GCCGCTAGCTCATTCTTCATCTGTG-3'). The PCR fragment was digested with *Bg*III and *Nhe*I and cloned as a fusion to the C termini of the GFP-coding region in plasmid pTD125 (Doyle and Botstein, 1996), which had been digested with *Bam*HI and *Xba*I. For the GST-MTK1 expression construct (pTY132) the *MTK1* coding region (aa 22–1607) was PCR amplified from plasmid pcDNA1-MTK1 with primers TYO-YMTK1-*Xma*I (5'-GCCCGCCGGGA ATGGAGGAGCCGCCGCCACCG-3') and TY-YMTK1-*Xho*I (5'-CCGTGCTC GAGTCATTCTTCATCTGTGCAAC-3'). The PCR fragment was digested with *Xma*I and *Xho*I and cloned as a fusion to the C-terminal coding region of the glutathione S-transferase (GST) gene in plasmid pEG(kit) (Mitchell *et al.*, 1993), which had been digested with *Xma*I and *Sal*I.

Microscopy and Rhodamine-Phalloidin Staining

GFP-Ssk2p and GFP-MTK1 localization in normal osmotic medium and after 20 min of osmotic stress (0.9 M NaCl) was visualized without fixation on an Axioskop 2MOT (Carl Zeiss, Thornwood, NY) by using a Plan-APOCHROMAT 100×/1.4 numerical aperture objective. Images were captured with an ORCA-ER camera (Hamamatsu Photonics, Bridgewater, NJ) and visualized in Open Lab (Improvision, Lexington, MA). Rhodamine-phalloidin staining of actin was performed as described previously (Bi *et al.*, 1998).

Cell Synchronization

For G_1 synchronization, cells at a density of 1×10^7 cells/ml were grown in selective synthetic medium in the presence of 1 μ g/ml α -factor (Sigma-Aldrich, St. Louis, MO) for 2 h at 25°C. Cells were pelleted and released into selective medium + 0.9 M NaCl. For mitotic arrest assays, cells at a density of 1×10^7 cells/ml were grown in selective synthetic medium in the presence of 15 mg/ml hydroxyurea (HU) for 3 h at 25°C. Cells were pelleted, washed once with YPD to remove residual HU and released into fresh medium. After a 1-h recovery, an equal volume of 1.8 M NaCl was added to a final concentration of 0.9 M NaCl. Cells were fixed in 3.7% formaldehyde before osmotic stress and 60, 90, 120, 150, and 180 min after osmotic stress. In each case >100 cells were counted. Numbers reported are averages calculated from two to five independent experiments.

Coprecipitation Assay

Cells were grown in selective synthetic medium in the presence of 2% galactose to a density of 1×10^7 cells/ml. Osmotically stressed cells (0.9 M NaCl) were quick frozen in liquid nitrogen, and then melted on ice and harvested by centrifugation. Cells were resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 15 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 5 μ g/ml aprotinin, 2 μ g/ml chymostatin, 2.5 μ g/ml antipain, 150 mM NaCl) and lysed using glass beads. Cell extracts (750 μ l in buffer A) were incubated with 100 μ l of glutathione-Sepharose beads for 50 min at 4°C. The beads were washed five times with 1 ml of buffer A, resuspended in reducing sample buffer and separated by electrophoresis through a 10% SDS-polyacrylamide gel.

Immunoblotting was done with an anti-GST monoclonal antibody at a 1:500 dilution (Pharmacia, Piscataway, NJ), the mouse anti-actin monoclonal antibody C4 at a 1:400 dilution (ICN Biomedicals, Irvine, CA) and anti-Spa2p antibody (kindly provided by Mike Snyder, Yale University, New Haven, CT) at a 1:1000 dilution.

RESULTS

Ssk2p Promotes Actin Polarization and Bud Emergence in Osmotically Stressed Cells

We have previously shown that Ssk2p, one of the MAP-KKKs of the HOG pathway, promotes actin reassembly to the neck after osmotic stress, thereby facilitating cell cycle completion (Yuzyuk *et al.*, 2002). Given the prominent localization of Ssk2p to the incipient bud site and small bud cortex, we surmised that the kinase might have a similar role in recovery of actin polarization early in the cell cycle in osmotically stressed cells. To address this question, wild-type (FY23) and *ssk2Δ* (TYYD6B) cells were synchronized in G_1 by a 2-h treatment with α -factor and immediately released from α -factor treatment into selective medium containing 0.9 M NaCl. Samples of the cells were stained with rhodamine-phalloidin over time to examine the organization of the actin cytoskeleton. Before the application of osmotic stress (time 0), the actin cytoskeleton was well polarized into the mating projections of both wild-type and *ssk2Δ* cells (Figure 2 A). In contrast, after 60 min of osmotic stress all actin cables were disassembled and the actin cortical patches were randomly distributed over the cortex of both cell types (Figure 2A). However, 120 min after osmotic stress there was a robust polarization of actin structures in 57% of wild-type cells (Figure 2, A and B), whereas only 12% of the *ssk2Δ* cells showed significant polarization of the actin cytoskeleton (Figure 2, A and C). Even after 180 min of osmotic shock, actin polarization was observed in only 32% of the *ssk2Δ* cells (Figure 2A). As a likely consequence of delays in actin polarization, the *ssk2Δ* cells were considerably delayed in bud emergence compared with the wild-type strain. After 180 min of osmotic stress, almost all wild-type cells were budded compared with only 20% of *ssk2Δ* cells (our unpublished observations). Note that *ssk2Δ* cells released into normal osmotic medium after α -factor synchronization were not delayed in bud formation showing that *ssk2Δ* cells do not have defects in recovery from α -factor treatment. A congenic *ssk22Δ* strain (TYY3Da) displayed normal actin recovery to the bud site after osmotic shock (Figure 2C). Therefore, even though Ssk2p and Ssk22p are highly homologous and redundant for transmission within the HOG pathway (Maeda *et al.*, 1995), they do not share roles in mediating actin recovery to the bud site. Cells lacking Ssk1p (TYY7Da) the activator of Ssk2p kinase activity in the HOG pathway were not defective for reassembly of a polarized actin cytoskeleton at the bud site, showing that Ssk1p is not required to activate Ssk2p for its function in actin recovery.

We next showed that a GFP-Ssk2p fusion protein on a CEN plasmid (pTY111L) could complement the *ssk2Δ* strain for actin recovery to the bud site (Figure 2A). In contrast, kinase dead (GFP-*ssk2*^{K1295N}; Figure 2A) and phosphorylation defective (GFP-*ssk2*^{T1460A}; our unpublished data) mutants were indistinguishable from the *ssk2Δ* strain in comparable experiments. We previously isolated a mutant of *ssk2* (*ssk2Δ*LD, a 40 amino acid deletion in the actin inter-

Figure 2. Ssk2p promotes actin repolarization to the bud site after osmotic stress. Wild-type (FY23), *ssk2Δ* (TYYD6B), *ssk1Δ* (TYY7D), *ssk22Δ* (TYY3D), *ssk2ΔLD* (TYY47) strains and an *ssk2Δ* (TYYD6B) strain expressing GFP-Ssk2p (pTY111L), GFP-Ssk2p^{K1295N} (pTY113L), and GFP-Ssk2ΔLD (pTY119L) were synchronized with α -factor, released into selective medium containing 0.9 M NaCl, and stained with rhodamine-phalloidin. (A) Percentages of cells with a polarized actin cytoskeleton are shown before osmotic shock (time 0) and at different times after osmotic shock. $N \geq 100$. Actin cytoskeleton organization is shown in wild-type FY23 cells (B) and *ssk2Δ* cells (C) 120 min after osmotic stress.

acting region) that was unable to form a complex with actin in response to osmotic stress and unable to mediate actin recovery to the neck of osmotically stressed cells (Yuzyuk *et al.*, 2002). Surprisingly, the GFP-*ssk2ΔLD* fusion protein was able to complement actin recovery defects to the bud site and facilitate bud formation in *ssk2Δ* cells (Figure 2A). However, as soon as *ssk2Δ* cells expressing GFP-*ssk2ΔLD* reached a large-budded size, the mutant was not able to promote cell separation in osmotic conditions. After 4 h of osmotic stress 80% of *ssk2Δ* cells expressing GFP-*ssk2ΔLD* were large budded compared with 23% of *ssk2Δ* cells expressing wild-type GFP-Ssk2p (our unpublished observation). Careful analysis of expression levels showed that both GFP-Ssk2p and GFP-*ssk2ΔLD* expressed from a CEN plasmid are comparably overexpressed sixfold above Ssk2p-GFP expressed from an

integrated allele under the control of the *SSK2* promoter (Yuzyuk *et al.*, 2002).

We theorized that this approximate sixfold overexpression could be suppressing defects of the *ssk2ΔLD* mutant in bud emergence but not cell separation. In agreement, a strain carrying a single, integrated copy of the *ssk2ΔLD* allele (TYY47) was found to have defects in actin polarization to the bud site after osmotic shock that were comparable to the *ssk2Δ* strain (Figure 2A).

Osmotic Stress Induces Ssk2p to Concentrate to the Presumptive Bud Site and Small Bud Cortex

We have previously documented neck localization of Ssk2p as induced by osmotic stress and the role of Ssk2p late in the

Figure 3. Polarized localization of GFP-Ssk2p and GFP-ssk2 Δ LD in osmotically stressed cells. An wild-type strain (FY23) expressing (A–C) GFP-Ssk2 from plasmid pTY111L or (D–F) GFP-ssk2 Δ LD from plasmid pTY119L was synchronized with α -factor, released into selective medium to allow cell cycle reentry, and osmotically stressed with 0.9 M NaCl. Cells were examined by fluorescence microscopy 40 min after α -factor release followed by 20 min of osmotic stress (A and D), 60 min after α -factor release followed by 20 min of osmotic stress (B and E), and 90 min after α -factor release followed by 20 min of osmotic stress (C and F).

cell cycle (Yuzyuk *et al.*, 2002). In contrast, Ssk2p localization early in the cell cycle has not been rigorously studied. To examine this aspect of Ssk2p function, wild-type cells (FY23) expressing GFP-Ssk2p on a low copy CEN vector were synchronized with α -factor, released from α -factor for different periods of time, and then osmotically stressed for 20 min. Forty minutes after α -factor release, GFP-Ssk2p was concentrated at the incipient bud site on the tips of the former mating projections in $79 \pm 3\%$ of cells (Figure 3A). Sixty min after α -factor release buds had emerged in almost all cells, and GFP-Ssk2p was localized to the bud tip of $78 \pm 4\%$ of small and medium-budded cells and to the neck of $75 \pm 3\%$ of all budded cells (Figure 3B; Table 3). By 90 min, nearly all cells had medium to large-sized buds, bud tip localization of GFP-Ssk2p was no longer apparent and GFP-Ssk2p was focused in the neck of $81 \pm 3\%$ of these cells (Figure 3C). Note that at all cell cycle stages GFP-Ssk2p was uniformly distributed throughout the cytosol in nonosmotically stressed cells (our unpublished observation).

We previously showed that a 40 amino acid deletion in the actin interacting region of Ssk2p (ssk2 Δ LD) abolished neck

localization and the ability of Ssk2p to form a complex with actin. However, in this study we observed very weak localization of GFP-ssk2 Δ LD at the incipient bud sites in some osmotically stressed wild-type cells 40 min after release from α -factor (Figure 3D). In contrast, later in the cell cycle polarized localization of GFP-ssk2 Δ LD was not apparent at either the bud tip or the neck (Figure 3, E and F).

In agreement with our studies on neck localization for Ssk2p, the catalytically inactive GFP-ssk2^{K1295N} and phosphorylation defective GFP-ssk2^{T1460A} mutants localized as well as wild-type Ssk2p to incipient bud sites and to the tips of small-medium buds of ssk2 Δ cells released from α -factor (our unpublished observations). In addition, incipient bud site and bud tip localization of GFP-Ssk2p was observed in α -factor synchronized and osmotically stressed strains defective for transmission through the HOG pathway. In this regard, we tested an ssk1 Δ strain (TYY7Da) lacking the known activator of Ssk2p kinase activity, a pbs2 Δ strain (TYY1Ba) lacking the MEK substrate of Ssk2p, and a msb2 Δ sho1 Δ sln1 Δ ssk1 Δ strain (TYY146) lacking the three known putative membrane sensors of the HOG pathway (our un-

Table 3. Percentages of osmotically stressed cells with polarized localization of GFP-Ssk2p

Strain	Incipient bud site ^a	Bud tip ^b	Mother/bud neck ^b
Wild type	79 ± 3	78 ± 4	75 ± 3
<i>spa2</i>	67 ± 3	16 ± 4	27 ± 2
<i>shs1</i>	77 ± 4	71 ± 4	4 ± 1
<i>spa2 shs1</i>	45 ± 5	10 ± 5	2 ± 1

^a α -Factor-synchronized cells were released into normal osmotic medium for 40 min and osmotically stressed with 0.9 M NaCl.

^b α -Factor-synchronized cells were released into normal osmotic medium for 60 min and osmotically stressed with 0.9 M NaCl.

published data). These results lead us to conclude that no known components of the HOG pathway, either upstream or downstream of Ssk2p, play a role in translocation of Ssk2p to sites of polarized growth at the bud site or in the neck.

Spa2p Is Involved in Ssk2p Localization to Sites of Polarized Growth

Spa2p is a large scaffold protein that is involved in the localization of many regulators of polarized actin assembly, including Pea2p and Bni1p to the bud cortex (Valtz and Herskowitz, 1996; Fujiwara *et al.*, 1998; Ozaki-Kuroda *et al.*, 2001). To examine Spa2p dependence for GFP-Ssk2p localization, a *spa2Δ* strain (1H1) expressing GFP-Ssk2p on a CEN vector was synchronized with α -factor and osmotically stressed as described above. Identifying incipient bud sites in *spa2Δ* cells was complicated by defects in shmoo formation (Gehring and Snyder, 1990). Therefore, GFP-Ssk2p localization before bud emergence was only scored in cells with a discernible polarized morphology. We observed no defect in GFP-Ssk2p localization at the incipient bud sites in *spa2Δ* cells (Figure 4 A and Table 3). In contrast, the number of small- and medium-budded *spa2Δ* cells with GFP-Ssk2p localization to the bud tip was significantly reduced compared with wild-type cells (Figure 4B and Table 3). Note that in those *spa2Δ* cells in which we identified positive Ssk2p bud tip localization, the GFP signal at the bud tip was more diffuse as compared with the GFP signal in the wild-type cells.

The neck localization of GFP-Ssk2p was also lost in a majority of *spa2Δ* cells (Figure 4, B and C, and Table 3). Strikingly, at all stages of bud development the great majority of synchronized *spa2Δ* cells had enlarged bud necks with wide septin rings as visualized by GFP-Shs1p fluorescence (Figure 4G), indicating that *spa2Δ* cells have a defect in septin filament organization but no accompanying defects in Shs1p localization to the neck.

Decreases in the efficiency of polarized GFP-Ssk2p localization in α -factor-synchronized *spa2Δ* strains could reflect general defects in mating and mating projection formation in the *spa2Δ* cells. However, bud tip localization of Ssk2p was observed in <10% of small- and medium-budded *spa2Δ* cells from an asynchronous culture as compared with 65 ± 3% to that of wild-type cells. GFP-Ssk2p localized to the neck in 24 ± 4% of all budded *spa2Δ* cells compared with 77 ± 5% of all budded wild-type cells. These results demonstrated

that Spa2p is involved in efficient localization of Ssk2p at sites of polarized growth.

It has been previously shown that polarized localization of Ssk2p is dependent on its binding partner Pea2p (Valtz and Herskowitz, 1996). Consistent with this, we found that GFP-Ssk2p localization in osmotically stressed *pea2Δ* cells (49G5) was as defective as in *spa2Δ* cells. This indicates that Spa2p polarization is essential for efficient polarized localization of Ssk2p and suggests that Spa2p could be a scaffold for Ssk2p. Therefore, we next asked whether Ssk2p interacts with Spa2p in osmotically stressed cells. Cells expressing GST or GST-Ssk2p were osmotically stressed with 0.9 M NaCl for 20 min, and GST proteins were precipitated from cell extracts with glutathione-agarose beads. Western Assays confirmed that Spa2p coprecipitated with GST-Ssk2p in osmotically stressed cells but not with GST (Figure 5).

Shs1p Is Required for Ssk2p Localization to the Neck

Previous work from our laboratory showed that localization of GFP-Ssk2p to the neck is lost in the majority of *cdc12-6* temperature-sensitive mutant cells after a 20-min shift to nonpermissive temperature and in *cdc10Δ* cells (Yuzyuk *et al.*, 2002). Decreased efficiency of Ssk2p neck localization in *cdc12-6* and *cdc10Δ* strains probably reflects general defects in septin ring structure. It has been reported that *cdc12-6* cells shifted to nonpermissive temperature for 30 min lack all septins from the mother/bud neck (Byers and Goetsch, 1976; Haarer and Pringle, 1987; Kim *et al.*, 1991), and mutant *cdc10Δ* cells do not contain detectable neck filaments as shown by electron microscopy (Frazier *et al.*, 1998). However, we were still able to see neck localization of the septin Cdc3p fused to GFP in some *cdc12-6* cells after 20-min temperature shift and in a *cdc10Δ* cells, indicating that disruption of the septin ring was not complete in either strain. Therefore, although we were able to conclude that an intact septin ring structure is important for proper neck localization of Ssk2p, we did not identify the key septin responsible.

Recent studies identified an additional septin protein called Shs1p as a Spa2p interacting protein in a yeast two-hybrid screen (Mino *et al.*, 1998). Shs1p localizes to the mother/bud neck and is believed to play a role in cytokinesis and Gin4p kinase activation (Mino *et al.*, 1998; Mortensen *et al.*, 2002). This suggested to us that Shs1p could be a likely candidate for regulating Ssk2p localization. We found that Ssk2p localized to the neck in only 4 ± 1.8% of all budded

Figure 4. Spa2p and Shs1p are involved in polarized localization of Ssk2p. *spa2Δ* (1HI) and *shs1Δ* (38F1) strains were transformed with plasmid pTY111L expressing GFP-Ssk2p. Localization of GFP-Ssk2p was examined in *spa2Δ* (A–C) and *shs1Δ* cells (D–F) synchronized with α -factor and osmotically stressed for 20 min with 0.9 M NaCl 40, 60, and 90 min after release from α -factor arrest. (G) Localization of Shs1-GFP expressed from plasmid pTY20 in *spa2Δ* cells and in wild-type (FY23) cells (H) synchronized with α -factor and osmotically stressed 90 min after release from α -factor arrest.

shs1Δ cells (38F1) compared with 77 + 5% of wild-type (FY23) cells. In contrast, GFP-Ssk2p localized to the small and medium bud tip in *shs1Δ* cells as well as in wild-type cells (68 + 2 compared with 65 + 3%).

Figure 5. Ssk2p interacts with Spa2p in osmotically stressed cells. The protease deficient strain JTY143 was transformed with plasmids expressing GST [pEG(kt); Mitchell *et al.*, 1993] or GST-Ssk2p (pTY118). Cells were osmotically stressed with 0.9M NaCl for 15 min, cell extracts were prepared, and the complex was precipitated with glutathione-Sepharose beads. Western blot assays were performed to detect GST and Spa2p.

To investigate further, we examined Ssk2p localization to sites of polarized growth in *shs1Δ* cells synchronized with α -factor. GFP-Ssk2p localization to the neck was lost in synchronized *shs1Δ* cells compared with wild-type cells (Figure 4, E and F, and Table 3). Therefore, Shs1p seems to be important for neck localization of Ssk2p. Weak localization of Ssk2p to the neck in *shs1Δ* cells may be attributable to the septin Cdc10p, with which Ssk2p shows a two-hybrid interaction (Yuzyuk *et al.*, 2002). Interestingly, incipient bud site and bud tip Ssk2p localization were also unaffected in synchronized *shs1Δ* cells (Figure 4D and Table 3). Furthermore, actin recovery and bud formation in α -factor synchronized and osmotically stressed *shs1Δ* cells were comparable with those in wild-type cells (our unpublished observation), confirming little role for Shs1p in Ssk2p localization and activation early in the cell cycle.

We next addressed whether *shs1Δ* cells that are defective in Ssk2p neck localization have defects in completion of the cell cycle after osmotic stress. *shs1Δ* cells were synchronized with HU and treated with 0.9 M NaCl or an equal volume of low osmotic medium. Unfortunately, even in normal osmotic conditions we observed a considerable delay in the separation of large-budded *shs1Δ* cells after HU synchronization, limiting our ability to study Ssk2p function in osmotically stressed *shs1Δ* cells.

Because Shs1p and Spa2p localization overlap at the incipient bud site, and Ssk2p localization at the bud site is normal in *shs1Δ* and *spa2Δ* cells, we theorized that Shs1p and Spa1p could be redundant for mediating localization of Ssk2p early in the cell cycle. However, Ssk2p localized at the incipient bud site in 45 + 5% of *shs1Δ spa2Δ* osmotically stressed cells (Table 3), suggesting additional factors must facilitate Ssk2p localization at the incipient bud site.

spa2Δ Cells Have Osmotic Stress-induced Defects in Actin Recovery and Bud Emergence That Can Be Suppressed by Overexpression of Ssk2p

If localization of Ssk2p at sites of polarized growth is important for its function in actin recovery, then *spa2Δ* cells

should have defects in actin recovery from osmotic stress. To address this question, *spa2Δ* cells were synchronized with α -factor, released into medium + 0.9 M NaCl, and actin recovery overtime was analyzed by rhodamine-phalloidin staining. Right after α -factor release but before osmotic stress, we observed actin polarization into the mating projections of the *spa2Δ* cell, as reported previously (Gehring and Snyder, 1990). Disassembly of actin cables and depolarization of actin patches in the *spa2Δ* cells was complete 60 min after osmotic stress (Figure 6A). However, actin recovery and bud formation were significantly delayed in *spa2Δ* cells compared with the wild-type control (Figure 6, A–C). Note that the timing of bud emergence was the same for wild-type and *spa2Δ* cells after release from α -factor arrest into low osmotic medium. Therefore, the observed delay in *spa2Δ* cells does not reflect general defects in actin polarization or recovery from mating factor arrest. Delays in actin recovery and bud emergence in *shs1Δ spa2Δ* cells were comparable with that observed in *spa2Δ* cells (our unpublished observation).

Given our observation that *spa2Δ* cells are defective for neck localization of Ssk2p, we asked whether this strain is also defective for cell cycle completion. However, even in low osmotic condition HU synchronized *spa2Δ* cells were significantly delayed for cell division (our unpublished observation), making it difficult to study Ssk2p function in osmotically stressed *spa2Δ* cells. This result and our previous observation of abnormally wide necks suggests that *spa2Δ* cells have general defects in septin organization, leading to defects in cell separation.

We previously confirmed that the plasmid-borne copy of GFP-Ssk2p leads to an approximate sixfold increase in the cellular concentration of Ssk2p (Yuzyuk *et al.*, 2002). As reported above, localization of overexpressed GFP-Ssk2p was observed at the incipient bud site of *spa2Δ* cells (Figure 4A). We therefore asked whether overexpression of Ssk2p could suppress *spa2Δ* defects in actin recovery to the bud site after osmotic stress. *spa2Δ* cells expressing wild-type GFP-Ssk2p (pTYY111L), catalytically inactive GFP-*ssk2*^{K1295N} (pTYY113L) and the GFP-*ssk2Δ*LD (pTYY119L) mutants from a CEN vector were synchronized with α -factor and osmotically stressed. The delay in actin recovery and bud emergence of *spa2Δ* cells expressing GFP-*ssk2*^{K1295N} was comparable to delays in *spa2Δ* cells (Figure 6, A, B, and D). In contrast, *spa2Δ* cells expressing wild-type GFP-Ssk2p or GFP-*ssk2Δ*LD were not delayed in either reassembly of a polarized actin cytoskeleton or in bud formation (Figure 6, A, B, E, and F). Overexpression of GFP-Ssk2p also complemented actin recovery and bud emergence defects of the *spa2Δ shs1Δ* strain (our unpublished observation). However, a comparable fusion of GFP to Ssk22p (the close homologue of Ssk2p) was unable to suppress the actin recovery defects of the *spa2Δ* strain despite its ability to complement the osmosensitivity of a *sho1Δ ssk2Δ ssk22Δ* strain (our unpublished observation).

The observed bypass suppression by Ssk2p of the *spa2Δ* allele suggests that Ssk2p acts downstream of Spa2p in mediating actin recovery and that Spa2p probably functions to concentrate the kinase at its appropriate site of action. Overexpression of Ssk2p would seem to provide sufficient concentrations of the kinase in the bud to find and activate the appropriate cytoskeletal substrate(s).

The Human MEK Kinase MTK1 Can Facilitate Actin Recovery in Osmotically Stressed Yeast Cells

The human homolog of Ssk2p, MTK1 can functionally replace Ssk2p/Ssk22p for transmission within the HOG pathway of *S. cerevisiae* (Takekawa *et al.*, 1997). Therefore, we asked whether MTK1 could perform the specialized functions of Ssk2p in actin recovery from osmotic stress we have described in this and our previous report. First, we examined localization of MTK1 expressed in yeast cells as a GFP fusion from plasmid pTY131. On low osmotic medium GFP-MTK1 was uniformly distributed throughout the cytoplasm (Figure 7 A) but after a shift into 0.9 M NaCl GFP-MTK1 localized at sites of polarized growth (Figure 7B). These localization patterns were indistinguishable from that of GFP-Ssk2p.

We next sought to determine whether MTK1 would complement the actin recovery defects of osmotically stressed *ssk2Δ* and *spa2Δ* cells. To address this question, *ssk2Δ* (TYYD6B) and *spa2Δ* (1HI) cells expressing GFP-MTK1 were synchronized with α -factor in G₁ and then osmotically stressed as described above. Both *ssk2Δ* and *spa2Δ* cells expressing GFP-MTK1 reassembled a polarized actin cytoskeleton to the bud much faster than cells of parallel cultures not expressing GFP-MTK1 (Figure 7C). GFP-MTK1 also complemented the actin recovery defects of HU synchronized, osmotically stressed *ssk2Δ* cells. After 90 min of osmotic stress, there was an impressive repolarization of filamentous actin to the neck in 55% of *ssk2Δ* cells expressing GFP-MTK1 compared with 22% of *ssk2Δ* cells not carrying the GFP-MTK1 plasmid (our unpublished observations).

These experiments suggest that despite a tremendous evolutionary distance, many of the cytoskeletal interactions of Ssk2p have been conserved in MTK1. We directly tested this hypothesis by constructing and expressing a GST-MTK1 fusion in yeast and performing GST pull downs. Neither actin nor Spa2p were detectable in GST-MTK1 precipitates from unstressed cells but a short treatment with 0.9 M NaCl did lead to the association of both actin and Spa2p with MTK1 (Figure 7D).

DISCUSSION

Many environmental stresses such as heat shock, mechanical stress, and changes in external osmolarity elicit a common set of cellular responses in *S. cerevisiae*, including an arrest in the cell cycle and disassembly of a polarized actin cytoskeleton (Gustin *et al.*, 1998; Hohmann, 2002), and in some cases inhibition of translation initiation (Uesono and Toh-e, 2002). Recovery from an osmotic stress requires accumulation of glycerol and reassembly of a polarized actin cytoskeleton (Chowdhury *et al.*, 1992; Gustin *et al.*, 1998; Hohmann, 2002). Up-regulation of glycerol synthesis is known to be under the control of the HOG MAP kinase pathway. In contrast, regulation of actin cytoskeleton recovery during osmotic stress adaptation is poorly understood. We previously identified Ssk2p, a MAPKKK of the HOG pathway, as an actin interacting protein and found that Ssk2p is required for efficient actin recovery from osmotic stress, but that its functions in actin recovery are regulated from outside the HOG pathway. Data presented here provides further insights into the mechanisms that drive recovery of the actin cytoskeleton and polarized growth after osmotic stress. In addition, we

Figure 6. Overexpression of catalytically active GFP-Ssk2p suppresses the actin recovery defects of *spa2Δ* cells. A *spa2Δ* strain expressing GFP-Ssk2p (pTY111L), GFP-ssk2p^{K1295N} (pTY113L), and GFP-Ssk2ΔLD (pTY119L) were synchronized with α -factor, released into selective medium containing 0.9 M NaCl, and stained with rhodamine-phalloidine overtime. (A) Percentages of cells with a polarized actin cytoskeleton are shown before osmotic stress (time 0) and at different times after osmotic stress. $N \geq 100$. (B) Percentages of small- and medium-budded cells are shown before osmotic stress (time 0) and different times after osmotic stress. $N \geq 100$. Actin cytoskeleton organization after 120 min of osmotic stress is shown in *spa2Δ* cells (C), *spa2Δ* cells expressing GFP-ssk2p^{K1295N} (D), *spa2Δ* cells expressing GFP-Ssk2p (E), and *spa2Δ* cells expressing GFP-ssk2ΔLD (F).

Figure 7. MTK1 localizes at sites of polarized growth, interacts with actin and Spa2p, and suppresses the actin recovery defects of osmotically stressed *ssk2Δ* and *spa2Δ* cells. (A and B) *ssk2Δ* cells (TYD6B) were transformed with plasmid pTY131 expressing GFP-MTK1 and were examined under normal osmotic conditions (A), 15 min after osmotic stress (B). (C) *ssk2Δ* and *spa2Δ* strains with or without plasmid pTY131 expressing GFP-MTK1 were synchronized with α -factor, released into selective medium containing 0.9 M NaCl, and stained with rhodamine-phalloidin. The percentages of cells with a polarized actin cytoskeleton are shown before osmotic stress (time 0) and at different times after osmotic stress. (D) Strain JTY143 expressing GST-MTK1 from plasmid pTY132 was incubated in the presence (+) or absence (–) of 0.9 M NaCl for 15 min. Cell extracts were prepared and the complex was precipitated with glutathione-Sepharose beads. Western blot assays were performed to detect GST, actin, and Spa2p.

show that this actin recovery pathway is likely to be conserved.

How Does Ssk2p Sense a Rise in External Osmolarity?

The response of Ssk2p to osmotic stress can be temporally divided into two phases. Within minutes of cells experiencing osmotic stress, Ssk2p forms 1:1 complex with actin and localizes to sites of polarized growth (Yuzyuk *et al.*, 2002; Figure 3). Ssk2p polarization persists until osmotic balance is restored (~60–90 min) at which time polarized actin assembly is reinitiated in a process that requires polarized localization and the kinase activity of Ssk2p. It is unclear how Ssk2p, as measured by changes in its localization, senses rises in external osmolarity. The simplest model would posit that Ssk2p localization is under the control of the HOG pathway. However, we have shown that Ssk1p is not required for Ssk2p localization or actin recovery. In fact, activation of the HOG pathway seems to play no role because a *pbs2Δ* strain and an *msb2Δ sho1Δ sln1Δ ssk1Δ* strain (lacking the putative plasma membrane sensors of the HOG pathway) have no defects in Ssk2p localization (Yuzyuk *et al.*, 2002).

It has been previously proposed that actin disassembly could act as an osmosensor (Hohmann, 2002) and with respect to Ssk2p regulation such a model has merit. For example, we previously showed that actin disassembly as induced by latrunculin A treatment, even in the absence of osmotic stress, activates Ssk2p to localize to the mother/bud neck and small bud cortex (Yuzyuk *et al.*, 2002). Furthermore, there is a temporal correlation between the ability of Ssk2p to form a 1:1 complex with actin and its relocalization to sites of polarized growth. Moreover, the *ssk2ΔLD* mutant that is unable to interact with actin is also defective in localization to the neck (Yuzyuk *et al.*, 2002). Given the structural and functional similarities between Ssk2p and MTK1, we could theorize that binding of actin monomer to the N-terminal region of either kinase triggers their polarized localization, for example, by activating their Shs1p- and/or Spa2p-interacting sites. Actin binding could also induce activation of the kinase activities of Ssk2p or MTK1 by disrupting the autoinhibitory interaction between the N-terminal regulatory and the C-terminal kinase domains. Activation of MTK1 in mammalian cells is controlled by GADD45-like proteins that disrupt the interaction between the N-terminal autoinhibitory and the C-terminal kinase

domains of MTK1, thereby releasing the kinase domain for further interactions with its substrates (Mita *et al.*, 2002). Homologs of GADD45 proteins have not been found in budding yeast cells, and MTK1 cannot be activated within the HOG pathway unless its autoinhibitory domain is deleted (Mita *et al.*, 2002). However, our observations that MTK1 localizes at polarized growth sites, interacts with actin and Spa2p, and promotes actin recovery in osmotically stressed *ssk2Δ* and *spa2Δ* yeast cells strongly suggests the mechanism of MTK1 activation in the actin recovery pathway is highly conserved among eukaryotes.

In apparent conflict with models that invoke kinase regulation by actin binding, we have shown herein that the GFP-*ssk2ΔLD* mutant weakly localizes to the incipient bud site upon osmotic stress and can complement the bud emergence defects of both *ssk2Δ* (Figures 2A and 3D) and *spa2Δ* strains (Figure 6). However, when integrated the *ssk2ΔLD* mutant strain had delays in actin recovery and bud emergence that were comparable with the *ssk2Δ* strain. The ability of overexpressed *ssk2ΔLD* to suppress the actin recovery defects of *ssk2Δ* cells at the bud site but not in the neck may reflect differences in mechanisms of Ssk2p localization to the neck and to the incipient bud site. However, we believe that different threshold amounts of kinase are required at these locations to activate critical cytoskeletal substrates. For example, actin assembly must occur over a much broader area in the neck as opposed to the bud site. In fact, Ssk2p levels may normally be kept low so that tight spatial regulation and function can be maintained. However, the ability of the *ssk2ΔLD* mutant to support actin recovery in the bud when overexpressed would seem to indicate that actin binding is not required for kinase activation, merely kinase localization.

Shs1p Is Required for Ssk2p Localization at the Mother/Bud Neck

We previously demonstrated that efficient Ssk2p localization to the mother/bud neck was compromised but not completely blocked in *cdc12-6* cells at nonpermissive temperature and in *cdc10Δ* cells (Yuzyuk *et al.*, 2002). However, both mutations are known to cause general defects in septin organization (Kim *et al.*, 1991; Frazier *et al.*, 1998), and yet these defects seem to be incomplete because we found that a GFP-Cdc3p reporter was able to localize to the neck in the same percentage of *cdc12-6* and *cdc10Δ* cells as Ssk2p in parallel experiments. We previously reported a two-hybrid interaction between Ssk2p and Cdc10p and yet residual localization of Ssk2p in *cdc10Δ* cells indicated other neck proteins must be involved.

An additional, nonessential septin called Shs1p was recently identified (Mino *et al.*, 1998). During mitosis the Gin4p kinase forms a complex with the septins by binding Shs1p and is thereby recruited to the neck. Binding to Shs1p leads to oligomerization of Gin4p, autohyperphosphorylation of Gin4p, and phosphorylation of Shs1p (Mortensen *et al.*, 2002). The role of Shs1p in localization and activation of the Gin4p kinase led us to investigate its role in neck localization of Ssk2p. Our results suggest that like for Gin4p, Shs1p is the major septin involved in regulation of Ssk2p neck localization. It will be interesting to determine whether the analogy extends to regulation of Ssk2p kinase activity. For example, Shs1p binding by Ssk2p could induce autophosphorylation

of Ssk2p on Thr1460 and activation of the kinase toward cytoskeletal substrates that are colocalized at the neck. In this scenario Shs1p would function both to concentrate the kinase and to activate the kinase in a spatially restricted manner.

What Is the Role of Spa2p in Ssk2p Localization?

Our data suggest that two proteins are primarily involved in localization of Ssk2p to sites of polarized growth: Shs1p is required for Ssk2p localization at the neck, and Spa2p is largely involved in efficient localization of Ssk2p at the bud tip of small and medium-budded cells. However, our observation of Ssk2p localization at the incipient bud site in *shs1Δ spa2Δ* cells suggests the involvement of a third protein in Ssk2p localization at the incipient bud site.

Surprisingly, Ssk2p localization to the neck was also affected in *spa2Δ* cells. There are several possible explanations for this observation. Spa2p may be bridging an interaction between Ssk2p and the septins Cdc10p and Shs1p. However, the fact that Spa2p localizes at the neck later in the cell cycle than Ssk2p (Snyder, 1989; Arkowitz and Lowe, 1997) argues against this possibility. We and others (Snyder *et al.*, 1991; Zahner *et al.*, 1996; Sheu *et al.*, 2000) have observed that the mother/bud necks in *spa2Δ* cells are wider than those in wild-type cells (compare Figure 4G to H). Moreover, *spa2Δ* cells synchronized with HU were delayed in cell separation after release into low osmotic medium compared with wild-type cells, indicating that *spa2Δ* cells have general defects in cytokinesis and cell separation. Despite these defects, a GFP-Shs1p fusion protein was able to localize to the neck of *spa2Δ* cells. These data suggested that not only the presence of Shs1p at the septin ring but also proper organization of the septin filaments are important for neck localization of Ssk2p.

Could the Targets of the Ssk2p Kinase Be Members of the "Polarisome" Complex?

Spa2p is a large scaffolding protein that has been shown to display two-hybrid interactions with many important regulators of cell polarity and polarized actin assembly, including the formin Bni1p, Aip3p/Bud6p, and Pea2p (Sheu *et al.*, 1998). All of these proteins, including Spa2p have been shown to localize at the incipient bud site, at the tips of small and medium buds, and at the neck of cells undergoing cytokinesis (Snyder, 1989; Valtz and Herskowitz, 1996; Amberg *et al.*, 1997; Evangelista *et al.*, 1997). Localization of Bni1p at the bud tip is largely dependent on Spa2p though *spa2Δ* cells are not defective for Bni1p localization at the incipient bud site (Fujiwara *et al.*, 1998; Ozaki-Kuroda *et al.*, 2001), this is believed to be controlled by Cdc42p (Ozaki-Kuroda *et al.*, 2001). In contrast, Aip3p localization does not require Spa2p but is Cdc42 dependent (Jaquenoud and Peter, 2000; Jin and Amberg, 2000). Spa2p, Aip3p, Pea2p, and Bni1p have been shown to comigrate in velocity gradients (Sheu *et al.*, 1998) and are believed to form a complex (the polarisome) at sites of polarized growth that regulates the actin cytoskeleton by linking Rho GTPase signaling to actin filament assembly (Sheu *et al.*, 1998). Recent work has shown that Bni1p drives actin cable formation by nucleating filaments and that Aip3p is required for Bni1p-mediated polarized actin cable formation in vivo (Evangelista *et al.*, 2002; Sagot *et al.*, 2002a). Therefore, the polarisome complex

would seem to be a likely cytoskeletal substrate for Ssk2p and MTK1. Our observation that MTK1 is able to promote actin recovery in *ssk2Δ* and *spa2Δ* osmotically stressed cells suggests that the actin-related substrate of MTK1 is a conserved protein. Therefore, among the polarisome proteins, the formin Bni1p seems to be the strongest candidate to be a substrate for Ssk2p/MTK1 phosphorylation. Proteins of the formin family are highly conserved in all eukaryotes. In contrast, there have not been human or mouse homologs reported for Spa2p, Aip3p, or Pea2p. However, Spa2p was originally identified as a yeast protein reactive to antisera from a scleroderma patient, suggesting a human homolog may still exist (Snyder, 1989). Because Spa2p is a component of the polarisome complex, we hypothesize that in yeast it recruits Ssk2p/MTK1 to the bud cortex after osmotic stress thus bringing catalytically active kinase in close molecular contact with other members of the polarisome complex. Defects in actin recovery and bud emergence observed in *spa2Δ* cells might be attributable to both a failure to properly localize the kinase but more importantly a failure to recruit Ssk2p into the polarisome complex where it can find its substrate(s). Interestingly, Spa2p has been shown to interact with Ste11p, another MAPKKK of the HOG pathway (Sheu *et al.*, 1998), and to be required for localization of Mkk1p and Mpk1p, MAP kinases of the cell integrity pathway, to sites of polarized growth (van Drogen and Peter, 2002). Importantly, our data are the first demonstration of the functional significance of the scaffolding activity of Spa2p in a signaling pathway.

In summary, we have extended our preliminary analysis of the role of the Ssk2p kinase in actin recovery from osmotic stress showing that the kinase seems to be critical for efficient actin polarization at early and late stages of the cell cycle. In particular, we now have a good understanding of the proteins involved in regulation of Ssk2p localization at the neck (Shs1p and Cdc10p) and at the bud tip (Spa2p). Moreover, *spa2Δ* cells are as defective in actin recovery as the *ssk2Δ* cells, suggesting the two proteins cooperate within the same pathway. Bypass suppression of *spa2Δ* defects in actin recovery by overexpression of Ssk2 is consistent with Ssk2p acting downstream of Spa2p possibly by regulating other components of the polarisome complex such as Bni1p and Aip3p. The ability of the human MEK kinase, MTK1, to facilitate actin recovery in yeast indicates that we have uncovered a novel and yet conserved pathway for direct kinase regulation of actin cytoskeleton organization.

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