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Protein-Protein Interaction Affinity Plays a Crucial Role in Controlling the Sho1p-Mediated Signal Transduction Pathway in Yeast

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Summary

Protein-protein interactions are required for most cellular functions, yet little is known about the relationship between protein-protein interaction affinity and biological activity. To investigate this issue, we engineered a series of mutants that incrementally reduced the affinity of the yeast Sho1p SH3 domain for its in vivo target, the MAP kinase kinase Pbs2p. We demonstrate a strong linear correlation between the binding energy of these mutants and quantitative in vivo outputs from the HOG high-osmolarity response pathway controlled by Sho1p. In addition, we find that reduction in binding affinity for the correct target within this pathway causes a proportional increase in misactivation of the related mating pheromone response pathway and that strong binding affinity alone does not guarantee efficient biological activity. Our experiments also indicate that a second binding surface on the Sho1p SH3 domain is required for its proper in vivo function.

Introduction

All aspects of cell growth, differentiation, and death are profoundly influenced by protein-protein interactions. For this reason, tremendous effort has been expended in recent years to use proteomic methods to define all the protein-protein interactions within a given cell type (Gavin et al., 2002; Giot et al., 2003; Ho et al., 2002; Ito et al., 2001; Tong et al., 2002; Uetz et al., 2000). These studies have revealed a highly complex network of interactions with most proteins interacting with many partners. A current hope is that these interaction networks will provide the basis for accurate computational modeling of cellular processes and prediction of the outputs from biological pathways under a given set of conditions. However, fundamental knowledge about the roles of binding affinity and specificity in directing these interaction networks must be obtained before this goal can be realized. For example, it is difficult to gauge the significance of a detected interaction between two proteins without knowing the affinity of the interaction and how that affinity compares to the affinities of interactions that the two proteins might make with other potential binding partners. To investigate the importance of protein-protein interaction affinity for the functioning of a signal transduction pathway, we have undertaken a comprehensive quantitative analysis of the relationship between binding affinity and biological response for a single protein-protein interaction domain in yeast.

The model system for our studies is the SH3 domain of the yeast protein Sho1p. SH3 domains, which are generally comprised of 55–60 residues, are found in many different types of proteins and mediate protein-protein interactions in a variety of signaling pathways in eukaryotic cells (reviewed in Dalgarno et al., 1997; Mayer and Eck, 1995; Pawson and Scott, 1997). Their important biological role, small size, and amenability to biophysical techniques make SH3 domains an attractive subject for structural, folding, and functional studies. SH3 domains bind to short peptides (9–15 residues), which usually contain a PXXP motif (where X can be any residue). Bound peptides contact a surface region on the domain rich in conserved aromatic residues (Feng et al., 1994; Lim et al., 1994; Musacchio et al., 1994) (Figures 1A and 1B). Less conserved polar residues in the RT-Src and N-Src loops, which flank the binding surface, also play important roles in binding.

Sho1p is a 367 residue membrane protein with a cytoplasmically located SH3 domain at its C terminus. The sequence of this SH3 domain and homologs from other yeast species are shown in Figure 1C. Sho1p functions in the HOG mitogen-activated protein kinase (MAPK) pathway in yeast, which responds to hyperosmotic stress (Brewster et al., 1993). The HOG pathway can be fully activated by two different transmembrane osmosensors: Sln1p (Maeda et al., 1994; Ota and Varshavsky, 1993) and Sho1p (Maeda et al., 1995); thus, both branches of the pathway must be inactivated to result in an osmosensitive phenotype. In the Sho1p-dependent branch, upon hyperosmotic stress, the PAK family kinase Ste20p activates the MAPKKK Ste11p, which in turn activates Pbs2p (the MAPKK) and Hog1p (the MAPK) (Figure 2A; reviewed in O'Rourke et al., 2002). Activation of Hog1p by phosphorylation leads to its translocation to the nucleus, where it stimulates the expression of numerous genes involved in the response to high osmolarity. A major role of Sho1p in the HOG pathway is to bind Pbs2p via its SH3 domain. Mutations that disrupt either the Sho1p SH3 domain or the proline-rich region of Pbs2p to which it binds interfere with recruitment of Pbs2 to the membrane and result in sensitivity of cells to hyperosmotic stress (Maeda et al., 1995; Reiser et al., 2000). Thus, the Sho1p SH3 domain-mediated interaction with Pbs2p is critical for pathway activation, and the functionality of Sho1p SH3 domain mutants can be easily assayed by measuring their sensitivity to hyperosmotic stress.

In addition to its role in the HOG pathway, Sho1p is also involved in the activation of other yeast MAPK pathways, such as the invasive growth pathway (O'Rourke and Herskowitz, 1998), and a related pathway that is induced by defects in protein glycosylation (Cullen et al., 2000). In strains that lack a functioning HOG pathway (e.g., *pbs2*Δ or *hog1*Δ strains), hyperosmotic stress causes an inappropriate Sho1p-dependent activation of the MAPK pathway that controls the mating response

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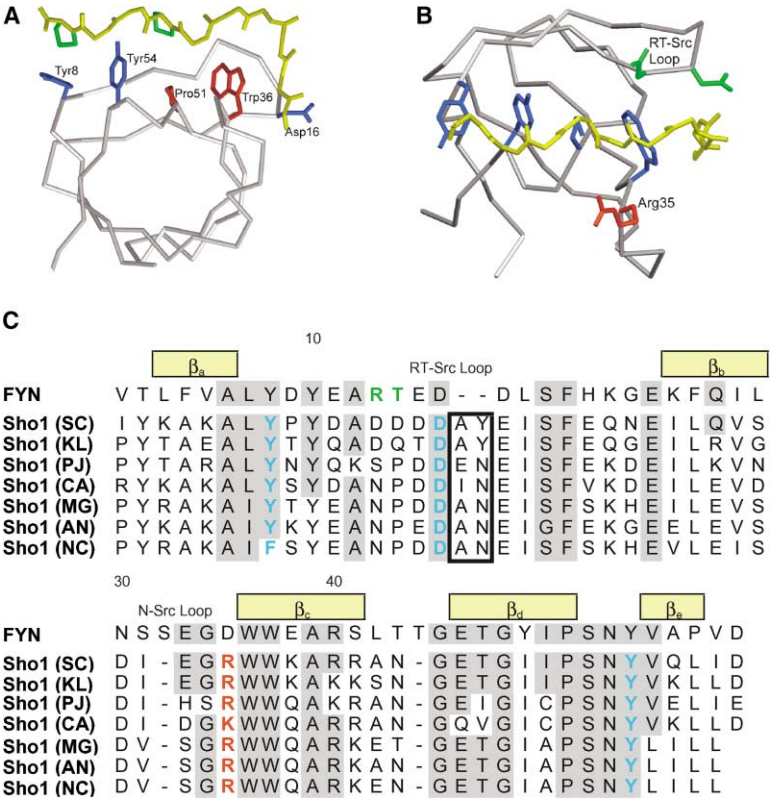


Figure 1. SH3 Domain Structure and the Role of the Sho1p SH3 Domain

(A) The structure of the Fyn SH3 domain bound to a PXXP-containing target peptide colored in yellow is shown here (PDB ID, 1FYN; Musacchio et al., 1994). Residues playing an important role in the binding reaction that were mutated in the Sho1p SH3 domain are shown in blue. Other conserved residues important for binding are shown in red. The Pro residues of the PXXP motif in the target peptide are shown in green. Amino acid positions in the SH3 domains discussed here are numbered using a standardized SH3 domain numbering system (Larson and Davidson, 2000). Molecular structure figures were created using SETOR (Evans, 1993).

(B) This Fyn SH3 domain:peptide complex is rotated approximately 90° around the *x* axis from its position in (A). Arg has been modeled at position 35 (red), and the residues in the RT-Src Loop between which the Sho1p SH3 domain AY insertion would be located are shown in green. Conserved residues involved in PXXP-containing peptide binding are shown in blue, and the bound peptide is shown in yellow.

(C) An alignment of Fyn SH3 domain and the Sho1p SH3 domain and its homologs from *S. cerevisiae* (SC), *Kluyveromyces lactis* (KL), *Pichia jadinii* (PJ), *Candida albicans* (CA), *Magnaporthe grisea* (MG), *Aspergillus nidulans* (AN), and *Neurospora crassa* (NC) is shown. The Arg 35 position is shown in red, and the unusual AY insertion is boxed. Conserved residues directly involved in PXXP binding that were mutated in the Sho1p SH3 domain to reduce affinity for Pbs2p are indicated in blue. The positions in Fyn that were mutated to increase binding affinity to the Pbs2p target peptide are shown in green. Residues that are identical in the Fyn and Sho1p (SC) SH3 domains are shaded.

(O'Rourke and Herskowitz, 1998). The crosstalk between these MAPK pathways in yeast is likely a result of their sharing a single MAPKKK, Ste11p, and other upstream pathway components, such as Cdc42p, Cdc24p, and Ste50p (Posas and Saito, 1997; Posas et al., 1998; Raitt et al., 2000; Reiser et al., 2000). Clearly, the regulation of these intertwined pathways, such that a given stimulus produces only the correct response, requires finely tuned control of the interactions between their protein components.

Since MAPK cascades are highly conserved signaling pathways in eukaryotes for responding to extracellular stimuli (reviewed in Toone and Jones, 1998; Waskiewicz and Cooper, 1995), and SH3 domains are one of the most ubiquitously utilized protein-protein interaction modules, the Sho1p system provides a very suitable representative model system for the study of signal transduction. The great advantage of this system over many others is that its output, resistance to high osmolarity, can be easily quantitated. In the work described here, we have exploited the Sho1p system to investigate the relationship between protein-protein interaction affinity and signaling pathway output. Our approach has involved the construction of amino acid substitutions to alter the affinity of the interaction between the Sho1p SH3 domain and its target sequence in Pbs2p. The effects of these substitutions have been accurately quantitated both in vitro by determining dissociation constant (K_d) values for each mutant and in vivo with a variety of phenotypic assays measuring outputs from the HOG and mating pathways. We have also tested the effect of replacing the Sho1p SH3 domain with a different SH3 domain that was engineered to bind to Pbs2p with high affinity. These experiments demonstrate the importance of protein-protein interaction affinity in determining the output level of a response and in preventing misactivation of related pathways.

Results

The Effects of Reduced Sho1p-Pbs2p Binding Affinity on HOG Pathway Activity In Vivo

Several Sho1p SH3 domain positions that were predicted to contact target peptide (Figures 1A and 1C) were substituted with a variety of amino acids in order to create a set of SH3 domain mutants with varying binding affinities. Each mutant SH3 domain was expressed and purified from *E. coli*, and its affinity for the PXXP-containing binding site from Pbs2p was quantitated using an in vitro Trp fluorescence assay as previously described (Maxwell and Davidson, 1998). The Asp16 position, a moderately conserved residue, was substituted with seven different residues (Phe, Cys, His, Ile, Ser, Asn, and Thr) with the expectation that the diverse properties of these amino acids would result in mutants with a wide range of binding affinities. Somewhat surprisingly, all of these mutants except D16H displayed virtually the same dissociation constant (K_d)

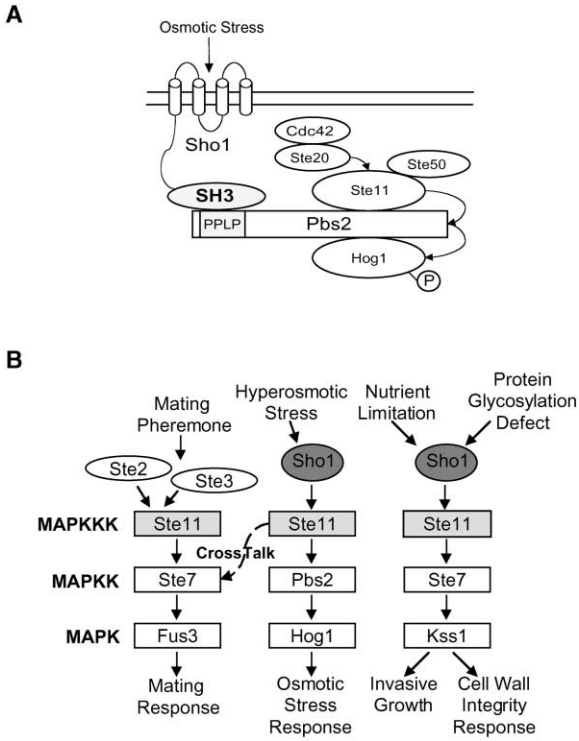


Figure 2. Yeast MAPK Pathways Involving Sho1p

(A) In the yeast high-osmolarity glycerol (HOG) MAPK pathway, the SH3 domain of the membrane-bound osmosensor Sho1p binds to a PXXP-containing site in the MAPK kinase (MAPKK) Pbs2p upon exposure to high osmolarity. As indicated, several other proteins, which interact with one another, are also required for the activation of this pathway. Arrows indicate the cascade of protein phosphorylation events occurring in the pathway that culminate in the phosphorylation of the MAPK Hog1p.

(B) The response to mating pheromone in yeast is also mediated by a MAPK pathway, which shares its MAPK kinase kinase (MAPKKK), Ste11p, with the HOG pathway. Exposure of *pbs2*Δ or *hog1*Δ strains to high osmolarity results in an aberrant "crosstalk" activation of the mating response, as indicated by the dashed line. Sho1p is also required for activation of the invasive growth response, and a similar response induced by protein glycosylation defects (Sho1p→ Ste12p response).

value of approximately 3 μM, which is 4-fold increased from wt (Table 1). For this reason, only the D16H and D16I single mutants were used in further studies. Substitutions at Tyr8 and Tyr54, which are highly conserved positions on the SH3 domain binding surface (Figure 1A), produced more substantial reductions in binding affinity (Table 1). A further diversity of K_d values was generated by combining the Y8A substitution with several Asp16 substitutions (Table 1). Since changes in the functional activity of the mutant SH3 domains characterized here could potentially be caused by thermodynamic destabilization, in vitro thermal unfolding experiments were performed on each mutant using circular dichroism (CD) spectroscopy. Most of the amino acid substitutions examined altered the temperature midpoint of the unfolding transition (T_m) by less than 10°C as compared to the wt (Table 1). Since the in vivo experiments described below were performed at 30°C, all of the mutants shown in Table 1 are expected to be fully folded under these conditions.

Table 1. In Vitro Binding and Stability Data for Sho1p and Fyn SH3 Domain Mutants

	K_d (μM)[a]	$\Delta\Delta G_{bind}$ (kcal mol^{-1})[b]	ΔT_m (°C)[c]
Sho1p SH3 Domain			
wt	0.8 ± 0.1	0.00 ± 0.11	0.0
R35L	0.4 ± 0.1	−0.42 ± 0.08	0.0
Y54F	1.0 ± 0.1	0.13 ± 0.08	−12.9
ΔAY	1.5 ± 0.1	0.37 ± 0.09	13.1
D16I	3.2 ± 0.1	0.81 ± 0.08	−1.1
D16H	6.3 ± 0.2	1.21 ± 0.08	4.6
Y8A/D16S	12.3 ± 0.4	1.61 ± 0.08	−1.7
Y8A	13.8 ± 1.9	1.68 ± 0.12	−7.3
Y8A/D16N	16.2 ± 2.5	1.77 ± 0.12	−0.4
Y8A/D16P	38.4 ± 5.4	2.28 ± 0.12	−10.7
Y54M	38.9 ± 5.4	2.29 ± 0.12	−5.6
Y54I	198 ± 27	3.25 ± 0.11	2.2
Y54A	283 ± 18	3.46 ± 0.09	−9.3
Fyn Sh3 Domain			
wt	17.1 ± 2.0	1.81 ± 0.11	0.0
R13D/T14D	1.3 ± 0.4	0.29 ± 0.19	−4.9
R13E/T14E	1.5 ± 0.1	0.35 ± 0.09	−5.1
R13E	4.3 ± 0.3	0.99 ± 0.09	0.8
T14D	6.7 ± 1.6	1.26 ± 0.16	−1.8
T14E	8.7 ± 0.4	1.41 ± 0.09	−2.4
T14R	54.8 ± 10.0	2.49 ± 0.13	2.4
Y54C	nd[d]	–	−2.8

[a] K_d values for the Sho1p target peptide from Pbs2p are shown. Values were averaged from two to four repetitions of each experiment. The errors shown are the standard deviations from the average values.

[b] $\Delta\Delta G_{bind} = -RT \ln (K_d^{wt}/K_d^{mutant})$. Larger values indicate weaker binding. Errors shown were propagated from the errors in the K_d values.

[c] $\Delta T_m = T_m^{mutant} - T_m^{wt}$. Negative values indicate less thermodynamically stable domains. The T_m values for the wt Sho1p and Fyn SH3 domains are 55.3°C and 80.1°C, respectively.

[d] This mutant displayed no detectable peptide binding.

The effects of reduced Sho1p-Pbs2p binding affinity on in vivo HOG pathway activity were studied by expressing in yeast each of the SH3 domain mutants described above in the context of the entire Sho1p protein from low copy number plasmids. Sho1p in these constructs was produced under the control of its native promoter with green fluorescent protein (GFP) fused at its C terminus. Normal cellular localization and expression levels of all the mutants were confirmed using fluorescence microscopy (Figure 5D) and immunoblotting with antibodies directed against GFP (Figure 3H). All assays described below were performed in strains where the Sln1p-mediated pathway for HOG pathway induction was inactivated by mutations, so that the growth of these strains in high osmolarity was dependent on the Sho1p-Pbs2p interaction.

Spotting a selection of Sho1p SH3 domain mutant constructs onto plates containing 0.8 M NaCl clearly demonstrated that the strength of the Sho1p-Pbs2p binding interaction significantly influences the activity of the HOG pathway (Figure 3A). For example, the growth of the Y8A/D16N mutant, with a K_d value of 16.2 μM, was severely impaired, and growth was almost completely abolished in the Y8A/D16P mutant, which had a K_d value of 38.4 μM. To gain a quantitative measure of HOG pathway output in the Sho1p mutant strains, expression

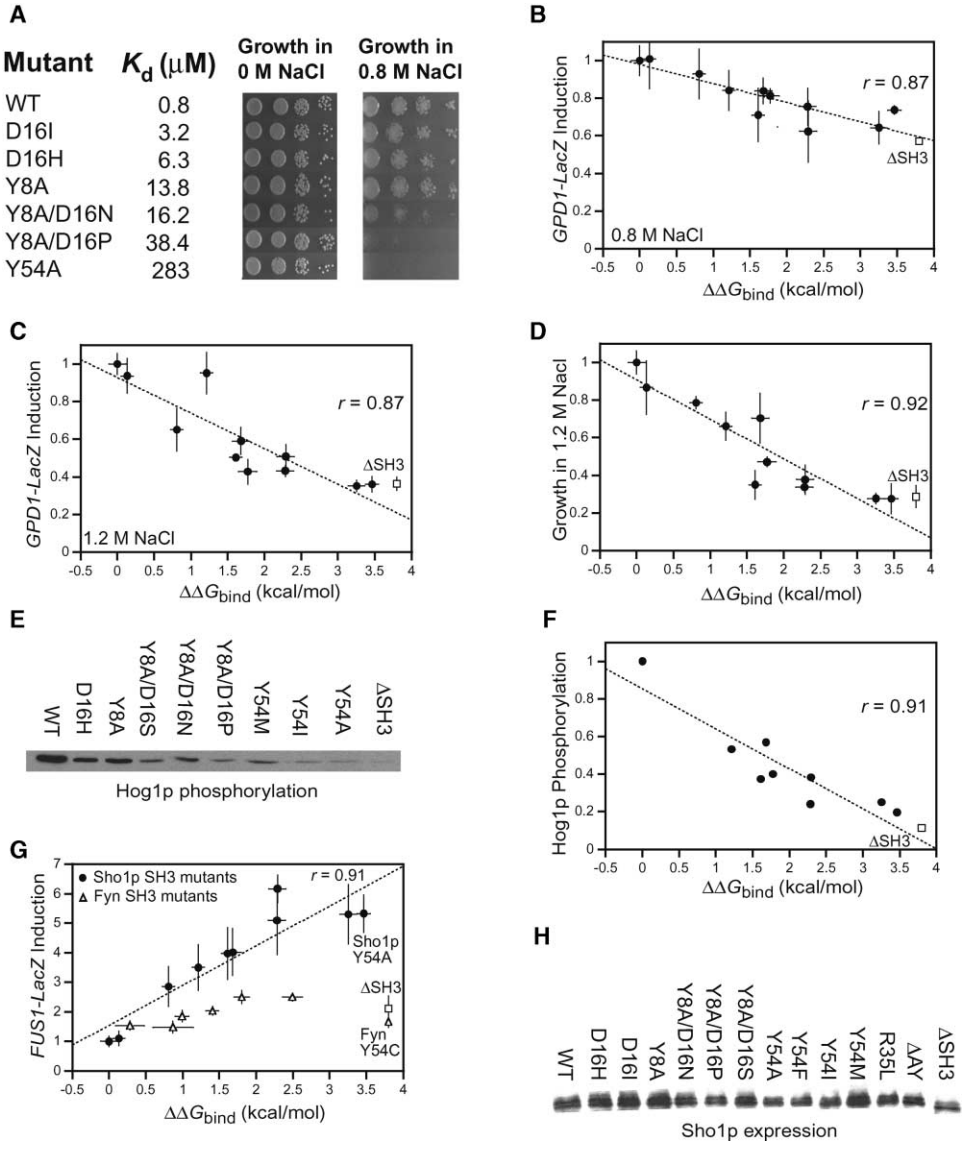


Figure 3. The Effects of SH3 Domain Mutants on HOG and Mating Response Pathway Output

(A) Serial 10-fold dilutions of cells were spotted on media with or without NaCl and grown at 30°C. A representative subset of the mutants tested are shown with their K_d values.

(B) The level of induction of *GPD1-LacZ*, a HOG pathway transcriptional reporter, after 3 hr in 0.8 M NaCl is plotted against the $\Delta\Delta G_{bind}$ of each Sho1p SH3 domain mutant shown in Table 1. β-galactosidase activity was normalized with the value for the wt Sho1p construct being set at 1.0. A correlation coefficient (*r* value) is shown for the straight line fit. In all graphs shown, Y error bars represent the standard deviation of at least three independent experiments, and the X error values are from Table 1. The ΔSH3 mutant, represented as an open square in all graphs, does not have a $\Delta\Delta G_{bind}$ value but was placed at a value of 3.8 for representative purposes here. Its value was not included in any of the linear fits.

(C) The same experiment described in (B) was performed in 1.2 M NaCl. In (B), the actual enzyme activity units varied from 438 for the ΔSH3 mutant to 765 for wt, while the range was 201–551 in (C). The higher basal activity in the lower salt conditions may be due to activation of the HOG response through a third yeast osmosensor, Msb2p (O'Rourke and Herskowitz, 2002).

(D) The level of growth of after 20 hr in 1.2 M NaCl is plotted against the $\Delta\Delta G_{bind}$ of each Sho1p SH3 domain mutant. Growth was assessed by measuring OD_{600}, and the value for the wt Sho1p construct was set at 1.0.

(E) Western blots using an antibody specific for the phosphorylated form of Hog1p were used to measure the level of Hog1p phosphorylation in cells carrying Sho1p mutants suspended in 1.2 M NaCl for 5 min. Western blots using an antibody directed against Hog1p that was not specific for the phosphorylated form demonstrated that the expression level of Hog1p was the same as wt in all of the mutant strains (data not shown).

(F) The band intensities in (E) were quantitated and plotted against the $\Delta\Delta G_{bind}$ values for each mutant.

(G) The ability of Sho1p mutants to induce the crosstalk response to the mating pathway was assessed by measuring induction of *FUS1-LacZ*. The level of induction after 4 hr in 1.2 M NaCl was plotted against the $\Delta\Delta G_{bind}$ for the wt and mutants of the Sho1p SH3 domain (closed circles) and wt and mutants of the Fyn SH3 domain (open triangles). The linear fit and *r* value were calculated for the Sho1p SH3 domain constructs only. The $\Delta\Delta G_{bind}$ value of the Fyn Y54C mutant, which showed no detectable peptide binding activity, was arbitrarily set at 3.8.

(H) The expression levels of the yeast Sho1p constructs carrying each SH3 domain mutant were assessed with Western blots using an anti-GFP antibody. The data shown are from a single experiment. In three repetitions of these assays, no consistent band intensity variations from wt were seen for any mutants.

of a HOG pathway-responsive β-galactosidase (β-gal) fusion gene, *GPD1-LacZ*, was assessed by measuring β-gal activity in cell lysates. We plotted the normalized level of β-gal activity measured for strains containing each Sho1p SH3 domain mutant grown in 0.8 M NaCl against the change in free energy of binding of each mutant as compared to the wt domain ($\Delta\Delta G_{bind} = -RT \ln [K_d^{wt}/K_d^{mutant}]$, weaker binding produces a larger value; Figure 3B). A remarkably good inverse linear correlation (*r* value, 0.87) was observed, and the same correlation was observed when the experiment was repeated at 1.2 M NaCl (Figure 3C). Notably, the slope of the linear fit in the 1.2 M NaCl experiments was considerably steeper than in the 0.8 M NaCl experiments, indicating that the repercussions of decreased binding activity are more pronounced when the severity of the stress is increased. The observed decreases in transcriptional activation were mirrored by the growth rates in high salt of the mutant strains, which also displayed a strong inverse correlation with the $\Delta\Delta G_{bind}$ values (Figure 3D). To obtain a direct and rapid measurement of the effect of the Sho1p SH3 domain mutants on HOG pathway activation, the extent of phosphorylation of Hog1p after exposure of cells to high salt was measured by Western blotting. The maximal level of phosphorylation of Hog1p occurred by 5 min after exposure to salt in all mutants and the wt strain (data not shown). The level of phosphorylation observed at this time point for a selection of mutants (Figure 3E) was quantitated and an excellent inverse correlation (*r* value, 0.91) with the $\Delta\Delta G_{bind}$ values was again observed (Figure 3F).

Is Binding Affinity the Only Crucial Determinant of Sho1p SH3 Domain Function?

To ascertain whether binding affinity to Pbs2p was the only determinant influencing Sho1p SH3 domain function in vivo, the Fyn SH3 domain, which shares 47% sequence identity with the Sho1p SH3 domain (Figure 1C), was engineered to bind the PXXP-containing site from Pbs2p with an affinity similar to that of the wt Sho1p SH3 domain. Although the Fyn and Sho1p SH3 domain binding sites are quite similar, the wt Fyn SH3 domain bound the Pbs2p target sequence relatively weakly with a K_d value of only 17 μM as compared to the K_d value of 0.4 μM with which it bound its own site (Figure 4A). Mutagenesis of the Fyn and Sho1p SH3 target sites at the P_{-3} position demonstrated that the Fyn SH3 domain strongly prefers Arg at this position as opposed to the Lys found in the Sho1p target site from Pbs2p (Figure 4A). For example, an Arg to Lys substitution in the Fyn target site reduced the affinity of the Fyn SH3 domain for the site by 30-fold. In contrast, the Sho1p SH3 domain did not discriminate between the Arg- and Lys-containing sites. Previous structural work on the Crk-N SH3 domain had shown that specific recognition of Lys at the P_{-3} position of SH3 domain binding sites was mediated by specific acidic residues in the RT-Src loop (Wu et al., 1995). Thus, we introduced acidic residues at homologous positions in the RT-Src loop of the Fyn SH3 domain (residues 13 and 14) in an attempt to increase its binding affinity for the Lys-containing Sho1p binding site. As we had predicted, the R13E, T14D, and T14E substitutions in the Fyn SH3 domain all increased its affinity for the Sho1p binding site by 2- to 4-fold (Table 1). The double mutants R13E/T14E and R13D/T14D displayed K_d values for the Sho1p site that were within 2-fold of the K_d value of the wt Sho1p SH3 domain.

The Fyn SH3 domain mutants were expressed and assayed in yeast in the context of intact Sho1p as described above. Spotting experiments on plates containing 0.8 M NaCl demonstrated that replacement of the Sho1p SH3 domain with the wt Fyn SH3 domain resulted in very poor growth (Figure 4B). The increased affinity of the designed Fyn SH3 domain mutants for Pbs2p did improve their ability to mediate growth in high salt, but growth was still not as robust as that seen for wt Sho1p. Surprisingly, when the Fyn SH3 domain constructs were grown in 1.2 M NaCl, they were unable to induce transcription of the *GPD1-LacZ* reporter gene or grow above the background level displayed by the ΔSH3 mutant (Figure 4C). Even in 0.8 M NaCl, the *GPD1-LacZ* expression was below what would be expected as compared to the activities seen for Sho1p wt domain and the Y8A/D16N mutant. These data demonstrate that ability of an SH3 domain to bind to the Pbs2p PXXP site with a similar strength as the wt Sho1p SH3 domain is not sufficient for the wt level of HOG pathway activation in vivo.

Sho1 SH3 Domain Mutants Cause Misactivation of the Yeast Mating Response

In strains lacking a functional HOG pathway due to *PBS2* or *HOG1* deletions, crosstalk through Sho1p to the mating response pathway leads to inappropriate high-osmolarity-induced activation of genes involved in mating (O'Rourke and Herskowitz, 1998). Since many of the Sho1p SH3 domain mutations constructed here reduce HOG pathway activation, we wondered whether a concomitant increase in crosstalk to the mating pathway would be observed. Strains carrying our mutant Sho1p constructs were transformed with a reporter plasmid bearing a gene in which the promoter region of *FUS1*, a gene induced by the mating response, was fused to the coding region of *LacZ*, so that crosstalk activation of the mating response could be quantitated by measuring β-galactosidase activity in cell lysates. It can be seen in Figure 3G that increases in the $\Delta\Delta G_{bind}$ values of the Sho1p SH3 domain were strongly correlated with increased induction of the crosstalk response (*r* value, 0.93). Strikingly, the Y54A mutant, which binds to the Pbs2p-derived PXXP-containing peptide very weakly (Table 1), induced a robust crosstalk response (Figure 3G). On the other hand, the Sho1pΔSH3 mutant was unable to activate the crosstalk response or the HOG pathway. In addition, none of the Fyn SH3 domain mutants induced a strong crosstalk response. These data indicate that the ability of the Sho1p SH3 domain to bind PXXP-containing peptides is not required for the activation of the crosstalk response but that some other features of the Sho1p SH3 domain are critical.

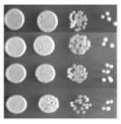
Sho1p SH3 Domain Mutants that Affect Crosstalk Activation but Not Pbs2p Binding

The data described above suggested that the Sho1p SH3 domain may possess some unique sequence features that allow it to activate the mating response. In

A

	P_{-5} P_{-4} P_{-3} P_{-2} P_{-1} P_0 P_1 P_2 P_3	K_d (μM) Sho1p	Fyn
Fyn Target	A R **R** P L P P L P	2.7	0.4
Fyn Target (R→K)	A R **K** P L P P L P	2.1	12
Sho1p Target (Pbs2p)	V N **K** P L P P L P	0.8	17
Sho1p Target (R→K)	V N **R** P L P P L P	1.6	3

B

Fyn Mutant	K_d (μM)	Growth in no NaCl	Growth in 0.8 M NaCl
WT	17		
T14R	50		
R13E	4.3		
R13D/T14D	1.3		

C

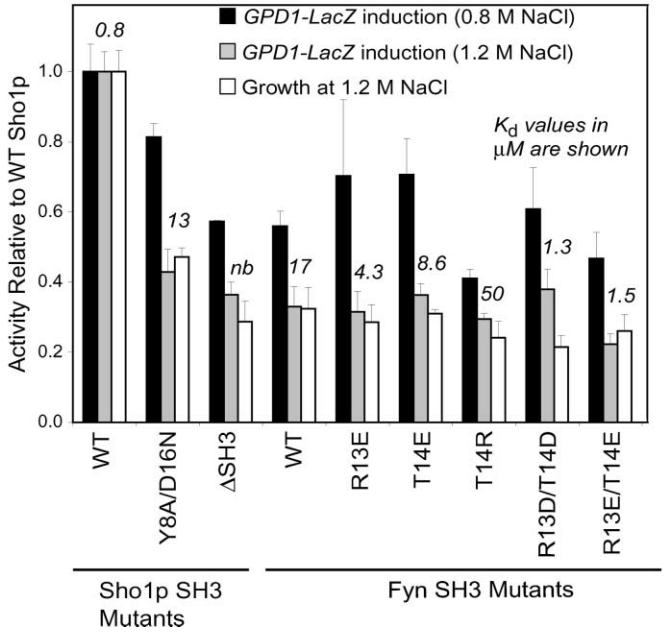


Figure 4. Activity of the Fyn SH3 Domain in the HOG Pathway

(A) The Sho1p target sequence shown is from Pbs2p and the Fyn target sequence was isolated by phage display (Rickles et al., 1995). Binding assays were performed with these sites and sites bearing single substitutions at the P_{-3} position. The K_d values of the wt Sho1p and Fyn SH3 domains for these sites are shown.

(B) Growth of a *sho1*Δ strain of yeast transformed with plasmid constructs expressing full-length Sho1p with its SH3 domain replaced by the wt or mutant Fyn SH3 domains was assessed. Serial 10-fold dilutions of the indicated mutants were spotted on media with or without NaCl and grown at 30°C.

(C) In vivo HOG pathway activation by Sho1p bearing the wt and mutant Fyn SH3 domains was quantitated by measuring *GPD1-LacZ* activation and cell growth as described in Figure 2. The K_d values of each mutant for the PXXP-containing site in Pbs2p are shown. The activity of mutants in each assay was normalized to the activity of the wt Sho1p construct, which was assigned an activity of 1.0. Each experiment for each mutant was repeated at least three times, and the errors shown are the standard deviations from the averages.

examining an alignment of Sho1p SH3 domain homologs from *S. cerevisiae* and six other yeast species, we observed features that were very unusual for SH3 domains in general (Larson and Davidson, 2000) yet were conserved among the Sho1p homologs (Figure 1C). Most striking was a two residue insertion in the functionally important RT-Src loop. In contrast to the Sho1p homologs, more than 85% of SH3 domains have an RT-Src loop that is the same size as the Fyn SH3 domain shown in Figure 1C. Another striking feature of the Sho1p homologs is that they display only Arg or Lys at position 35. In our alignment, Sho1p was the only SH3 domain out of 266 analyzed to possess a positively charged residue at this position. We hypothesized that these unusual residues in the Sho1p SH3 domain might be involved in crosstalk activation of the mating response. For this reason, we tested the effects of an R35L substitution and a deletion of the two extra residues in the RT-Src Loop (ΔAY).

It can be seen in Table 1 that the ΔAY and R35L substitutions had small effects on binding affinity with R35L displaying a 2-fold lower K_d value than wt and ΔAY displaying a 2-fold higher value. Neither substitution caused destabilization of the domain, and the ΔAY substitution was actually significantly stabilizing. Since the ΔAY and R35L mutant SH3 domains of Sho1p-bound Pbs2p target peptide at levels comparable to the wt SH3 domain, they were not expected to activate the crosstalk response in the presence of a functioning HOG pathway. Thus, these mutants were placed in a *pbs2*Δ strain in which a crosstalk response is observed even in the presence of wt Sho1p (O'Rourke and Herskowitz, 1998). Strikingly, both mutants exhibited dramatic reductions in their ability to activate the crosstalk re-

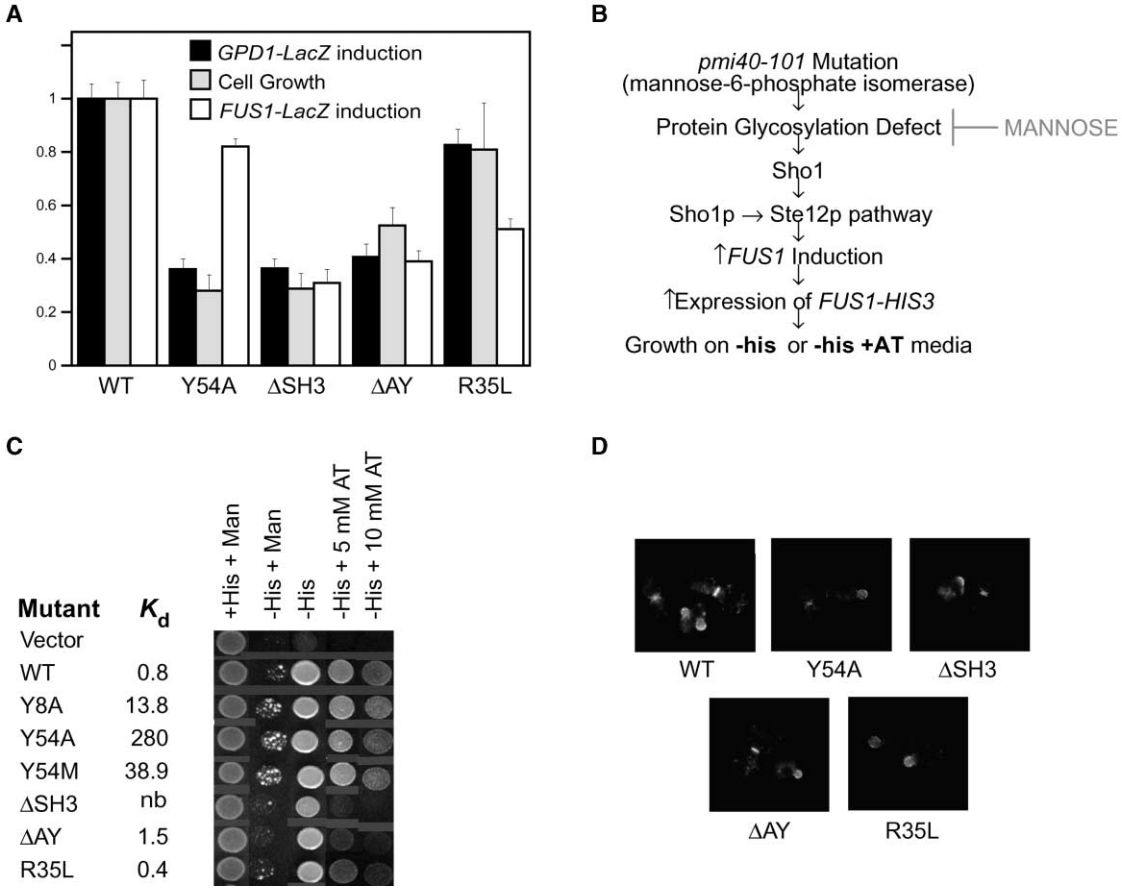


Figure 5. Activity of the R35L and ΔAY Sho1p Mutants

(A) The ability of mutants to activate the HOG pathway was assessed as described in Figure 3. Induction of the crosstalk response to the mating pathway was assessed by measuring induction of *FUS1-LacZ* in a *pbs2*Δ strain, a background in which the wt and SH3 domain mutants that maintained strong Pbs2p binding capability would be expected to activate the response.

(B) Diagram of the Sho1p→Ste12p pathway that leads to *FUS1* activation as the result of a protein glycosylation defect. This pathway can be shut off by the addition of 50 mM mannose.

(C) A strain containing the *pmi40-101* (glycosylation defect) and *sho1*Δ mutations was transformed with wt and a selection of mutant Sho1p constructs and assayed for induction of a *FUS1-HIS3* reporter. Expression of the reporter gene was detected by spotting on plates without His and with added AT. Growth was for 3 days at 30°C. The K_d value in μM of each mutant tested for binding to Pbs2p is shown.

(D) Fluorescence images of cells used in the experiments described here, which express Sho1p-GFP, are shown. wt Sho1p localizes to the bud and the neck region between mother and bud (Reiser et al., 2000). No differences in localization were observed for any of the characterized Sho1p mutants. Four examples are shown here.

sponse as compared to wt (Figure 5A). The ΔAY mutant was also much more severely impaired in activating the HOG response than would be expected from its K_d value for Pbs2p, while the R35L showed a small but consistent reduction in HOG pathway activation.

To assess the effects of our mutants on the induction of a more physiologically relevant pathway that is also activated independently of Pbs2p, we investigated a Sho1p-mediated response induced by protein glycosylation defects (Cullen et al., 2000). Our collection of mutant Sho1p constructs was placed in a strain deleted for *SHO1* that also carried a mutant allele of mannose-6-phosphate isomerase (*pmi40-101*). The protein glycosylation defect resulting from this mutation leads to constitutive activation of a Sho1p→Ste12p pathway, which can be suppressed by the addition of mannose (Figure 5B). The level of activation of this pathway was quantitated in our studies using a fusion between the coding region of *HIS3* and the promoter of *FUS1*, which is induced by the pathway. Transcription of this fusion gene allows the cells to grow on minimal medium plates without histidine, and higher level expression of the gene also confers resistance to aminotriazole (AT) (Cullen et al., 2000). Figure 5C shows that Sho1p is required for activation of the Sho1p→Ste12p pathway as the vector control cannot grow on the plate without His. The inability of the ΔSH3 mutant to grow well on AT indicates that the SH3 domain is required for optimal activation of the pathway. Reduced binding affinity of Sho1p for Pbs2p did not affect activation of the Sho1p→Ste12p pathway, as even the very weakly binding Y54A mutant was still able to fully activate this response (i.e., the cells are resistant to 10 mM AT). Remarkably, the ΔAY mutant induced the response almost as weakly as the deletion of the whole SH3 domain, and the R35L mutant was also debilitated in induction of the response, in that very

little growth was seen on 10 mM AT (Figure 5C). These data imply that the AY insertion and Arg 35 are part of a functional interface of the Sho1p SH3 domain that is distinct from the PXXP-containing peptide binding surface but is required for the activation of both the crosstalk and Sho1p→Ste12p pathways.

Discussion

Protein-Protein Interaction Affinity Plays a Crucial Role in Controlling Signaling Pathway Outputs

The results presented here highlight the importance of binding affinity for the proper functioning of a signal transduction pathway. We have rigorously demonstrated a strong linear correlation between the binding energy of the Sho1p SH3 domain-Pbs2p interaction as measured in vitro and output from the HOG pathway as quantitated in vivo by measuring growth rate, transcriptional activation, and the degree of phosphorylation of Hog1p (Figure 3). While the HOG pathway still functions at a low level even when the strength of the Sho1p-Pbs2p interaction is diminished 20-fold (Figure 3A), full activation of the response requires close to the wt binding affinity. Our results agree with a recent study by Zarrinpar et al. where a correlation between binding affinity and biological activity, as assessed by growth of cells on plates with 1 M KCl, was observed in the Sho1p system (Zarrinpar et al., 2003). Our study also shows that the strength of the interaction becomes more critical when the severity of the stress is heightened (Figures 3B and 3C). The crucial role of binding affinity for the proper functioning of this pathway is further emphasized by our observation that reducing the affinity of the Sho1p-Pbs2p interaction causes a proportional misactivation of the mating response (Figure 3G). Since the mating pathway leads to cell cycle arrest, its misactivation in the presence of salt would be detrimental to cell growth. We speculate that the wt level of interaction between Sho1p and Pbs2p may play a dual role of both producing a robust HOG response and preventing any crosstalk activation of the mating response.

The linear correlation between the binding energies of mutants measured in vitro, where the tested components are diffusing freely at relatively low protein concentrations, and the in vivo functional behavior of these mutants is striking. Sho1p and Pbs2p appear to behave as freely diffusible entities inside the cell even though the in vivo situation is expected to be much more complicated. For example, Sho1p is membrane bound, and the Sho1p-Pbs2p interaction likely occurs within a multiprotein complex where modulation of the Sho1p-Pbs2p interaction by other associated proteins could occur (Figure 2A). The Sho1p-Pbs2p interaction also must be somehow yield limiting for the pathway because if either Pbs2p or Sho1p were present in excess compared to the other reactants in the pathway, we would have seen little change in the biological response as binding affinity was reduced until some threshold K_d value was reached. It should be noted that there could be subtle threshold-dependent responses buried within our observed linear response that we could not detect due to the unavoidable degree of error in our biological assays. One would expect that some components in any pathway would be present in excess; thus, it will be of great interest in future studies similar to this one to determine which components of pathways are in excess and which are not.

Linear relationships between the binding affinity of mutants and biological activity have been observed in other studies, particularly in the examination of receptor-ligand interactions (Pearce et al., 1999; Piehler et al., 2000; Warner et al., 2002; Zhu et al., 2003). However, very few studies have examined intracellular signal transduction pathways as has been done here. One comparable study examined the interaction between Ras and Raf, which is required to recruit Raf to the plasma membrane and subsequently activate a MAPK pathway. The ability of ten Raf mutants with incrementally reduced binding affinity for Ras to activate transcription in vivo was assessed, and a very strong linear correlation to the logarithm of the K_d was revealed (Block et al., 1996). This result is the same as we have observed with the Sho1p system, which indicates that other signal transduction pathways are likely to behave in this manner. However, there is no reason to think that all protein-protein interaction systems will behave this way, since some may display a switch-like response, and other interactions may not control a yield-limiting step in a pathway.

Another intriguing aspect of our results is that each assay of HOG pathway output that we performed, growth, transcription, and Hog1p phosphorylation, showed the same linear response with a similar slope (Figures 3C, 3D, and 3F) even though these measurements were made 3 hr, 20 hr, and 5 min, respectively, after the addition of salt. These results imply that the magnitude of the HOG response is determined very quickly after the application of stress and that the effects of the early events in the response persist for a long period of time. Interestingly, the speed with which Hog1p becomes phosphorylated was not affected by reduction in the affinity of the Sho1p-Pbs2p interaction (data not shown), only the level of phosphorylation was changed. Interactions between other proteins involved in the pathway likely determine the rate of pathway activation.

While we were able to successfully engineer the Fyn SH3 domain to bind to the Pbs2p PXXP site with a K_d close to that of the wt Sho1p SH3 domain (Table 1), neither the wt nor mutant Fyn SH3 domains were able to mediate a significant HOG response when a strong stress was applied (Figure 4C). This result demonstrates that other factors, which might include altered on and off rates for binding or reduced binding specificity, can modulate the functional capacity of a protein-protein interaction module inside the cell. It is also possible that a second binding surface on the Sho1p SH3 domain, which is not present in the Fyn SH3 domain, also plays a role in activation of the HOG pathway. In any case, the Fyn SH3 domain data clearly illustrate that relatively strong in vitro binding between two proteins participating in a signal transduction pathway does not necessarily guarantee a normal biological response.

An SH3 Domain Surface Distinct from the PXXP Binding Surface Is Important for Sho1p Function

Sho1p has been shown to play a role in activating other pathways besides the HOG pathway, including crosstalk

to the mating response when Hog1p or Pbs2p are absent, and a Sho1p→Ste12p pathway in the presence of protein glycosylation defects, which resembles the invasive growth response (Cullen et al., 2000; O'Rourke and Herskowitz, 1998). However, the region or regions of Sho1p involved in activating these pathways was not previously identified. Since a Sho1p construct lacking its SH3 domain is unable to effectively activate either the mating pathway or Sho1p→Ste12p pathways (Figures 5A and 5C), the SH3 domain is clearly required for this function. Surprisingly, the debilitation of the PXXP binding activity of the Sho1p SH3 domain seen in mutants, such as Y54A, does not reduce the level of activation of these pathways, indicating that a surface of the SH3 domain that is distinct from the PXXP binding surface must be involved (Figures 5A and 5C).

The ΔAY and R35L mutants were unable to activate the crosstalk or Sho1p→Ste12p response even though they still bound to the PXXP-containing site of Pbs2p at close to wt levels (Figures 5A and 5C) and were also significantly impaired in their abilities to activate the HOG pathway. These data imply that the residues affected by these mutations are located on a distinct binding surface of the Sho1p SH3 domain that is required for full activation of the HOG, mating response, and Sho1p→Ste12p pathways. The existence of a second interaction interface on the Sho1p SH3 domain could explain why the Fyn SH3 domain fails to activate the HOG pathway as well as Sho1p and also fails to activate the mating response (Figures 3G and 4C). A shared component of both the HOG and Sho1p→Ste12p pathways, for example Cdc42p, Ste20p, Ste50p, or Ste11p, may be bound by this surface. As shown in Figure 1B, position 35 and the RT-Src loop where the AY insertion occurs in Sho1p are close enough to be part of a single protein-protein interface. However, these two regions are also involved in PXXP binding in most SH3 domains (Larson and Davidson, 2000), and without detailed structural information it is difficult to formulate a mechanism by which the Sho1p SH3 domain could simultaneously bind a PXXP-containing peptide and another protein using this interface. The existence of two different functioning binding surfaces on a single SH3 domain has been described in several other cases (Chan et al., 2003; Douangamath et al., 2002; Nishida et al., 2001). Interestingly, the Pex13 SH3 domain, which is one of the domains shown to possess two distinct protein-protein interaction surfaces, also possesses an insertion in its RT-Src loop.

Conclusion

By performing a quantitative analysis in vitro and in vivo, we have been able to answer fundamental questions about the role of protein-protein interaction affinity in the function of the Sho1p SH3 domain in the process of signal transduction. Reduction of the binding energy of the Sho1p-Pbs2p reaction both decreased HOG pathway response and increased aberrant mating pathway activation in a linear fashion. We also showed that binding affinity for Pbs2p is not the only factor important for the function of the Sho1p SH3 domain and that a second binding surface unique to this domain appears to be required for its optimal function both in the HOG pathway and in other pathways that do not involve Pbs2p. This work vividly demonstrates the complexities inherent in intracellular signal transduction pathways, which share some components, yet respond to different stimuli and produce unique outputs. It is clear that a key to understanding the mechanisms controlling these pathways lies in defining the affinities of interactions within them and determining how modulating these affinities will alter the output response. Future quantitative studies on other proteins and pathways will surely reveal that cells have evolved a wide variety of strategies to control their response to the environment.

Experimental Procedures

Plasmids, Protein Purification, and Mutagenesis

To express the Sho1p SH3 domain in *E. coli* for the purpose of purification, DNA encoding the Sho1p SH3 domain (residues 302–367) was amplified from plasmid CBP3485, which contains the *SHO1* gene (kindly provided by the laboratory of C. Boone). The amplified DNA was then ligated between the NcoI and XhoI sites of pET21d (Novagen) in a manner such that the SH3 domain was expressed with a 6-His C-terminal tag. The Fyn SH3 domain was also expressed from a pET21d-derived vector as previously described (Maxwell and Davidson, 1998). All mutations in the Sho1p and Fyn SH3 domains were constructed using standard PCR-based methods.

To perform in vitro binding assays, DNA encoding the region of Pbs2p containing the Sho1p SH3 domain binding site (residues 88–104, QQIVNKPLPPLPVAGSS) was synthesized and ligated into a previously described *E. coli* expression vector in which the target peptide is expressed with the DNA binding domain of bacteriophage lambda at its N terminus and a 6-His tag at its C terminus (Maxwell and Davidson, 1998). The lambda repressor does not interact with the SH3 domain and does not contain any Trp residues. To measure binding to the Fyn target sequence, a similar lambda repressor fusion construct was used, which carried the sequence VSLARRPLPPLP, a tight binding sequence for the Fyn SH3 domain isolated by phage display (Rickles et al., 1995). This construct was used in previous work (Maxwell and Davidson, 1998).

All proteins were expressed in *E. coli* BL21 STAR (λDE3) (Novagen), which contains a deletion of the RNaseE gene (*rne131*) to increase protein expression levels. Cells were grown to an OD_{600} of 0.6–0.8 and induced with 1 mM IPTG for 3 hr. All protein purifications were carried out in 6 M GuHCl using Ni-NTA (Qiagen) affinity chromatography as previously described (Maxwell and Davidson, 1998). Once purified, proteins were refolded by dialysis into 50 mM phosphate buffer (pH 7.0) with 100 mM NaCl. All in vitro assays were performed in this buffer.

For in vivo studies in yeast, the complete *SHO1* gene fused to the gene for GFP was carried on plasmid pAZ301 (kindly provided by A. Zarrinpar and W. Lim), which was derived from the low copy number vector pRS316 (Sikorski and Hieter, 1989). Mutant SH3 domains were amplified by PCR from the expression vectors described above and subcloned into pAZ301 using unique EcoRI and BamHI sites present on the vector, which resulted in removal of the wt domain. The Sho1pΔSH3 mutant was made by inserting the oligonucleotide linkers into the BamHI and EcoRI sites of pAZ301.

In Vitro Peptide Binding and Protein Stability Assays

Peptide binding by SH3 domains was quantitated using Trp fluorescence as previously described (Maxwell and Davidson, 1998). Protein concentrations were determined from absorbance at 280 nm, with extinction coefficients calculated from the amino acid composition of the protein (Pace et al., 1995). Experiments to assess the thermal stability of mutant SH3 domains were carried out using circular dichroism spectroscopy as previously described (Maxwell and Davidson, 1998).

In Vivo Assays of Sho1p Function

All assays of HOG pathway function and mating response crosstalk were carried out in yeast strains AZ116 (*sho1::hisG ssk2::HIS3-Cg*,

ssk22::HIS3-Cg) or AZ117 (*sho1::hisG*, *ssk2::HIS3-Cg*, *ssk22::HIS3-Cg*, *pbs2::KAN*), which were derived from W303 (*ade2-1 can1-100 his3-11 leu2-3 trp1-1 ura3-1*). These strains were kindly provided by A. Zarrinpar and W. Lim.

To assay growth on solid media, cells were grown to mid-log phase in selective media, diluted to 5×10^6 cells/mL, and serial 10-fold dilutions were spotted on selective plates with 0.8 M NaCl. Plates were incubated for 3 days at 30°C. Endpoint growth assays in liquid culture were carried out by growing cells to mid-log phase in selective media, then diluting them in fresh media with 1.2 M NaCl to a final OD_{600} of 0.2. The cells were then grown for 20 hr at 30°C, with the maximum OD_{600} reaching approximately 0.8. Some of the mutant strains were also investigated by obtaining complete growth curves over a 24 hr period, and comparable results were obtained (data not shown). These growth curve experiments demonstrated that the cells were still growing exponentially after 20 hr in 1.2 M NaCl; thus, the results in Figure 3D do not reflect the behavior of cells in stationary phase.

For analysis of *GPD1* induction by HOG pathway activation, cells were cotransformed with the plasmid, p3162, which contains 800 bp of the *GPD1* promoter controlling expression of the *LacZ* gene (Harris et al., 2001). Cells were grown to mid-log phase in selective media, then diluted into fresh media containing 0.8 M or 1.2 M NaCl for 3 hr. β-galactosidase assays were then carried out by harvesting 1 ml of cells and washing with 1 ml of Z buffer (150 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$). Cells were resuspended in 150 μl of Z buffer + 0.27% v/v β-mercaptoethanol and permeabilized by addition of 20 μl of 0.1% SDS and 50 μl of chloroform, followed by vortexing for 15 s. Reactions were initiated by addition of 700 μl of prewarmed 1 mg/mL ortho-nitrophenol-β-D-galactopyranoside in Z buffer + 0.27% v/v β-mercaptoethanol and incubated at 30°C for 10 min. Reactions were stopped by addition of 0.5 ml of 1 M Na_2CO_3, and β-galactosidase activity was calculated according to the formula A_{420}*1000/[OD_{600}*$time_{(min)}$*$volume_{(mL)}$]. Activation of the crosstalk mating response was assessed in the same way except that cells were cotransformed with the reporter plasmid p3058, which contains 834 bp of the *FUS1* promoter controlling *LacZ* gene expression (Roberts et al., 2000). The reporter plasmids used were both kind gifts from the laboratory of C. Boone. In all of these experiments, the level of activation displayed by the weakest binding mutants (Y54I and Y54A) and by a mutant with its SH3 domain deleted (ΔSH3) was similar to that displayed by cells carrying the vector plasmid with no Sho1p gene at all (data not shown). Residual activation of the HOG pathway seen in these cases was likely due to the presence of another membrane osmosensor in the these cells, Msb2p, which can activate the HOG pathway to low levels in the absence of Sho1p (O'Rourke and Herskowitz, 2002).

Assays to measure activation of the Sho1p→Ste12p induced by glycosylation defects were carried out in strain 506 (*ste4*, his3::FUS1-*HIS3*, *pmi40-101*, *sho1::URA3*, *ssk1*), which was kindly provided by P. Cullen and G. Sprague (Cullen et al., 2000).

Western Blots

Western blots were used to quantitate the level of phosphorylated Hog1p in cells after addition of salt. Cells were grown to mid-log phase, and 1.0 OD_{600} unit of cells was harvested and concentrated in 1 ml of selective media lacking uracil prior to addition of 1.2 M NaCl for the indicated times. Protein extracts were prepared as previously described (O'Rourke and Herskowitz, 1998). Samples were run on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST + 5% skim milk powder for 1 hr at room temperature, incubated with mouse monoclonal (phospho-p38 antibody 28B10 [NEB]) at 4°C overnight, and visualized using the ECL detection system (Amersham) using horseradish peroxidase-conjugated anti-mouse secondary antibodies. The level of phosphorylation of Hog1 was quantitated by scanning exposed films and using Adobe Photoshop 7.0 to quantitate the pixel density of each band. Several different exposure times were used for each blot to ensure that the intensity of the each band was within the linear exposure range of the film. The accuracy of this quantitation procedure was verified by assessing the intensity of bands in lanes with the same extract diluted by defined amounts. In control blots, a polyclonal antibody raised to the C terminus of Hog1 (y-215) (Santa Cruz Biotech) was used to determine the total level of Hog1 in cells, and a monoclonal anti-GFP antibody (B-2) (Santa Cruz Biotech) was used to quantitate the level of Sho1p-GFP present in cells under investigation. These blots were repeated at least three times to ensure reproducibility and equal loading of extract into each lane.

Microscopy

Cultures were grown at 30°C to mid-log phase in synthetic medium (SD) with plasmid selection. Strains were examined using phase-contrast microscopy and viewed through a fluorescein isothiocyanate filter to observe GFP fluorescence. Photographs were taken with a Micromax 1300y high-speed digital camera (Princeton Instruments, Trenton, NJ) mounted on a Leica DM-LB microscope. Images were analyzed using Metaview software (Universal Imaging, Media, PA). The localization of Sho1p does not change in high-osmolarity conditions (Reiser et al., 2000); thus, these assays were performed without addition of salt.

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