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# Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator

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**Exposure of yeast cells to increased extracellular osmolarity induces the HOG1 mitogen-activated protein kinase (MAPK) cascade, which is composed of SSK2, SSK22 and STE11 MAPKKKs, PBS2 MAPKK and HOG1 MAPK. The SSK2/SSK22 MAPKKKs are activated by a 'two-component' osmosensor composed of SLN1, YPD1 and SSK1. The SSK1 C-terminal receiver domain interacts with an N-terminal segment of SSK2. Upon hyperosmotic treatment, SSK2 is autophosphorylated rapidly, and this reaction requires the interaction of SSK1 with SSK2. Autophosphorylation of SSK2 is an intramolecular reaction, suggesting similarity to the mammalian MEKK1 kinase. Dephosphorylation of SSK2 renders the kinase inactive, but it can be re-activated by addition of SSK1 *in vitro*. A conserved threonine residue (Thr1460) in the activation loop of SSK2 is important for kinase activity. Based on these observations, we propose the following two-step activation mechanism of SSK2 MAPKKK. In the first step, the binding of SSK1 to the SSK1-binding site in the N-terminal domain of SSK2 causes a conformational change in SSK2 and induces its latent kinase activity. In the second step, autophosphorylation of SSK2 renders its activity independent of the presence of SSK1. A similar mechanism might be applicable to other MAPKKKs from both yeast and higher eukaryotes.**

*Keywords*: MAP kinase/phosphorylation/signal transduction/stress response/two-component sensors

## Introduction

MAP kinase cascades are common signaling modules found in both higher and lower eukaryotic cells. A typical MAP kinase cascade is composed of three tiers of protein kinases, a MAP kinase (MAPK), a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (Waskiewicz and Cooper, 1995). The budding yeast (*Saccharomyces cerevisiae*) has several MAP kinase cascades that transduce distinct extracellular stimuli (e.g. mating pheromone, high osmolarity, low osmolarity and nitrogen starvation) (Herskowitz, 1995). Yeast cells respond to increases in extracellular osmolarity by activating the HOG1 MAP kinase (Figure 1). Because a major function of the activated HOG1 MAPK is to elevate the synthesis of glycerol, the signaling pathway that leads to HOG1 activation is referred to as the HOG (high osmolarity glycerol response) pathway (Brewster *et al.*, 1993). The HOG pathway is essential for the survival of yeast in high osmolarity environments (Boguslawski, 1992; Brewster *et al.*, 1993; Albertyn *et al.*, 1994). Extracellular hyperosmolarity in yeast is detected by two independent transmembrane osmosensors, SHO1 and SLN1. The SHO1 osmosensor has four predicted transmembrane segments and a C-terminal cytoplasmic region containing an SH3 domain. SHO1 activates the PBS2 (MAPKK) through the STE11 (MAPKKK) (Maeda *et al.*, 1995; Posas and Saito, 1997). Once activated by phosphorylation, PBS2 activates the HOG1 MAPK, which induces glycerol synthesis and other adaptive responses.

The SLN1 osmosensor is a homolog of prokaryotic two-component signal transducers. Typical bacterial signaling pathways consist of two components: a sensor molecule (a transmembrane protein with a cytoplasmic histidine kinase domain) and a response regulator (a cytoplasmic protein with an aspartate-phosphorylated receiver domain). SLN1 is a transmembrane protein containing both a cytoplasmic histidine kinase domain and a receiver domain (Ota and Varshavsky, 1993; Maeda *et al.*, 1994). In two-component systems, an activated histidine kinase phosphorylates a histidine residue near the kinase domain, and this phosphate subsequently is transferred to an aspartic acid residue in the receiver domain. The phosphorylation of the receiver domain modulates its effector function, such as transcription regulation. Thus, the essence of any two-component system is the His–Asp phosphotransfer reaction (phospho-relay) (Bourret *et al.*, 1991; Stock *et al.*, 1991; Parkinson and Kofoid, 1992; Wurgler-Murphy and Saito, 1997). However, more complex two-component systems, such as the *Bacillus subtilis* Spo0 sporulation regulatory pathway, are known that involve multi-step phospho-relay reactions, typically in the order of His→Asp→His→Asp (Burbulys *et al.*, 1991). The yeast SLN1 osmosensor also utilizes a multi-step phospho-relay mechanism that involves His and Asp phosphorylation sites within SLN1, another His phosphorylation site in the intermediary protein YPD1 and an Asp in the receiver domain protein SSK1 (Maeda *et al.*, 1994; Posas *et al.*, 1996). Our genetic and biochemical characterization of the SLN1–YPD1–SSK1 phospho-relay suggested that under high osmolarity conditions, SLN1 histidine kinase activity is suppressed, resulting in accumulation of non-phosphorylated SSK1 (Maeda *et al.*, 1994; Posas *et al.*, 1996). It was also implied that non-phosphorylated SSK1 activates a MAPK cascade that is composed of SSK2/SSK22 (MAPKKKs), PBS2 (MAPKK) and HOG1 (MAPK).

Typical bacterial two-component systems are self-contained signal transduction mechanisms, because the response regulators themselves have effector functions

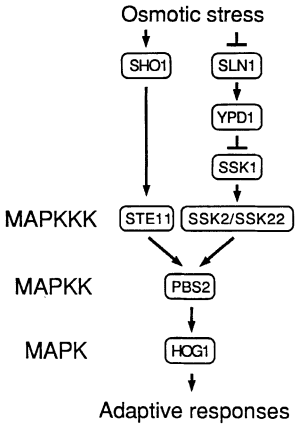


**Fig. 1.** Schematic diagram of the yeast HOG osmoregulatory signal transduction pathway. A current model of the pathway is shown indicating the name of the different elements. The two-component osmosensor is composed of SLN1, YPD1 and SSK1, whereas SHO1 is an alternative osmosensor. The arrows do not necessarily indicate direct interactions.

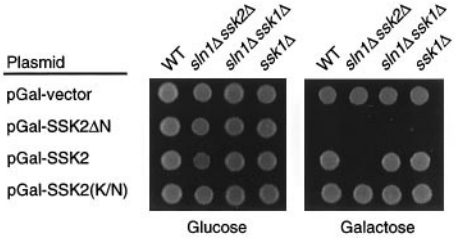


**Fig. 2.** Effect of various *SSK2* alleles on the HOG pathway. Host cells were transformed with plasmids carrying various *SSK2* alleles under the control of a $P_{GALS}$ promoter. The relevant genotypes of the host cells are indicated. Approximately $10^3$ cells were spotted on SC-Ura plus glucose or SC-Ura plus galactose plates. Growth was scored after 3 days at 30°C.

such as transcriptional activation (Bourret *et al.*, 1991; Stock *et al.*, 1991; Parkinson and Kofoid, 1992). In contrast, the yeast SLN1—YPD1—SSK1 two-component osmosensor is only the initial segment of a more extensive signaling pathway that regulates the HOG1 MAP kinase cascade (Maeda *et al.*, 1994; Posas *et al.*, 1996). Similar regulation of MAP kinase cascades by a two-component regulator was proposed for other systems. In the fission yeast *Schizosaccharomyces pombe*, the Mcs4 response regulator (a possible homolog of SSK1) is involved in the activation of a MAP kinase cascade in response to environmental stresses including high osmolarity (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). The plant *Arabidopsis thaliana* ethylene response pathway may also involve a similar signal transduction mechanism, because CTR1 (a kinase of the MAPKKK family) has been found to function downstream of a sensor histidine kinase (ETR1) (Kieber *et al.*, 1993). ETR1 is a transmembrane ethylene sensor with structural similarity to the yeast SLN1 osmosensor. However, it is not known how a two-component signal transducer regulates a MAPK cascade in eukaryotic cells.

Our previous genetic analyses have placed the SSK1 two-component response regulator upstream of the SSK2/SSK22 MAPKKKs (Maeda *et al.*, 1994; Posas *et al.*, 1996). However, the precise relationship of these molecules remained ambiguous. Here we demonstrate that: (i) SSK1 binds an N-terminal segment of SSK2; (ii) SSK2 becomes phosphorylated upon osmotic shock, by an autophosphorylation mechanism; (iii) SSK2 kinase activity is correlated with its phosphorylation state; and (iv) interaction between SSK1 and SSK2 is required for the SSK2 phosphorylation. Based on these observations, we propose a mechanism for the activation of SSK2 by the SSK1 response regulator.

## Results

### *The SSK1 response regulator is essential for SSK2 activation*

The SSK1 response regulator was proposed to act as an activator of the SSK2 MAPKKK based on genetic epistasis tests (Maeda *et al.*, 1994; Posas *et al.*, 1996). However, the underlying mechanism by which SSK1 activates SSK2 is not clearly established. To address this question, we designed a test to evaluate SSK2 activity *in vivo* based on the observation that constitutive activation of the HOG1 MAPK cascade is deleterious for cell growth (although the precise mechanim of this lethality is not known, prolonged activation of the HOG1 MAPK leads to lysis of multi-budded cells) (Maeda *et al.*, 1994, 1995). The accumulation of unphosphorylated SSK1 in *sln1*Δ mutant cells causes cell lethality due to constitutive activation of the SSK2 MAPKKK, which acts upstream of HOG1 MAPK. This lethality caused by *sln1*Δ can be suppressed by inactivation of downstream signaling elements: SSK1 (response regulator), SSK2 (MAPKKK), PBS2 (MAPKK) or HOG1 (MAPK).

Expression of wild-type *SSK2* in an *sln1*Δ *ssk2*Δ mutant strain is lethal because the HOG1 MAPK cascade is hyperactivated, whereas expression of the same wild-type *SSK2* gene in a wild-type ($SLN1^+$) strain is not toxic (Figure 2). The SSK2 toxicity in *sln1*Δ mutants requires the active *SSK1* gene, because *sln1*Δ *ssk1*Δ double mutants are resistant to the toxic effect of *SSK2* expression. Furthermore, SSK2 toxicity requires its kinase activity because the expression of a catalytically inactive mutant protein, SSK2(K/N) (K/N denotes Lys1295→Asn mutation), was not toxic in any of the strains tested. An SSK2 mutant protein lacking the N-terminal non-catalytic domain (SSK2ΔN) is also toxic in the presence or absence of upstream regulators (SLN1 and SSK1), suggesting that SSK2ΔN is constitutively active. Thus, the N-terminal domain of SSK2 probably plays a negative regulatory role in controlling kinase activity. These data, as well as our previous observations using a temperature-sensitive *sln1* mutant (Maeda *et al.*, 1994), indicate that SLN1 acts as a negative regulator of the SSK2 MAPKKK, whereas SSK1 is a positive regulator of SSK2.

### *The SSK1 receiver domain binds to an SSK1-binding site near the N-terminus of SSK2*

Previously, it was shown by two-hybrid analysis that SSK1 binds the N-terminal, non-catalytic domain of SSK2 (Maeda *et al.*, 1995). We extended the two-hybrid analysis to identify the specific regions in SSK1 and SSK2 that are essential for their interaction. To determine the SSK1-binding site in SSK2, various segments of the SSK2 N-terminal domain were fused to the GAL4 activator domain, and their interaction with the full-length SSK1 fused to the LexA DNA-binding domain was tested.

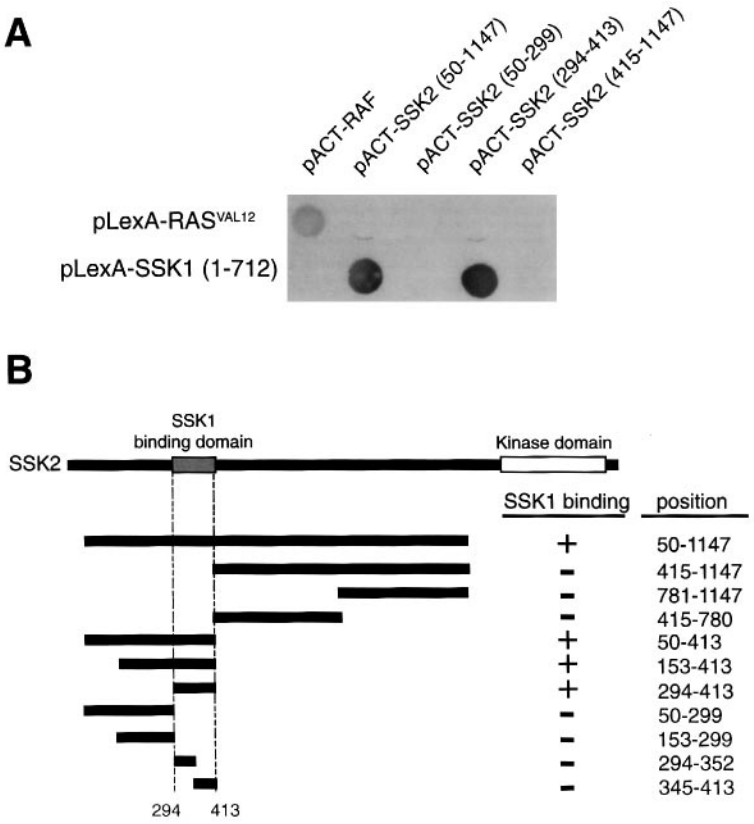


**Fig. 3.** Two-hybrid analysis of the SSK1-binding domain in the SSK2 MAPKKK. (**A**) Interactions of various segments of SSK2 fused to the GAL4 activator domain with full-length SSK1 protein fused to the LexA DNA-binding domain were evaluated by the filter β-galactosidase assay (Vojtek *et al.*, 1993). Representative interactions between SSK1 and SSK2 are shown. pACT-SSK2 (50–1147) contains most of the SSK2 non-catalytic domain, whereas the pLexA-SSK1 (1–712) construct contains the entire SSK1 coding sequence. Proteins encoded by the control plasmids pLexA-RAS$^{V12}$ and pACT-RAF interact with each other, but not with SSK2 or SSK1. (**B**) Summary of the two-hybrid interaction analysis between SSK1 and SSK2. The positions of the SSK2 segments included in the activator domain constructs are shown schematically on the left, with their precise amino acid positions indicated on the right. The presence or absence of interactions is depicted by (+) and (–).

Figure 3A shows a typical result, and Figure 3B summarizes the results using various SSK2 deletion constructs. This analysis indicates that SSK2 residues 294–413 are sufficient for binding to SSK1. We have thus designated this segment as the SSK1-binding domain (SSK1BD).

To determine the region in SSK1 that is essential for its interaction with the N-terminal domain of SSK2, various SSK1 segments were fused to the LexA DNA-binding domain, and these constructs were tested for interaction with the SSK2 N-terminal domain (amino acids 50–1147) fused to the GAL4 activator domain. As shown in Figure 4, the C-terminal portion (receiver domain) of SSK1 (amino acids 475–670) is required and sufficient for binding to SSK2. However, the full-length SSK1 interacts with SSK2 more efficiently than does the receiver domain alone, suggesting that the SSK1 N-terminal region may have a role in enhancing the SSK1–SSK2 interaction. A mutant of SSK1 that does not have the conserved phosphorylation site at Asp554, SSK1(D/N), interacted with SSK2 as well as the wild-type SSK1 (data not shown), supporting the notion that unphosphorylated SSK1 activates SSK2. Although SLN1 also contains a conserved receiver domain (Ota and Varshavsky, 1993), no interaction was detected between SLN1 and SSK2 (data not shown).

The interaction of SSK1 with SSK2 was investigated further using an *in vitro* binding assay with GST fusion proteins containing either the entire SSK2 non-catalytic domain (GST–SSK2) or the same region but lacking the SSK1-binding domain (GST–SSK2$^{\Delta SSK1BD}$), and His-epitope-tagged SSK1 receiver domain (His-SSK1-Rec). The SSK1 receiver domain bound GST–SSK2, but not GST–SSK2$^{\Delta SSK1BD}$ (Figure 5A). The interaction of SSK1 and SSK2 could be also demonstrated by *in vivo* co-precipitation experiment. Yeast cells were co-transformed with a plasmid that express GST-tagged SSK2 (or control GST) and HA-tagged SSK1. Proteins were co-precipitated using glutathione–Sepharose beads, and HA-SSK1 in precipitates was probed using anti-HA monoclonal antibody. As shown in Figure 5B, GST–SSK2, but not GST, co-precipitated HA-SSK1. Thus, both *in vitro* and *in vivo* binding assays corroborated the conclusion from the two-hybrid analyses.

### *The SSK1 receiver domain alone is capable of activating SSK2*

Previously we observed that wild-type *SSK1* expressed in an *sln1*Δ *ssk1*Δ strain hyperactivates the HOG1 MAPK cascade resulting in cell lethality (Maeda *et al.*, 1994). This effect is dependent on SSK2, because *SSK1* expression is not toxic in an *sln1*Δ *ssk2*Δ strain. We thus examined whether expression of the SSK1 N-terminal domain alone (SSK1ΔC), or the receiver domain alone (SSK1-Rec) can similarly activate the HOG1 MAPK cascade. As shown in Figure 5C, expression of the SSK1 receiver domain

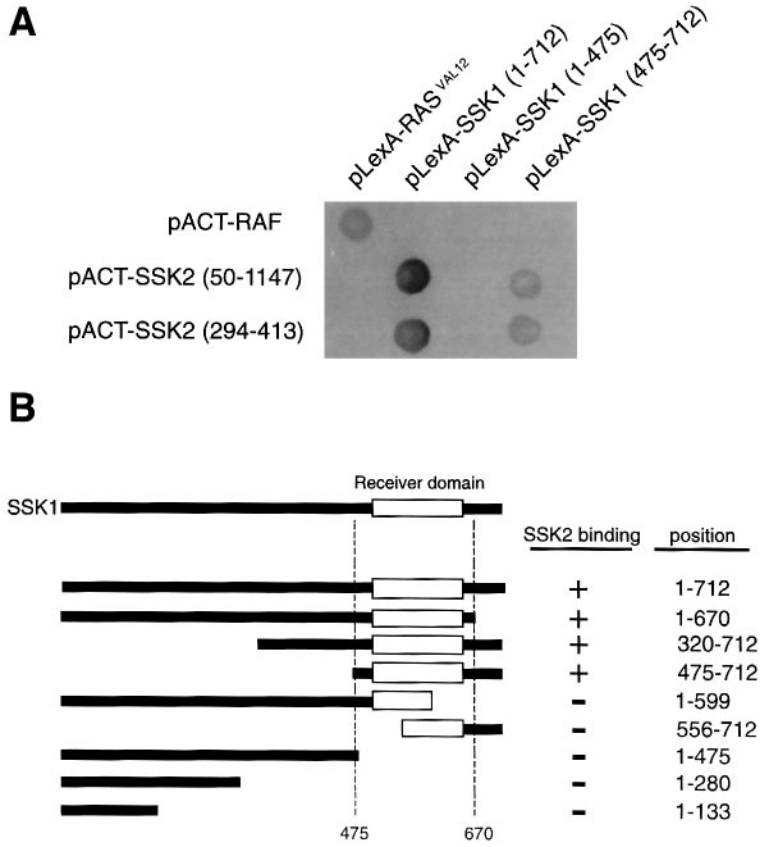


**Fig. 4.** Two-hybrid analysis of the SSK2-binding domain in the SSK1 response regulator. (**A**) Interactions of SSK1 fragments fused to the LexA DNA-binding domain with the SSK2 fragments fused to the GAL4 activator domain were examined. The positions of the SSK1 and SSK2 fragments included in the constructs are indicated in parentheses. Binding was assayed as in Figure 3. (**B**) Summary of the two-hybrid interaction analysis between SSK1 and SSK2. Positions of the SSK1 segments included in the DNA-binding domain constructs are shown schematically on the left, with their precise amino acid positions indicated on the right. The SSK2 activation domain construct contained the N-terminal domain of SSK2 (amino acids 50–1147). The presence or absence of interactions is depicted by (+) and (–).

alone activated the HOG1 MAPK cascade, albeit less efficiently than the full-length SSK1, whereas the expression of SSK1ΔC had no effect. Furthermore, both SSK1(D/N) and SSK1-Rec(D/N) mutants, in which the conserved phosphorylation site Asp554 has been mutated to Asn, could activate the HOG1 MAPK cascade as efficiently as their wild-type counterparts. Therefore, these results are qualitatively consistent with their binding abilities to SSK2 as seen in Figure 4, and support the notion that the SSK1–SSK2 interaction is an essential step in SSK2 activation. The receiver domain of SLN1, even when overexpressed using the strong $P_{GAL1}$ promoter, did not activate the HOG1 MAPK cascade (data not shown), further indicating the functional distinction between the SLN1 and SSK1 receiver domains.

### *Hyperosmotic stress induces SSK2 autophosphorylation*

Many protein kinases are activated by phosphorylation of their catalytic domains (Johnson *et al.*, 1996). To test the possibility that phosphorylation is required to activate SSK2, we examined if SSK2 becomes phosphorylated when cells are exposed to hyperosmotic stress. Cells expressing a GST–SSK2 fusion construct were grown in phosphate-depleted media, and pulse labeled with [$^{32}$P]orthophosphate, with or without a brief osmotic shock. GST–SSK2 was then purified by affinity chromatography using glutathione–Sepharose beads, and incorporation of $^{32}$P was detected by autoradiography. As shown in Figure 6, the level of the GST–SSK2 phosphorylation increased ~10-fold (9.5 ± 2.1, average of three independent experiments) upon hyperosmotic treatment. SSK1–SSK2 interaction appears essential for the induced SSK2 phosphorylation, because GST–SSK2$^{\Delta SSK1BD}$, which contains an SSK1-binding domain deletion, was not phosphorylated even after osmotic shock (Figure 6). Moreover, in *ssk1*Δ cells, there was no induction of SSK2 phosphorylation upon osmotic shock (data not shown). According to our current hypothesis, the SLN1 histidine kinase acts as a negative regulator of the SSK2/SSK22 MAPKKKs, by inactivating SSK1. Consistent with this model, SSK2 was constitutively phosphorylated in *sln1*Δ cells (Figure 6, right panel), and mutants deficient in *SLN1* have constitutively activated the HOG1 MAPK cascade (Maeda *et al.*, 1994; Posas *et al.*, 1996).

The osmotic stress-induced phosphorylation of SSK2 could be mediated by another protein kinase or by autophosphorylation. These two possibilities can be distinguished by examining whether a catalytically inactive SSK2 mutant protein is phosphorylated *in vivo*. If another protein kinase is responsible for SSK2 phosphorylation, a catalytically inactive SSK2 should still be phosphorylated upon osmotic shock. In contrast, if autophosphorylation is responsible for SSK2 phosphorylation, then the cata-

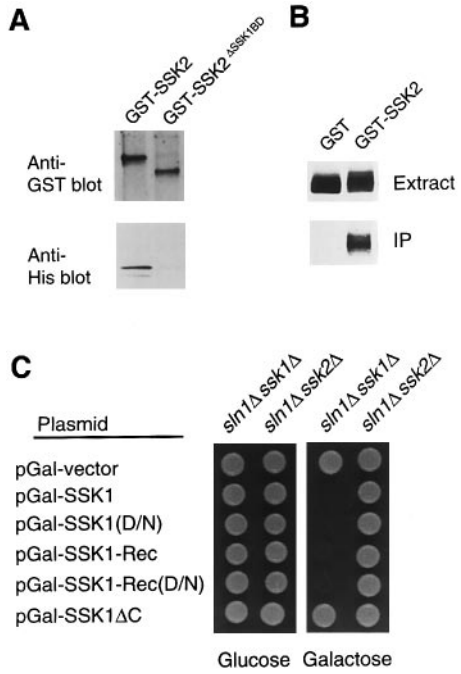


**Fig. 5.** (**A**) Binding of the SSK1 receiver domain to the SSK2 N-terminal region. Purified His-tagged SSK1 receiver domain (His-SSK1-Rec) was incubated with purified GST–SSK2 (containing the SSK2 N-terminal region; positions 1–1147) or GST–SSK2$^{\Delta SSK1BD}$ (deletion mutant of GST–SSK2 lacking amino acids 302–414) bound to glutathione–Sepharose beads. After incubation, beads were washed and subjected to immunoblot analysis with antibodies to the GST fusion proteins (anti-GST) or to the His-tagged protein (anti-His). (**B**) *In vivo* binding of SSK1 with SSK2. The FP69 (*sln1*Δ *pbs2*Δ) strain was co-transformed with a plasmid expressing GST–SSK2 fusion protein (or control GST) under the constitutive $P_{TEF1}$ promoter, and another plasmid expressing the HA-tagged full-length SSK1 (HA-SSK1) under the $P_{GAL1}$ promoter. GST proteins were precipitated using glutathione–Sepharose beads as described in Materials and methods, and HA-SSK1 in precipitates was detected by immunoblotting using anti-HA antibodies. Upper panel (Extract) shows an immunoblot of the whole extracts, while the lower panel (IP) shows the results from the co-precipitation experiments. (**C**) Activation of the HOG1 MAPK cascade by expression of the SSK1 receiver domain alone. The following segments of SSK1 were expressed under the $P_{GAL1}$ promoter: pGal-SSK1 (full-length SSK1; amino acids 1–712); pGal-SSK1-Rec (the receiver domain alone; amino acids 475–712); and pGal-SSK1ΔC (the N-terminal domain alone; amino acids 1–511). SSK1(D/N) and SSK1-Rec(D/N) contain the Asp554→Asn mutation that abolishes the conserved phosphorylation site. Plasmids were transformed into TM227b (*sln1*Δ *ssk1*Δ) or FP65 (*sln1*Δ*ssk2*Δ). The transformants were spotted on SC-Ura plus glucose or SC-Ura plus galactose plates. Growth was scored after 3 days at 30°C.

lytically inactive SSK2 will not be phosphorylated. Results using the catalytically inactive mutant kinase, SSK2(K/N), supported the latter possibility: SSK2, but not SSK2(K/N), is phosphorylated upon osmotic shock (Figure 6, left panel).

In summary, these data demonstrate that SSK2 is autophosphorylated rapidly after osmotic shock, and that this autophosphorylation is controlled by the SLN1–YPD1–SSK1 two-component osmosensor. Importantly, SSK2 autophosphorylation occurs only in conditions that result in the activation of SSK2. However, these results alone cannot establish whether SSK2 autophosphorylation is the cause or the consequence of its activation.

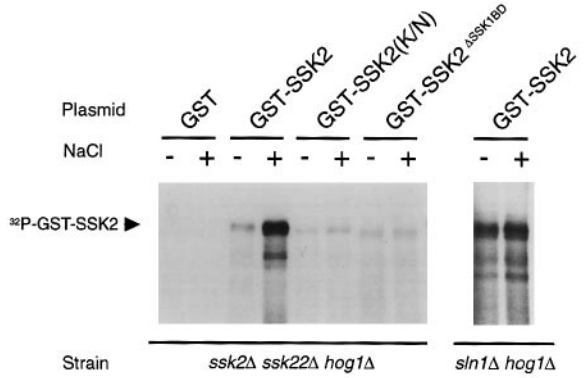


**Fig. 6.** Osmotic stress-induced phosphorylation of the SSK2 MAPKKK *in vivo*. The relevant genotypes of host yeast strains are shown below. Host strains were transformed with plasmids expressing GST-tagged SSK2. GST–SSK2 is the full-length SSK2 protein fused to GST. GST–SSK2(K/N) contains a catalytically inactive mutation (Lys1295→Asn), and GSTΔSSK2$^{\Delta SSK1BD}$ contains a deletion in the SSK1-binding domain (amino acids 302–414). Transformants were grown in phosphate-depleted medium in the presence of [$^{32}$P]orthophosphate, and subjected (+) or not (–) to a brief osmotic shock (0.4 M NaCl, 2 min). Proteins were purified as described in Materials and methods and detected by autoradiography.

### *Activation of SSK2 by SSK1 in vitro*

To test if SSK2 kinase activity is regulated by phosphorylation, we purified GST–SSK2 protein from yeast cells, and measured its kinase activity *in vitro* using GST–PBS2(K/M), a catalytically inactive form of PBS2, as the exogenous substrate (Posas and Saito, 1997). As seen in Figure 7, lane 1, purified GST–SSK2 phosphorylates GST–PBS2(K/M). Pre-treatment of the GST–SSK2 preparation with alkaline phosphatase (AP) blocked SSK2 activity, indicating that the dephosphorylation inactivates SSK2 kinase activity (Figure 7, lane 2). In control experiments using phosphatase inhibitors, SSK2 activity was not affected (lane 3). Thus, phosphorylation of SSK2 is required for its catalytic activity.

Taken together, our results suggest that SSK2 is activated by autophosphorylation, and that activated SSK2 phosphorylates its physiological substrate PBS2. However, if this is the case, how can the inactive (dephosphorylated) SSK2 kinase activate itself by auto-phosphorylation? Our genetic analyses indicated that SSK1 is essential for SSK2 activation (Maeda *et al.*, 1994, 1995). Furthermore, SSK1 binds SSK2. Thus, we tested the possibility that binding of SSK1 to SSK2 induces the latent SSK2 kinase activity. When SSK1 was added to dephosphorylated SSK2, re-activation of the SSK2 kinase was observed (Figure 7, lane 4). The SSK2$^{\Delta SSK1BD}$ mutant kinase, which lacks the SSK1-binding domain, could not be re-activated by SSK1 under similar conditions (data not shown), further supporting the role of SSK1 binding in the induction of the SSK2 kinase activity.

### *Autophosphorylation of SSK2 is an intramolecular reaction*

In the preceding section, we showed that SSK2 phosphorylation requires its own kinase activity. Indeed, purified GST–SSK2 phosphorylates both the exogenous substrate GST–PBS2(K/M) and itself (Figure 8A, lane 2). As expected, a catalytic site mutant of SSK2 [GST–SSK2(K/N)] does not autophosphorylate or trans-phos-

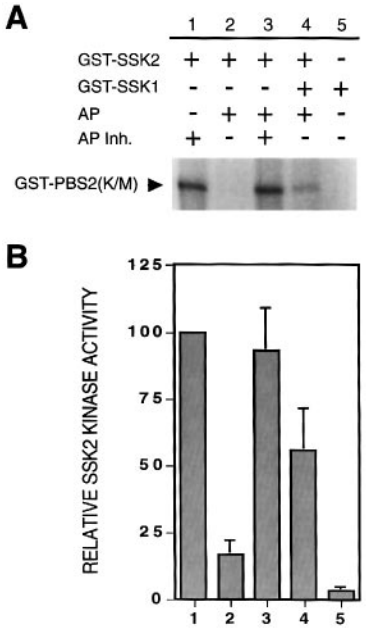


**Fig. 7.** Inactivation of SSK2 kinase activity by dephosphorylation. **(A)** Purified GST–SSK2 was bound to glutathione–Sepharose, incubated with (+) or without (–) alkaline phosphatase (AP), in the presence (+) or absence (–) of a mixture of phosphatase inhibitors (AP Inh.). After incubation, AP was removed by washing the beads, and the kinase activities of the treated GST–SSK2 were assayed using exogenous substrate GST–PBS2(K/M). Phosphorylated proteins were separated by SDS–PAGE and detected by autoradiography. The position of GST–PBS2(K/M) is indicated on the left. (**B**) The intensity of the GST–PBS2(K/M) phosphorylation was quantified by densitometry. Samples from four independent experiments were measured, and the intensity of each band was normalized to that of lane 1. Error bars indicate standard error of the mean.

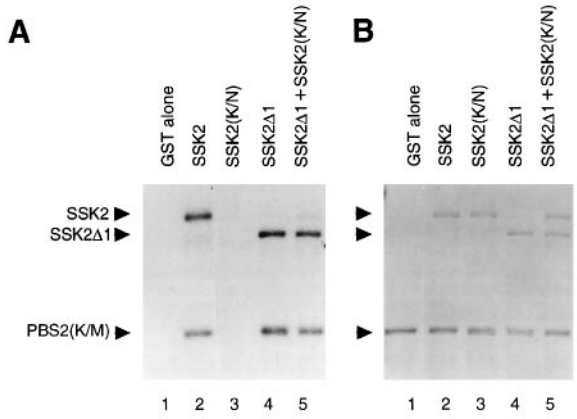


**Fig. 8.** Intramolecular autophosphorylation of SSK2. The constitutively active SSK2Δ1 kinase autophosphorylates, but fails to phosphorylate another SSK2 molecule *in trans*. A slight difference in the sizes of SSK2 and SSK2Δ1 allows for the detection of the intra- and intermolecular reactions. PBS2(K/M) was included in all reactions to confirm the activity of the different SSK2 kinase preparations tested. (**A**) Phosphorylated proteins were detected by autoradiography following SDS–PAGE. The positions of proteins are indicated on the left. All protein used were tagged with GST. (**B**) Coomassie blue staining of the same gel as in (A).

phorylate exogenous substrate (Figure 8A, lane 3). SSK2 autophosphorylation, however, could occur either by an intermolecular reaction or by an intramolecular reaction. In order to distinguish between inter- and intramolecular reactions, we constructed a small deletion mutant (GST–

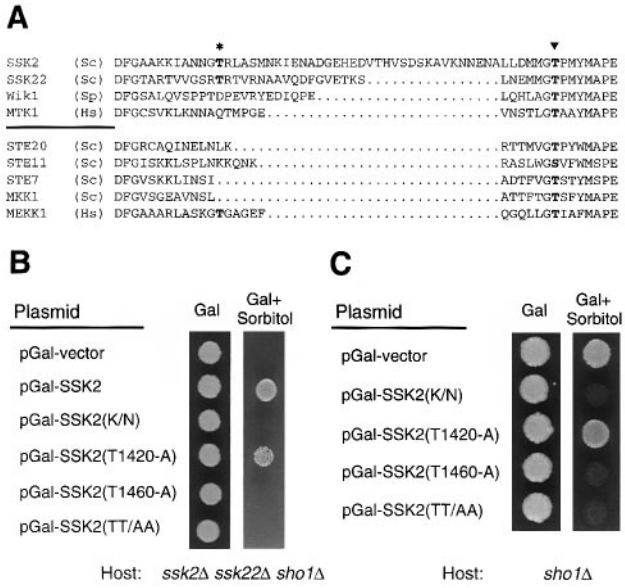


**Fig. 9.** Mutational analysis of threonine residues in the SSK2 activation loop. (**A**) Comparison of activation loop sequences (between subdomains VII and VIII) from kinases related to SSK2 (SSK22, Wik1 and MTK1) and several other kinases (STE20, STE11, STE7, MKK1 and MEKK1). Thr at position 1420 of SSK2 is indicated by (*) and Thr at position 1460 by (▼). Sc, *S.cerevisiae*; Sp, *S.pombe*; Hs, *Homo sapiens*. (**B**) The SSK2(T1460-A) mutant cannot complement the *ssk2*Δ defect. The indicated *SSK2* mutant alleles were expressed from the galactose-inducible $P_{GALS}$ promoter in the osmosensitive yeast strain MY007 (*ssk2*Δ *ssk22*Δ *sho1*Δ). Approximately $10^3$ cells were spotted on SC-Ura plus galactose plates with or without 1.5 M sorbitol. Successful complementation of the *ssk2*Δ mutation will allow the transformed cells to grow on the sorbitol plates. Growth was scored after 4 days. SSK2(TT/AA) contains the double mutation T1420-A/T1460-A. **(C**) Dominant-inhibitory effect of several *SSK2* mutant alleles. The constructs used in (B) were transformed into the yeast strain TM285 (*sho1*Δ), and osmosensitivity was tested on sorbitol plates. Catalytically impaired mutants [SSK2(K/N), SSK2(T1460-A) and SSK2(TT/AA)] dominantly inhibited the activation of the HOG1 MAPK pathway, rendering the transformed cells osmosensitive.

SSK2Δ1), which lacks a 191 amino acid segment near the N-terminus of the SSK2 sequence, and can be separated readily from GST–SSK2 by SDS–PAGE. Purified GST–SSK2Δ1 is catalytically active, phosphorylating both the exogenous substrate GST–PBS2(K/M) and itself (Figure 8A, lane 4). However, GST–SSK2Δ1 cannot phosphorylate the full-length GST–SSK2(K/N) *in trans*, indicating that autophosphorylation of SSK2 is an intramolecular reaction (Figure 8A, lane 5). Intramolecular autophosphorylation was also reported for MEKK1, a mammalian kinase of the MAPKKK family (Deak and Templeton, 1997). Thus, it seems likely that intramolecular autophosphorylation is a common activation mechanism for both yeast and mammalian MAPKKKs.

### *Thr1460 in the activation loop of SSK2 is important for kinase activity*

Phosphorylation of Ser and/or Thr residues in the kinase activation loop (between subdomains VII and VIII) is essential for activation of many protein kinases, including MAPK and MAPKK (Johnson *et al.*, 1996). As shown in Figure 9A, the SSK2 Thr1420 and Thr1460 residues located within the activation loop are also present in the SSK22 and MEKK1 MAPKKKs. To determine whether

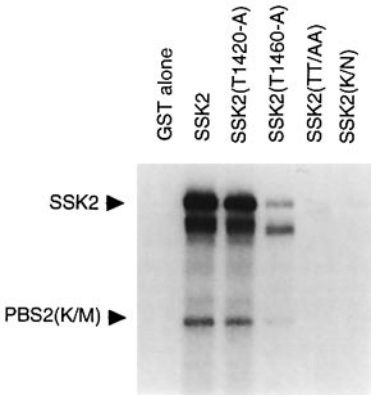


**Fig. 10.** *In vitro* kinase activity of SSK2 mutant proteins. The wild-type GST–SSK2 and the indicated mutant forms were purified from yeast, and kinase reactions were performed in the presence of [$\gamma$-$^{32}$P]ATP and the exogenous substrate GST–PBS2(K/M). Phosphorylated proteins were detected by autoradiography following SDS–PAGE. All proteins were GST tagged and their positions are indicated on the left.

these Thr residues are essential for SSK2 activity, we tested complementation of the *ssk2*Δ mutation by various *SSK2* alleles. The osmosensitivity of an *ssk2*Δ *ssk22*Δ *sho1*Δ triple mutant strain can be suppressed by expression of the wild-type *SSK2* gene, and by the *SSK2* T1420-A (Thr1420→Ala) mutant gene (Figure 9B). However, expression of the T1460-A mutant, as well as the catalytically inactive K1295-N (K/N), failed to complement the osmosensitive defect.

Expression of SSK2(K/N) protein in a *sho1*Δ strain resulted in inhibition of the HOG pathway, presumably by binding to either SSK1 and/or PBS2, and rendered the yeast cells osmosensitive (Figure 9C) (a *sho1*Δ mutation was necessary to inactivate the SSK2/SSK22-independent activation mechanism). The SSK2(T1460-A) mutant protein is likely to be intact, because it also functions as a dominant-inhibitory protein in *sho1*Δ cells.

Furthermore, as shown in Figure 10, T1420-A mutant protein has catalytic activity *in vitro* comparable with wild-type SSK2, whereas the T1460-A mutant protein has significantly lower specific activity. The T1420-A/T1460-A double mutant protein, as well as the K/N mutant, had essentially undetectable catalytic activity. Thus, both *in vivo* and *in vitro* experiments indicate that Thr1460, but not Thr1420, in the activation loop plays a major role in the activation of SSK2, perhaps as the site of autophosphorylation.

## Discussion

### *A two-step model for SSK2 kinase activation by the SSK1 response regulator*

The results reported here, together with our earlier findings, allow for speculation on the molecular mechanism of SSK2 activation by SSK1. It has been proposed for several MAPKKKs that their N-terminal non-catalytic domains are autoinhibitory for kinase activity (Minden *et al.*, 1994). This is also the case for SSK2, since an N-terminal domain deletion results in constitutive activation of the kinase (Maeda *et al.*, 1995; Posas and Saito, 1997). Although we have not mapped the SSK2 autoinhibitory domain precisely, a deletion of the N-terminal 191 amino acids was sufficient to activate the SSK2 kinase domain (F.Posas and H.Saito, unpublished observation). Thus, the autoinhibitory region may be close enough to the SSK1-binding domain (294–413), so that binding of SSK1 to SSK2 disrupts autoinhibition, and leads to activation of SSK2 kinase. The observation that expression of the SSK1 receiver domain (which coincides with the SSK2-binding domain) alone is sufficient to activate SSK2 is consistent with this possibility.

However, as purified SSK2 kinase is highly active *in vitro* without any SSK1, a simple model whereby SSK1 binding alone regulates SSK2 activity is unlikely to be correct. Activities of many protein kinases are regulated by phosphorylation (Johnson *et al.*, 1996). Indeed, the observation that SSK2 kinase activity is sensitive to phosphatase treatment indicates that phosphorylation is also important for SSK2 activity. The observation that SSK2 is phosphorylated rapidly upon osmotic shock of yeast cells is consistent with phosphorylation regulating SSK2 activity. Furthermore, SSK2 is constitutively phosphorylated in an *sln1*Δ strain, in which the HOG1 MAPK cascade is hyperactivated. SSK2 phosphorylation occurs mainly by autophosphorylation, because a catalytically inactive SSK2 mutant [SSK2(K/N)] is not phosphorylated under the conditions where the wild-type SSK2 protein is phosphorylated. Our data further indicate that SSK2 autophosphorylation occurs by an intramolecular reaction. These findings are similar to recent reports that autophosphorylation is crucial for activation of MEKK1 (a member of the MAPKKK family) and STE20 (an activator of STE11 MAPKKK, and structurally related to MAPKKKs) (Wu *et al.*, 1995; Deak and Templeton, 1997; Siow *et al.*, 1997). MEKK1 autophosphorylation also results from an intramolecular reaction (Deak and Templeton, 1997). An intramolecular phosphorylation mechanism suggests that dimerization might not be a critical step for the activation of MEKK1 and SSK2.

How do these two potential activation mechanisms, the binding of SSK1 to SSK2 and SSK2 autophosphorylation, relate to one another? Two lines of observations, one from the *in vivo* experiments and another from the *in vitro* observations, suggest that SSK1 binding is necessary for SSK2 autophosphorylation. For instance, the osmotic stress-induced SSK2 phosphorylation *in vivo* did not occur if the SSK1-binding domain was deleted in SSK2 (as in the SSK2$^{\Delta SSK1BD}$ mutant protein). Furthermore, the binding of SSK1 to dephosphorylated (inactive) SSK2 induces its latent kinase activity *in vitro*. Thus, it seems possible that SSK1 binding is needed to activate dephosphorylated SSK2 but, once phosphorylated, SSK2 does not require SSK1 for its catalytic activity. Probably the phosphorylated form of SSK2 is no longer sensitive to the autoinhibitory domain.

Thus, we propose the following two-step activation mechanism for SSK2. Initially, SSK2 is in a closed, non-active, form, with an N-terminal autoinhibitory domain interacting with the kinase catalytic site. Binding of SSK1 (unphosphorylated form) to SSK2 releases the autoinhibitory domain, effecting partial activation of SSK2. Partially activated SSK2 then autophosphorylates on Thr (and to a lesser extent Ser) residues, in the catalytic domain and/or the N-terminal non-catalytic domain. Phosphorylation in the catalytic domain, possibly at

Thr1460 in the activation loop, may increase catalytic activity. It is, however, equally possible that autophosphorylation in the non-catalytic domain causes a conformational change that obliterates the need for the SSK1 binding for continued activation.

A similar two-step activation mechanism might be applicable to other MAPKKKs, particularly those kinases involved in mammalian stress responses. The mammalian stress-induced p38 MAPK cascade is homologous to the yeast HOG1 MAPK cascade: the MAPKKs in the p38 and the HOG pathways (MKK3/MKK6 and PBS2) are very similar, as are the respective MAPKs (p38 and HOG1) (Boguslawski and Polazzi, 1987; Brewster *et al.*, 1993; Han *et al.*, 1994; Dérijard *et al.*, 1995). Mammalian p38 can functionally complement the osmosensitivity of the yeast *hog1*Δ mutation (Han *et al.*, 1994). Furthermore, we recently identified a human homolog of SSK2/SSK22, named MTK1, using a functional cloning method (Takekawa *et al.*, 1997). MTK1 complements the *ssk2*Δ *ssk22*Δ defect in yeast, and is involved in the mammalian stress signal transduction. The mouse homolog of MTK1 is known as MEKK4 (Gerwins *et al.*, 1997). Although the specific substrate of HOG1 MAPK is not yet defined, the substrate of its *S.pombe* homolog Spc1 was identified recently to be Atf1, a transcription factor containing a bZIP domain (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Remarkably, Atf1 is highly similar to ATF-2, a mammalian transcription factor that is a substrate of p38 (van Dam *et al.*, 1995; Raingeaud *et al.*, 1996). Thus, the human MTK1–MKK3/6–p38 pathway is very similar to the yeast SSK2/SSK22–PBS2–HOG1 pathway, both structurally and functionally. The MTK1 N-terminal non-catalytic domain sequence has significant similarity to the same region of SSK2/SSK22, suggesting a common activating mechanism. Furthermore, dominant inhibition by MTK1(K/R) (a catalytically inactive mutant) requires the N-terminal non-catalytic domain in addition to the catalytic domain, suggesting that titration of an upstream regulator molecule is involved in the dominant inhibition mechanism (Takekawa *et al.*, 1997). Although it is not known whether the hypothetical regulator of MTK1 is structurally related to SSK1, it seems reasonable to assume that SSK2 and MTK1 (and other MAPKKKs) use basically the same two-step activation mechanism.

## Materials and methods

### Yeast strains

The following yeast strains were used: TM141 (*MAT**a** ura3 leu2 trp1 his3*); TM188 (*MAT**a** ura3 leu2 trp1 ssk1::LEU2*); TM227b (*MAT**a** ura3 leu2 trp1 sln1::hisG ssk1::LEU2*); TM233 (*MATα ura3 leu2 trp1 his3 lys2 hog1::TRP1*); TM285 (*MATα ura3 leu2 trp1 his3 sho1::TRP1*); FP20 (*MATα ura3 leu2 trp1 his3 ssk2::LEU2 ssk22::LEU2 hog1::TRP1*); FP23 (*MAT**a** ura3 leu2 trp1 sln1::hisG hog1::TRP*); FP24 (*MATα ura3 leu2 trp1 his3 ssk1::LEU2 hog1::TRP1*); FP65 (*MAT**a** ura3 leu2 trp1 sln1::hisG ssk2::LEU2*); FP69 (*MAT**a** ura3 leu2 trp1 sln1::hisG pbs2::LEU2*); MY007 (*MAT**a** ura3 leu2 his3 ssk2::LEU2 ssk22::LEU2 sho1::HIS3*); L40 (*MAT**a** trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*)

### Buffers

Buffer A is 50 mM Tris–HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 5 μg/ml of leupeptin. Buffer B is 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 2 mM DTT. AP (alkaline phosphatase) buffer is 50 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10 mM $MgCl_2$. Phosphatase inhibitors cocktail contains 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate and 10 mM β-glycerophosphate. SDS loading buffer is 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol.

### Plasmids

The yeast expression vector p416GALS ($P_{GALS}$ promoter, *URA3*$^+$, centromeric) (Mumberg *et al.*, 1995) contains the $P_{GALS}$ promoter, which is an attenuated but still inducible version of the yeast $P_{GAL1}$ promoter. p426TEG1 ($P_{TEF1}$-*GST*, *URA3*$^+$, 2 μ) is a gift from M.Takekawa (unpublished). The $P_{TEF1}$-*GST* allows the expression of GST fusion proteins using the strong $P_{TEF1}$ promoter. Wild-type and several mutant alleles of the *SSK2* gene were cloned into p416GALS and p426TEG1. Site-directed mutants were generated by PCR-directed mutagenesis as described previously (Maeda *et al.*, 1995). Each mutation was verified by DNA sequencing. GST–SSK2$^{\Delta SSK1BD}$ containing a deletion (deleting amino acids 302–414) was also made by a PCR method. GST–SSK2Δ1 is a truncated version of GST–SSK2 in which the GST coding sequence is fused to SSK2 at Met192. Yeast expression plasmids for full-length SSK1, SSK1-Rec (containing amino acids 475–712) and SSK1ΔC (containing amino acids 1–511) were constructed using pYES2 ($P_{GAL1}$ *URA3*+, 2 μ) (Invitrogen).

### Two-hybrid analysis

The two-hybrid analysis was carried out essentially according to Durfee *et al.* (1993), using pACTII (Li *et al.*, 1994) and pBTM116 (Vojtek *et al.*, 1993) as the activation domain plasmid and the LexA DNA-binding domain plasmid, respectively. A systematic series of deletion constructs was made for the SSK1 and SSK2 coding sequences using the Erase-a-Base system (Promega), or subcloning of DNA fragments generated by restriction enzyme digestion or PCR. pBTM116-based plasmids that carry either full-length or fragments of the SSK1 coding sequence and pACTII-based plasmids carrying SSK2 sequences were co-transformed into the L40 reporter strain. Transformed cells (~$5\times10^6$) were spotted onto YEPD plates, incubated for 5 h at 30°C, and the cells were replicated onto nitrocellulose membrane. β-Galactosidase activity was visualized *in situ* using X-gal as described (Vojtek *et al.*, 1993).

### Expression and purification of epitope-tagged proteins

His-tagged SSK1 receiver domain protein was constructed using pRSET B (Invitrogen), expressed in *Escherichia coli* BL21(DE3) (Studier and Moffatt, 1986) and purified using TALON metal affinity resin (Clontech) according to the manufacturer's instructions. GST fusion proteins encoding SSK2ΔN (kinase domain), a catalytically inactive PBS2(K/M) and full-length SSK1 were constructed using pGEX-4T (Pharmacia), expressed in *E.coli* DH5, and purified using glutathione–Sepharose beads (Pharmacia) as described (Posas *et al.*, 1996).

### In vitro protein-binding assay

GST fusion proteins encoding the non-catalytic domains of SSK2 (GST–SSK2) and its variant that lacks the SSK1-binding domain (GST–SSK2$^{\Delta SSK1BD}$) were expressed in yeast and purified using glutathione–Sepharose beads. Beads were washed once with buffer A, and four times with buffer A plus 150 mM NaCl. Approximately 2 μg of GST fusion proteins bound to beads were incubated with 7.5 μg of purified His-tagged SSK1 receiver domain protein (His-SSK1-Rec) for 6 h at 4°C with tumbling. The beads were washed extensively with buffer A plus 150 mM NaCl, resuspended in SDS loading buffer and separated by SDS–PAGE. Proteins were transferred to nitrocellulose membrane by electroblotting. Immunoblotting was done using specific antibodies together with the ECL reagent (Amersham).

### In vivo SSK1–SSK2 binding assay

Cells were grown in the presence of galactose and extracts were prepared. Cell extract (750 μg in buffer A plus 150 mM NaCl) was incubated with 50 μl of glutathione–Sepharose beads overnight at 4°C. Beads were washed extensively with buffer A plus 150 mM NaCl, resuspended in loading buffer and separated by SDS–PAGE. Immunobloting was done using anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) together with ECL reagent (Amersham).

### In vivo [$^{32}$P]phosphate labeling of proteins

*In vivo* protein labeling was performed essentially as previously described (Posas *et al.*, 1996). Yeast cells were grown in phosphate-depleted medium (Warner, 1991), incubated with [$^{32}$P]orthophosphate (0.75 mCi/ml, 9000 Ci/mmol) for 15 min at 30°C, and treated with 0.4 M NaCl for 2 min immediately before harvesting. The wild-type or mutant forms

of GST–SSK2 were purified using glutathione–Sepharose beads in buffer A plus phosphatase inhibitors cocktail, followed by SDS–PAGE and autoradiography.

#### *In vitro kinase assay*

The wild-type or mutant forms of GST–SSK2 were expressed in yeast cells and purified using glutathione–Sepharose beads. Beads were resuspended in buffer B, mixed with 1 μg of purified GST–PBS2(K/M) and 50 μM [$\gamma$-$^{32}$P]ATP (50 Ci/mmol), and incubated at 30°C for 10 min. Reactions were terminated by the addition of 2× SDS loading buffer, and proteins were separated by SDS–PAGE. Autoradiograms were made either using dried gels, or after transferring proteins to Immobilon P membranes (Millipore). Bead-bound proteins were incubated with 40 U of calf-intestinal alkaline phosphatase (Boehringer Mannheim) in 50 μl of AP buffer for 20 min at 30°C, in the presence or absence of phosphatase inhibitors (10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate and 10 mM β-glycerophosphate). After alkaline phosphatase treatment, beads were washed with several column volumes of buffer A plus 150 mM NaCl and phosphatase inhibitors, and once with buffer B. The kinases were subjected to the kinase assay as described above.

#### *Phosphoamino acid analysis*

Phosphoproteins were separated by SDS–PAGE, transferred to an Immobilon P membrane, and individual bands were hydrolyzed in 6 M HCl at 110°C for 2 h. Samples were dried and separated by two-dimensional thin-layer chromatography (Duclos *et al.*, 1991).

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