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Sho1 and Pbs2 Act as Coscaffolds Linking Components in the Yeast High Osmolarity MAP Kinase Pathway

Short Article

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Summary

Scaffold proteins mediate efficient and specific signaling in several mitogen-activated protein (MAP) kinase cascades. In the yeast high osmolarity response pathway, the MAP kinase kinase Pbs2 is thought to function as a scaffold, since it binds the osmosensor Sho1, the upstream MAP kinase kinase kinase Ste11, and the downstream MAP kinase Hog1. Nonetheless, previous work has shown that Ste11 can be activated even when Pbs2 is deleted, resulting in inappropriate crosstalk to the mating pathway. We have found a region in the C terminus of Sho1 that binds Ste11 independently of Pbs2 and is required for crosstalk. These data support a model in which Sho1 has at least two separable interaction regions: one that binds Ste11 and mediates its activation, and one that binds Pbs2, directing Ste11 to act on Pbs2. Thus, a network of interactions provided by both Sho1 and Pbs2 appears to direct pathway information flow.

Introduction

Cellular behavior is controlled by a complex network of signal transduction pathways. Given that each cell contains many related signaling proteins, how are proper pathway connections maintained? Scaffold proteins play a central role in directing information flow (Morrison and Davis, 2003; Pawson and Nash, 2003). They have multiple protein interaction sites that allow them to bind and coordinate the activity of multiple proteins within a given pathway. They are also thought to increase local concentrations of signaling components, increasing their efficiency of interaction and excluding nonspecific interactions with related but improper components.

Some of the best-characterized scaffold proteins are found in yeast mitogen-activated protein (MAP) kinase pathways (Elion, 2001; Whitmarsh and Davis, 1998). MAP kinase pathways are cascades of three kinases that successively phosphorylate and activate one another, referred to as MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). In yeast there are at least four distinct MAP kinase pathways involved in different signaling responses (Gustin et al., 1998; Sprague, 1998).

The yeast MAPKKK Ste11 acts in at least three separate pathways: the mating (pheromone) response pathway, the filamentous growth pathway, and the high osmolarity response pathway. Nonetheless, in wild-type strains, there is no crosstalk between these distinct MAPK pathways, due at least in part to the role of scaffold proteins. For example, in the mating response pathway (Figure 1A), the scaffold protein Ste5 interacts with the G_β protein Ste4 as well as the kinases Ste11, Ste7, and Fus3 (MAPKKK, MAPKK, and MAPK, respectively) (Elion, 2001). Because the scaffold organizes these proteins into a distinct complex, Ste11 molecules activated by the pheromone input are specifically directed to activate the downstream mating kinases Ste7 and Fus3 (Harris et al., 2001).

Similarly, in one branch of the high osmolarity response pathway, the MAPKK Pbs2 acts as a scaffold as well as a kinase (Figure 1A) (Posas and Saito, 1997). It interacts with the osmosensor Sho1 through a proline-rich peptide in Pbs2 that is recognized by a Src homology 3 (SH3) domain in Sho1. In addition, Pbs2 interacts with the MAPKKK Ste11 and the MAPK Hog1. Thus, Ste11 molecules that are activated by high osmolarity stress through Sho1 are directed to activate the downstream kinases Pbs2 and Hog1. The importance of scaffolding interactions in directing information flow is demonstrated by the fact that chimeric scaffold molecules can be designed that redirect mating input to osmolarity response output (Park et al., 2003).

At least some steps in the high osmolarity response MAPK pathway, however, can take place even in the absence of the Pbs2 scaffold. Specifically, in strains deleted for either Pbs2 (MAPKK and scaffold) or Hog1 (MAPK), stimulation with high osmolarity stress is observed to result in crosstalk to the mating response (O'Rourke and Herskowitz, 1998). This mutation-induced crosstalk is consistent with a model in which the osmolarity response normally results in feedback inhibition of the osmosensor Sho1 by activated Hog1. Thus, if either downstream kinase is deleted, this feedback inhibition is lost, resulting in a build-up of activated Ste11 that can then activate the downstream mating MAPKK Ste7 (Figure 1B). Sho1 and Ste11 are essential for crosstalk. If Pbs2 is truly a scaffold that coordinates communication between the pathway components, then how can Sho1 activate Ste11 in the absence of Pbs2? We therefore hypothesized that the osmosensor Sho1 may be able to directly interact with and activate Ste11.

Here we have systematically searched for regions in Sho1 that are required for crosstalk from osmostress to mating response. We found a region in the cytoplasmic tail that is required for crosstalk and is also sufficient for direct interaction with the MAPKKK Ste11. These findings indicate that Sho1 participates in at least two functional interactions: one with Ste11 that can mediate its activation upon osmoshock and another with Pbs2 that directs Ste11 to activate Pbs2. Thus, Sho1 also functions as a scaffold, working together with Pbs2 through a network of protein interactions to direct a specific extracellular input to a specific response output.

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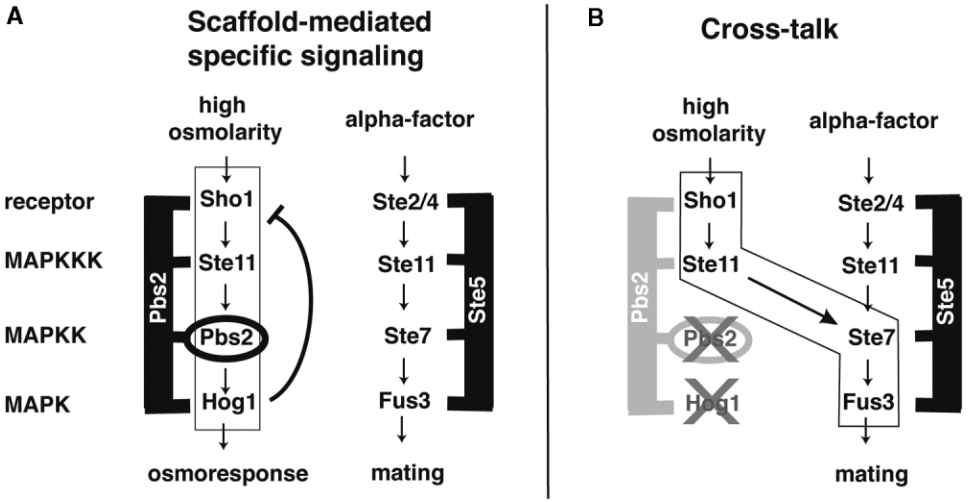

Figure 1. Scaffold Proteins in the Yeast High Osmolarity and Mating Response MAP Kinase Pathways

(A) The osmolarity MAPKK Pbs2 is thought to serve as a scaffold in the Sho1 branch of the osmolarity response pathway (Posas and Saito, 1997). Pbs2 interacts with the osmosensor Sho1, the MAPKKK Ste11, and the MAPK Hog1. The protein Ste5 serves as a scaffold in the mating response pathway (Elion, 2001). Ste5 interacts with the activated G_{β} protein Ste4 (activated by the G protein coupled receptor Ste2), the MAPKKK Ste11, the MAPKK Ste7, and the MAPK Fus3. These scaffolds prevent crosstalk between pathways, despite Ste11 being involved in both. Another mechanism thought to prevent crosstalk is negative feedback from Hog1 to Sho1, which may prevent excess activation of Ste11 (O'Rourke and Herskowitz, 1998). For clarity, several components of the pathways are not shown. First, the kinase Ste20 is not shown, although it is necessary for Ste11 activation. Scaffolding events upon activation are thought to recruit Ste11 in proximity of Ste20, which is membrane localized independently of the scaffold (Drogen et al., 2000). Second, an alternative branch of the osmolarity response pathway involving the two-component sensor Sln1 is not shown.

(B) When either Pbs2 or Hog1 is deleted, crosstalk from a high osmolarity stimulus to the mating response is observed. This crosstalk may occur because eliminating the negative feedback from activated Hog1 results in an excess buildup of activated Ste11, which can now activate Ste7. The fact that Ste11 activation takes place in the absence of the Pbs2 scaffold suggests that Sho1 may be able to independently interact with and activate Ste11.

Results

Identification of Regions in the Cytoplasmic Tail of Sho1 Required for Osmostress to Mating Response Crosstalk

If there were a region in Sho1 involved in direct interaction with and activation of Ste11, then mutation of that region should significantly impair crosstalk. However, this phenotype could also result from mutations involved in other general functions of Sho1, including proper folding and the ability to respond to high osmolarity. In addition, because Ste11 is directly activated by the upstream kinase Ste20 (Drogen et al., 2000), it is possible that this phenotype could also result from disruption of proper coordination between Ste20 and Ste11. We therefore reasoned that a way to screen for mutations that selectively interfere with Ste11 binding would be to compare effects on both osmoresponse and crosstalk. Mutation of a region involved in recruiting and activating Ste11, independently of Pbs2, might result in a significant loss of crosstalk from osmostress to mating response but a minimal or less significant loss in osmoresistance (since Pbs2 could still recruit Ste11). Such mutants would contrast with those that are important for interacting with Pbs2, which would show the opposite phenotype: a significant loss of osmoresistance and minimal loss of crosstalk.

We constructed mutants scanning through Sho1 and tested these mutants for the ability to rescue osmoresistance and mating crosstalk (Figure 2). Mutants were tagged with GFP to test for proper localization and expression. Sho1 has a short N-terminal cytoplasmic tail, four transmembrane regions, and a cytoplasmic C-terminal tail bearing a single SH3 domain. The mutant library included a deletion of the N terminus, mutation of all three loops between transmembrane regions (to equal length Gly-Ser repeats), mutation of the SH3 domain binding surface (SH3*; specific mutation W338F that is shown to disrupt SH3 binding [Zarrinpar et al., 2003], and scanning deletions of the cytoplasmic tail, including the SH3 domain [Figure 2A]).

We tested the ability of the Sho1 mutants to rescue osmoresistance in a strain deleted for Sho1 (Figure 2B). Because there is a redundant, Sho1-independent branch of the osmolarity response pathway, components of this pathway (the MAPKKKs Ssk2 and Ssk22) were also deleted to render the strain osmosensitive unless transformed with a functional *SHO1* gene (Maeda et al., 1994). Mutation of the SH3 domain surface or its deletion led to complete failure to rescue osmoresistance, consistent with the importance of this domain in mediating the Pbs2-Sho1 interaction. In contrast, deletions in the intervening loops or the remainder of the C terminus rescued partial to full osmoresistance (with the sole exception of the L2 mutation, which is neither expressed nor localized).

Mating crosstalk was tested in a strain deleted for Pbs2 (Figure 2C). This strain shows Sho1-dependent crosstalk from osmostress to the mating pathway be-

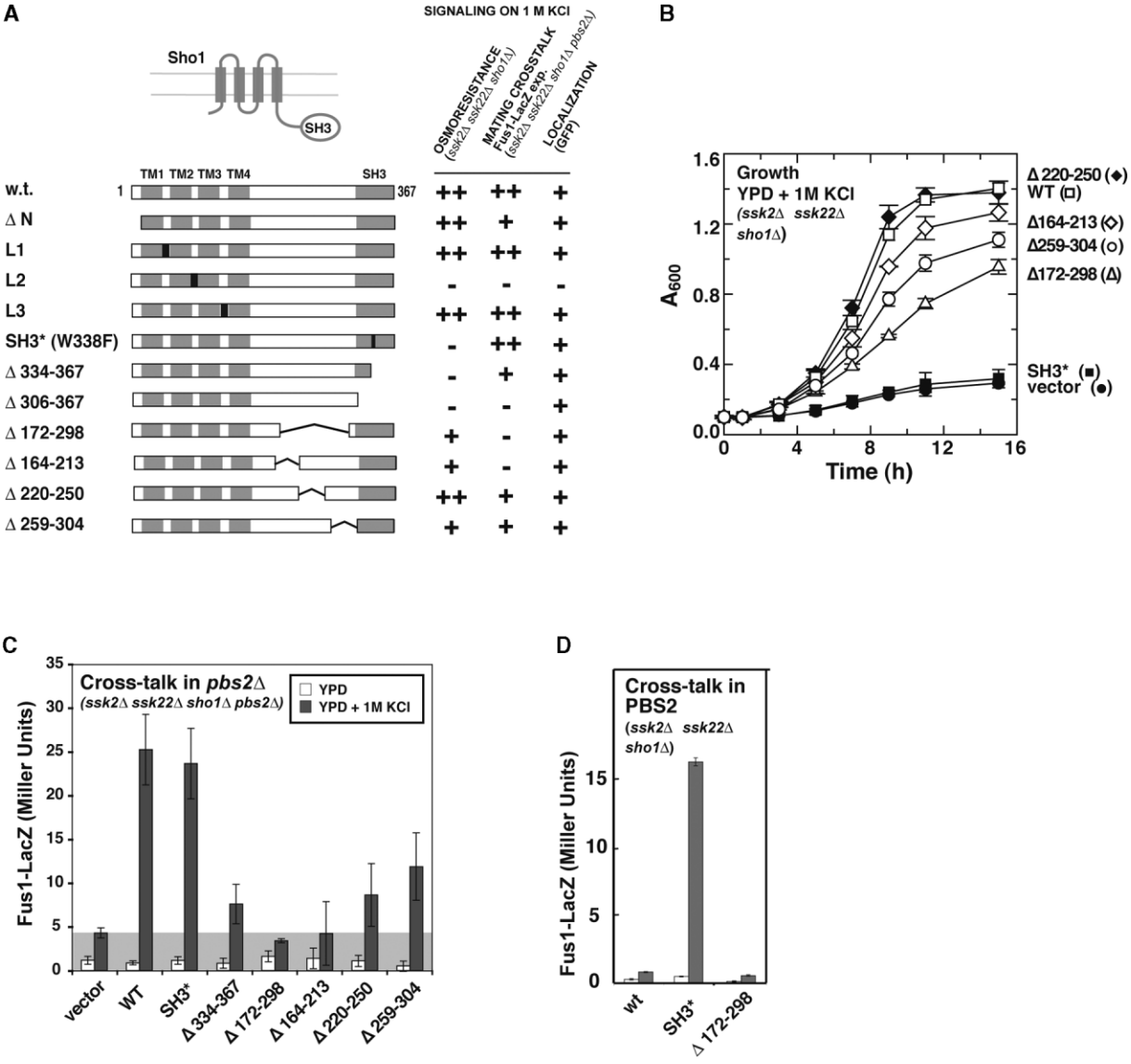

Figure 2. The Sho1 SH3 Domain Is Required for Osmoresistance, and the Intervening Region Is Required for Crosstalk

(A) Constructs used for functional analyses of Sho1 and a summary of results. Schematic of Sho1 (top) shows four putative transmembrane spanning regions and the cytoplasmic SH3 domain. Sho1 constructs here all contain a C-terminal GFP tag. In mutants L1, L2, and L3, the loops in black were replaced by equivalent length Gly-Ser repeats (L1, residues 61–63; L2, residues 88–92; L3, residues 120–121). The ability of the mutants to confer osmoresistance was tested by transforming them into SO355 (*ssk2*Δ *ssk22*Δ *sho1*Δ) and scoring growth on YPD + 1 M KCl. The ability of the mutants to rescue crosstalk was tested by transforming them into AZ113 (*sho1*Δ *pbs2*Δ) and testing for the induction of Fus1-lacZ (β-galactosidase assay). Localization and expression of the GFP fusions in SO355 was confirmed by fluorescence microscopy. (B) Complementation of osmoresponse in SO355 by Sho1 mutants: growth rates in YPD + 1 M KCl at 30°C were measured by following A_{600}. (C and D) Crosstalk (osmostress to mating response) by Sho1 mutants in (C) *pbs2*Δ and (D) *PBS2* strains (AZ113 and SO355, respectively). Yeast cells containing the indicated mutant Sho1 were assayed for β-galactosidase activity in the absence (white bars) or presence (gray bars) of 1 M KCl. The gray area in (C) indicates the background levels of crosstalk. Normally, the *PBS2* strain, when transformed with a wild-type *SHO1* copy, does not yield crosstalk.

cause activation of the downstream MAPK Hog1 is required for feedback inhibition of Sho1. Such crosstalk can be monitored by assaying the expression of a mating reporter gene (Fus1-lacZ) upon treatment with 1 M KCl. Sho1 bearing a mutation of the SH3 binding surface maintains high crosstalk, indicating that the SH3 binding surface is not required for Ste11 activation. Moreover, the SH3 mutant is still able to exhibit crosstalk even in a strain bearing wild-type Pbs2 and Hog1, consistent with the requirement for activated Hog1 to inhibit crosstalk (Figure 2D). Thus, the Sho1-Pbs2 interaction itself is not necessary for Ste11 activation, though the interaction does appear to be required to direct activated Ste11 to Pbs2.

Analysis of the remaining mutants identifies regions in the C terminus as critical for crosstalk but less important for osmoresponse; these regions are candidates for direct interaction with Ste11. Neither the N terminus

nor the intermembrane loops appear to be essential for crosstalk. Deletion of the SH3 domain (Δ306–367), or even parts of the domain (Δ334–367), impairs crosstalk. Thus, the SH3 domain structure itself may be important in Ste11 activation in a manner that is independent of the canonical SH3 interaction surface. For example, the SH3 domain may participate in interactions with Ste11 or with the upstream kinase Ste20. This model is consistent with observations that other surfaces on the SH3 domain are required for optimal osmoresistance (A. Davidson, personal communication). However, partial or full deletion of the SH3 domain also appears to slightly reduce expression levels of Sho1. Therefore, reduced crosstalk by these mutant proteins may be caused by protein instability.

Deletion of the intervening region of the Sho1 cytoplasmic tail (between the transmembrane regions and the SH3 domain, i.e., Δ172–298) results in clear loss of crosstalk with intermediate effects on osmoresistance. Although several regions distributed throughout the cytoplasmic tail appear to contribute to crosstalk, discrete regions appear to be particularly important. Specifically, Sho1 deleted for residues 164–213 shows nearly full osmoresistance (Figure 2B) but no detectable crosstalk (Figure 2C).

These findings suggest that the intervening cytoplasmic region of Sho1 plays an important role in crosstalk from osmostress to mating response, highlighting the region as a potential site for direct interaction with Ste11. In particular, Sho1 deleted for residues 164–213 may be impaired in its ability to bind Ste11 and, therefore, may not generate a level of Ste11 activation high enough to produce a significant crosstalk response. The recruitment of Ste11 via interactions with other parts of Sho1 and Pbs2, however, may still be sufficient to allow for a relatively unimpaired osmoresponse.

Identification of a Physical Interaction between the Osmosensor Sho1 and the MAPKKK Ste11

Current models posit that Ste11 recruitment to the membrane upon osmoshock allows its activation by the kinase Ste20, which in turn is activated by the membrane-localized GTPase Cdc42 (Drogen et al., 2000; Elion, 2001). Previously, Pbs2 was thought to mediate this recruitment event (Drogen et al., 2000; O'Rourke et al., 2002; Reiser et al., 2000). However, if Sho1 can activate Ste11 in a Pbs2-independent manner during crosstalk, it must be able to directly or indirectly recruit Ste11.

On the basis of the mutant analysis described above, we wanted to test the ability of the Sho1 cytoplasmic tail to physically bind Ste11 in a Pbs2-independent manner. We transformed a strain lacking Sho1 and Pbs2 with HA-tagged Sho1-cytoplasmic tail (C-tail), with or without Myc-tagged Ste11 (Figure 3A). The Sho1 cytoplasmic tail was immunoprecipitated by anti-Myc antibodies only in the presence of Ste11-Myc, indicating a physical interaction between Sho1 and Ste11 in the absence of Pbs2.

To map critical sites for the physical association between Sho1 and Ste11, we expressed several recombinant fusions of the Sho1 C-tail to glutathione S-transferase (GST) in *E. coli* and used these fusions as bait in assays to determine the ability to bind Myc-tagged Ste11 from *sho1*Δ *pbs2*Δ yeast lysates (Figure 3B). The Sho1 fusion variants included a point mutation of the SH3 binding surface (C-tail* = W338F), the SH3 domain alone, and a short fragment (145–211) encompassing most of the region identified as essential for crosstalk (Figure 2A). The full cytoplasmic tail is able to bind Ste11 from lysates, and mutation of the SH3 domain has no effect on this interaction. Moreover, even the 67 residue peptide shows a strong interaction with Ste11.

To determine whether this observed interaction between Sho1 and Ste11 is direct, we expressed fragments of both proteins in bacteria, purified them, and tested them for direct interaction. We expressed the Sho1 fragments as GST fusions and an N-terminal fragment of Ste11 (residues 1–200) as a His_6-tagged protein (full-length Ste11 is expressed very poorly in bacteria). This nonkinase region of Ste11 has been implicated in other interactions and the regulation of the kinase activity. This region of Ste11 was observed to bind directly to the Sho1 cytoplasmic tail (Figure 3C). Binding of Ste11 was also observed to a minimal fragment of Sho1 encompassing residues 172–211. Thus, we have identified a region of Sho1 that can directly and independently bind Ste11 and is functionally required for mating crosstalk (Figure 3D).

Sho1 Is Required for Osmoresistance of a Strain Bearing a Constitutively Active Ste11 Allele

If Pbs2 were to act as the sole scaffold in this pathway, then a constitutively active allele of Ste11 should be sufficient to confer osmoresistance to a strain deleted for Sho1. To test this hypothesis, we used the allele $Ste11^{S281D\ S285D\ T286D}$ (Ste11-CA), which mimics Ste11 that has been activated by Ste20 phosphorylation and has previously been shown to constitutively activate the mating response pathway (Drogen et al., 2000). We transformed this allele of *STE11* into strains with or without Sho1 (Figure 3E). An osmoresistant phenotype was only observed in the presence of Sho1, indicating that Pbs2 alone is insufficient to mediate its output to the osmolarity pathway. In addition, no Hog1 phosphorylation was observed in a *sho1*Δ strain with or without 1 M KCl stimulation (R.P.B., unpublished data). These findings are consistent with a possible scaffolding role for Sho1. If it played a scaffolding role, Sho1 would be required for proper signaling by a constitutively active allele of *STE11*; it could not simply be placed epistatically upstream from *STE11*.

Mapping the Physical Interaction between Pbs2 and Ste11

Although Ste11 was previously shown to bind Pbs2, little is known about this interaction site on Pbs2. A previous study identified regions of Pbs2 required for distinct branches of the osmoresponse pathway (Tatebayashi et al., 2003). Pbs2 residues 55–107 were identified as being required for the Sho1 branch but not the Sln1 branch of the pathway. Thus, this region could be involved in interaction with either or both of the components unique to that branch of the pathway—Sho1 or Ste11. Because this region includes the proline-rich peptide motif recognized by the Sho1 SH3 domain, it is not obvious whether this is also a site for Ste11 binding.

We used GST pull-down assays to determine whether this region could bind the bacterially expressed N-ter-

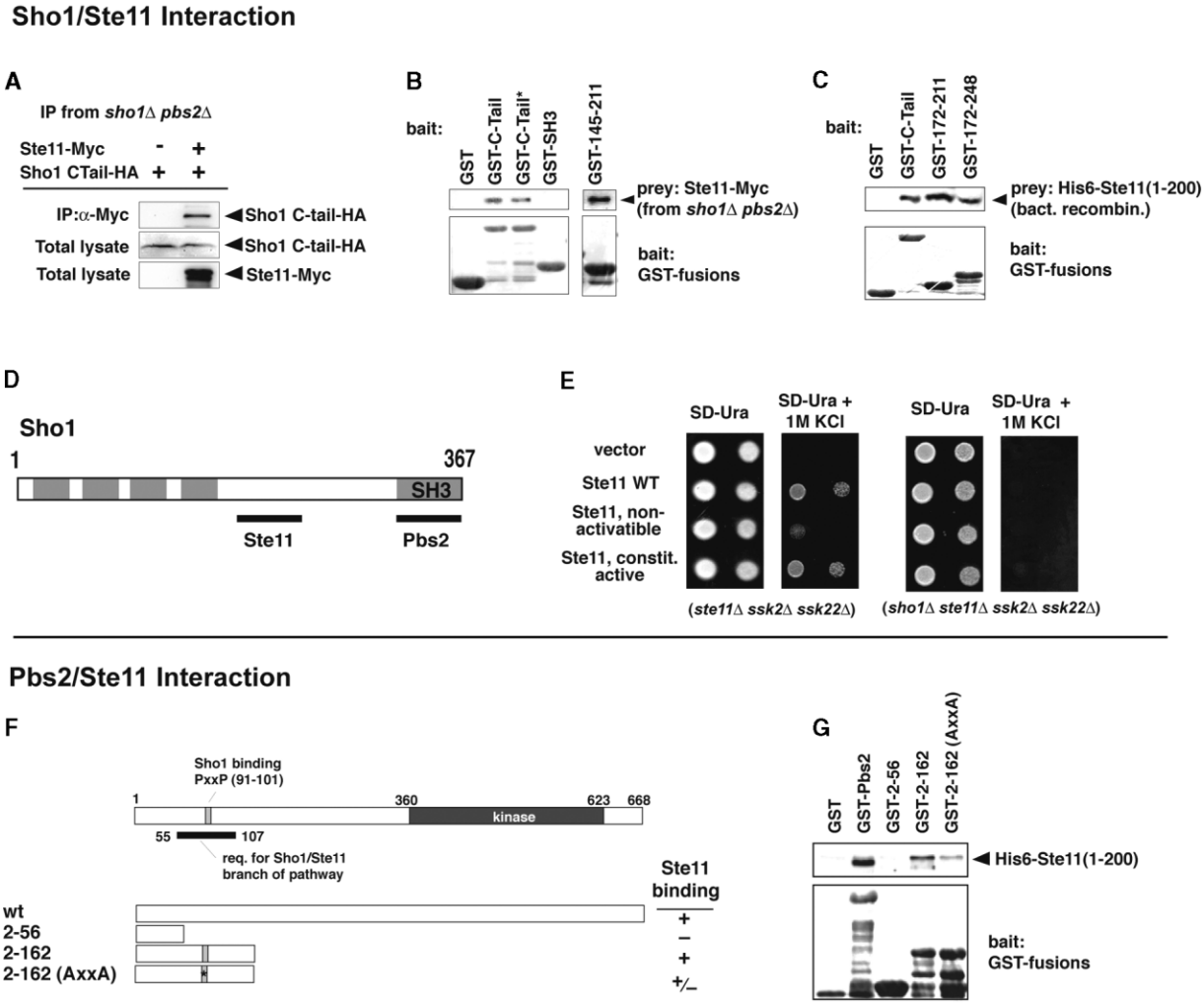

Figure 3. Physical Interactions between Sho1 and Ste11 (A–E) and between Pbs2 and Ste11 (F and G)

(A) Ste11 and the Sho1 C-tail (residues 145–367) coimmunoprecipitate from *sho1*Δ *pbs2*Δ yeast extracts. C-terminal Myc-tagged Ste11 expressed from a galactose-inducible promoter in a 2 μm vector (pRS424) was precipitated with agarose beads coated with antibody to Myc. The recruited Sho1 C-tail, expressed as a C-terminal HA tag fusion, was detected by immunoblot.

(B) Ste11 binds Sho1 independently of the SH3 domain. Sho1 mutants were constructed as N-terminal glutathione S-transferase (GST) fusions and expressed in *E. coli*. They were then purified from lysates by glutathione-conjugated agarose beads and incubated with yeast extract containing Ste11-Myc fusions expressed from a galactose-inducible promoter in a 2 μm vector. The bound proteins were analyzed by SDS-PAGE and either Coomassie stain (lower panel, total protein) or anti-Myc immunoblot (upper panel).

(C) Sho1 and Ste11 interact directly. GST pull-down assays were repeated with bacterially expressed GST-Sho1 fragments and His_6-tagged Ste11 (residues 1–200). The bound proteins were analyzed by SDS-PAGE and either Coomassie stain (lower panel, total protein) or anti-His immunoblot (upper panel).

(D) Schematic of the regions of Sho1 required for Ste11 and Pbs2 binding. The SH3 domain and its binding surface are required for Sho1/Pbs2 interaction (Posas and Saito, 1997; Zarrinpar et al., 2003). The Sho1 residues 172–211 comprise the minimal region sufficient for interaction with Ste11.

(E) Sho1 is also required for the transmission of signal from activated Ste11 to the osmo-response pathway. Ste11 variants, either wild-type, the nonactivatable allele (Ste11-NA: S281A, S285A, T286A), or the constitutively active allele (Ste11-CA: S281D, S285D, T286D), expressed in a *CEN/ARS* vector (pRS316) (or the empty vector alone), were transformed into strains with or without Sho1. The cells were then grown on SD-URA solid media with or without 1 M KCl in two different dilutions (left spots have 10-fold more cells than right spots). Only cells with Sho1 were osmo-resistant.

(F) Summary of the interaction between Ste11 and the N-terminal region of Pbs2. Two regions are highlighted: the PxxP motif required for binding to the Sho1 SH3 domain and a region that was previously identified as essential only for the Sho1 branch of the osmolarity pathway (residues 55–107; Tatebayashi et al., 2003).

(G) GST pull-down binding assays between Pbs2 and Ste11 fragments. Bacterially expressed GST fusions of Pbs2 fragments were incubated with bacterial lysates expressing His_6-Ste11(1–200). The bound proteins were analyzed by SDS-PAGE and either Coomassie stain (lower panel, total protein) or anti-His immunoblot (upper panel). The region important for Ste11 binding to Pbs2 overlaps that for Sho1 binding.

minal fragment of Ste11. The Ste11 fragment was able to bind both intact Pbs2 and a fragment (2–162) encompassing the region described above (Figures 3F and 3G). Pbs2 fragment 2–56 alone, which was shown to be dispensible for the Sho1/Ste11-dependent branch of the osmolarity pathway, did not bind Ste11. Identical results

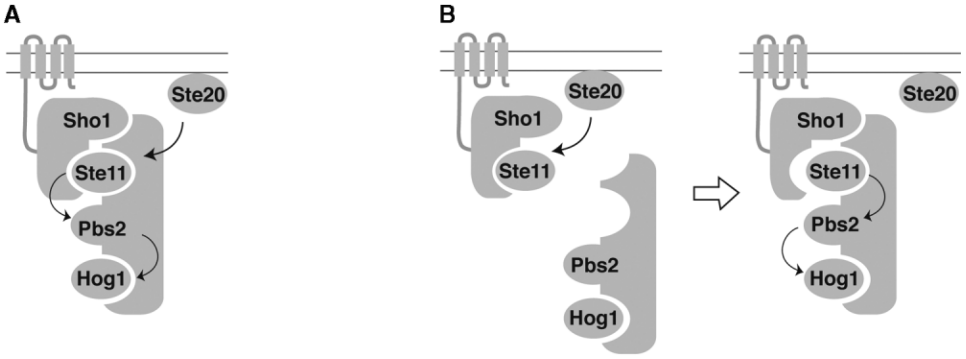

Figure 4. Models for How Sho1 and Pbs2 May Function Together to Direct Flow of Information in the Osmolarity MAPK Pathway

(A) Sho1 and Pbs2 may form a cooperative network of interactions that allows for the activation of Ste11 by Ste20, followed by the subsequent activation of Pbs2 and Hog1.

(B) Sho1 and Pbs2 may form sequential interactions with pathway components. For example, Sho1 may interact with Ste11 in a manner that allows for activation by Ste20. This initial complex may then transition to one in which active Ste11 is now transferred to a site on Pbs2, allowing for proper downstream events. This is one of several similar possible models.

were observed when these Pbs2 fragments were tested for interaction with full-length HA-tagged Ste11 expressed in yeast (data not shown). Interestingly, mutation of the proline-rich peptide in Pbs2 that binds the Sho1 SH3 domain also impairs but does not destroy interaction with Ste11. These data indicate that this N-terminal fragment of Pbs2 is able to physically interact with both Sho1 (via the SH3) and Ste11 and that the two proteins have some overlapping interaction requirements.

Discussion

Here we report that the MAPKKK Ste11 can directly bind a region in the cytoplasmic tail of the transmembrane osmosensor Sho1 (residues ~170 to ~210). Moreover, we show that this region is required to activate Ste11 in a Pbs2-independent manner: this region of Sho1 and not a functional SH3 domain (which binds Pbs2) is necessary to generate crosstalk to the mating pathway. Our analysis reveals that other regions of Sho1 also contribute to crosstalk, including the SH3 domain fold (but not the canonical binding surface) and other sites in the cytoplasmic tail. However, because these sites are equally important for crosstalk and osmosignaling, they cannot be uniquely implicated in Ste11 interaction. In principle, these other regions could be important for other aspects of Sho1 function, including stability, osmosensing, and coordination with Ste20. Finally, we show that Sho1 is required to transmit signal from a constitutive allele of *STE11* to the osmoresponse pathway. These findings are somewhat unexpected given that Pbs2 is viewed as the canonical scaffold of this branch of the osmolarity response pathway.

Although Pbs2 plays a key scaffolding role—organizing the components of this pathway and directing information flow—the current results suggest that Sho1 also plays an important scaffolding role. Sho1 physically binds two partners: Ste11 and Pbs2. Interaction with Ste11 most likely contributes to Ste11 activation by recruiting it to the proximity of active Ste20 at the cell membrane. Both Sho1 and Ste20 are localized to sites of polarization (Peter et al., 1996; Raitt et al., 2000; Reiser et al., 2000). Sho1's interaction with Pbs2, via the Sho1 SH3 domain, may then be required to direct the action of activated Ste11 to Pbs2. When the SH3 mediated interaction is disrupted, inappropriate crosstalk to the mating pathway (activation of MAPKK Ste7) occurs.

Together, these data suggest that it may be more appropriate to view Sho1 and Pbs2 as coscaffolds, cooperating to fulfill the role of Ste5 in the mating pathway. Previous studies demonstrated that when Ste11 and Pbs2 were covalently fused, activation of Ste11 led only to the osmoresponse and not the mating response (Harris et al., 2001). These data are consistent with a model in which Sho1 normally plays the role of an adaptor, functionally linking Ste11 and Pbs2.

It is possible that Sho1's interaction with Ste11 is one of the steps regulated by osmostress. However, it is difficult to see clear differences in the Sho1-Ste11 interaction by immunoprecipitation under osmotic stress conditions (A.Z., unpublished data), as cell lysis prior to precipitation may disrupt any such regulation.

This study indicates that a fairly small fragment of Sho1 (~170–210) is minimally sufficient for interaction with Ste11 in vitro. While it is clear that this fragment is energetically and functionally most critical for recruitment and crosstalk, respectively, other observations indicate that additional elements in Sho1 also contribute to Ste11 binding (A.Z., unpublished data). First, reduced but detectable levels of Ste11 binding are observed to the Sho1 C terminus even when residues 172–211 are deleted. Second, deletions of different regions (e.g., residues 258–304) significantly reduce interaction of the C terminus with Ste11. Thus, in the wild-type context, we suggest that a set of redundant interactions may work together to recruit Ste11 to the signaling complex—interactions with multiple regions of Sho1 as well as with Pbs2. These functionally redundant recruitment interactions may explain why these smaller deletions in Sho1 show only partial disruption of the osmoresistance phenotype (Figure 2B).

There are several distinct models for how Sho1 and Pbs2 may function together to direct kinase activation

events in the osmolarity response pathway. First, the two molecules and their coordinated interactions may function to cooperatively assemble the pathway members to mediate activation of Ste11 by Ste20 and the proper transmission of signal from Ste11 to Pbs2 to Hog1 (Figure 4A). However, alternative models are also possible in which Sho1 and Pbs2 coordinate sequential assembly events. For example, Sho1 may coordinate initial activation of Ste11 by Ste20 (Figure 4B), while subsequent interaction of Sho1 with Pbs2 may allow the transfer of activated Ste11 to a docking site on Pbs2 whereby it can communicate with downstream partners. This is only one of several possible sequential assembly models that are consistent with current data. Precisely which complexes can form remains somewhat unclear, as some interactions may be cooperative, whereas others may be mutually exclusive. For example, our studies suggest that Ste11 and Sho1 may partially overlap in their binding sites on Pbs2.

The Pbs2-independent scaffolding function of Sho1 may also be a result of evolutionary modularity. Sho1 has also been shown to be involved in a pathway that detects protein glycosylation (Cullen et al., 2000) and cell wall defects (Toh-e and Oguchi, 2001). This pathway appears to involve signaling from Ste11 to Ste7. Thus, it is conceivable that Sho1 first evolved to activate Ste11 in a Pbs2-independent manner, but then was later coopted through the SH3 interaction to transmit signals to Pbs2 and the osmolarity pathway.

Overall, these new findings indicate that the protein interactions that determine the flow of information in the Sho1 branch of the osmolarity pathway probably function as a coordinated network of interactions, rather than as a set of interactions mediated by only a single scaffolding molecule. Coassembly of interacting signaling molecules into a single complex has clearly emerged as an important mechanism for determining signaling specificity (Gavin et al., 2002), and there are apparently many ways in which to achieve this type of coassembly. Analysis of protein interaction data frequently identifies a network of crossinteractions between proteins involved in one pathway or process (Bader et al., 2001; Gavin et al., 2002; Wuchty et al., 2003). Thus, redundant, cooperative networks of interactions, as observed with Sho1 and Pbs2, may be a common mechanism of achieving specific complex assembly and consequently specific functional linkage.

Experimental Procedures

Plasmid/Strain Construction

All plasmids/strains are listed in Supplemental Table S1 at http://www.molecule.org/cgi/content/full/14/6/825/DC1. Sho1 mutants were constructed by PCR and expressed from the native promoter with either a GFP or HA3 C-terminal tag. Ste11 mutants were constructed by PCR mutagenesis and expressed from either the Ste11 native promoter or a *GAL1* promoter. (Note: Residue numbers for the Ste11 mutants differ from those cited [Drogen et al., 2000], but the mutations involved are identical. This problem arises because of discrepancies in the start codon chosen for Ste11 by different databases. Our residue numbers correspond with the *Saccharomyces* Genome Database entry.) Constructs used for coimmunoprecipitation were expressed with a Myc_{13} C-terminal tag.

Osmolarity and Crosstalk Assays

The ability of the Sho1 mutants to confer osmoresistance was tested by transforming them into SO355 (*ssk2 ssk22*Δ *sho1*Δ) and scoring growth at 30°C on YPD + 1 M KCl. Alternatively, for more quantitative comparisons, liquid culture growth rates were measured (A_{600}). The ability of Sho1 mutants to rescue crosstalk was tested by transforming them into SO356 (*sho1*Δ *pbs2*Δ) and assaying for induction of the mating reporter gene Fus1-lacZ (β-galactosidase assay). Cells at log phase were diluted to A_{600} 0.1 in media ± 1 M KCl, and after 4 hr were pelleted, washed, and lysed in a phosphate buffer containing 25% chloroform and 0.01% SDS. ONPG was added to 0.5 mg/ml, and after a 30 min incubation at 30°C the reaction was stopped by adding Na_2CO_3 to a final concentration of 0.4 M. β-galactosidase activity in Miller units was calculated by $(A_{420} \times 1000)/(A_{600} \times \text{minutes} \times \text{ml of culture})$. Crosstalk in the presence of wild-type Pbs2 was assayed by transforming the mutants into SO355 (*ssk2 ssk22*Δ *sho1*Δ).

Localization/Expression Analysis

Cells (SO355) bearing GFP fusions were grown in minimal media to mid-log phase and examined using a Nikon Microphot-SA fluorescence microscope with a 100× objective lens and a Princeton Instruments cooled charge-coupled device camera (RTE/CCD-1300-V).

Immunoprecipitation Binding Assays

Cells were grown at 30°C to mid-log phase in selective media containing 2% w/v glucose. Cells bearing *GAL1* promoter plasmids were grown in selective media containing raffinose (2%, w/v). At mid-log phase, galactose was added to 2% (w/v) for induction and cells were further grown for 6 hr at 30°C before harvesting. Fifty microliters of cells were harvested, resuspended in Y-PER lysis solution (Pierce, 3 ml/g cell pellet) containing protease inhibitors (leupeptin 5 μg/ml, chymostatin 5 μg/ml, pepstatin 5 μg/ml, PMSF 1 μg/ml), and incubated at 25°C for 20 min by gentle shaking. Lysates were cleared by centrifugation (18,000 g, 10 min), and 300 μl was mixed with 20 μl of anti-Myc agarose beads (Santa Cruz Biotechnology) and incubated at 4°C for 1 hr with gentle rocking. The beads were washed three to five times with 0.6 ml of ice-cold TBST buffer, resuspended in SDS sample buffer (50 μl) and 10 μl of each sample was used for immunoblotting. Blots were probed with HRP-conjugated anti-HA or anti-Myc antibodies (Santa Cruz Biotechnology) and developed by chemiluminescence.

GST Pull-Down Binding Assays

Recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3) RIL by growing cultures to $A_{600} = 0.7$ at 20°C and inducing with 1 mM IPTG for 3–6 hr. Cells were centrifuged, resuspended in PBS (50 mM sodium phosphate, 100 mM NaCl [pH 7.4]), and frozen at −80°C. Subsequently, cells were thawed and lysed by sonification. Lysates were cleared by centrifugation at 20,000 g. His_6 fusions were bound to Ni^{2+}-NTA resin (Qiagen) at 4°C, washed three times with PBS containing 20 mM imidazole, eluted with PBS containing 250 mM imidazole, and dialyzed three times into 100 mM NaCl, 10 mM HEPES (pH 8.0). GST fusions were bound to glutathione agarose at 4°C, washed three times with PBS + 2 mM dithiothreitol (DTT), and incubated directly with the prey at 4°C for 1 hr. The beads were then washed three times with PBS + 2 mM dithiothreitol (DTT) and resuspended in SDS sample buffer (50 μl), and 10 μl of each sample was used for SDS-PAGE and either Coomassie staining for total protein or immunoblotting using HRP-conjugated anti-His antibody (Santa Cruz Biotechnology).

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