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Yeast HOG1 MAP Kinase Cascade Is Regulated by a Multistep Phosphorelay Mechanism in the SLN1–YPD1–SSK1 "Two-Component" Osmosensor

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Summary

An osmosensing mechanism in the budding yeast (Saccharomyces cerevisiae) involves both a two-component signal transducer (Sln1p, Ypd1p and Ssk1p) and a MAP kinase cascade (Ssk2p/Ssk22p, Pbs2p, and Hog1p). The transmembrane protein Sln1p contains an extracellular sensor domain and cytoplasmic histidine kinase and receiver domains, whereas the cytoplasmic protein Ssk1p contains a receiver domain. Ypd1p binds to both Sln1p and Ssk1p and mediates the multistep phosphotransfer reaction (phosphorelay). This phosphorelay system is initiated by the autophosphorylation of Sln1p at His576. This phosphate is then sequentially transferred to Sln1p-Asp-1144, then to Ypd1p-His64, and finally to Ssk1p-Asp554. We propose that the multistep phosphorelay mechanism is a universal signal transduction apparatus utilized both in prokaryotes and eukaryotes.

Introduction

Yeast cells in their natural habitats must adapt to extremes of osmotic conditions such as the saturating sugar of drying fruits and the nearly pure water of rain (Mager and Varela, 1993). In budding yeast (Saccharomyces cerevisiae), glycerol synthesis appears to be particularly important for hyperosmolarity adaptation, because mutants defective in *GPD1* (NAD^+-dependent glycerol-3-phosphate dehydrogenase) cannot grow on hyperosmotic media (Albertyn et al., 1994). However, the molecular mechanisms by which yeast cells detect and respond to extracellular osmolarity changes have only been recently identified. It is now known that yeast has two osmosensors that independently regulate a common downstream signal-transducing mechanism, termed the HOG (high osmolarity glycerol response) pathway (Brewster et al., 1993; Maeda et al., 1994, 1995; see Figure 7B). One of the osmosensors is a transmembrane protein (Sho1p) with a cytoplasmic region containing an SH3 domain, while the other is homologous to the prokaryotic two-component systems. Here we describe the multistep phosphorelay mechanism in the yeast "two-component" osmosensor, which is actually composed of three proteins, Sln1p, Ypd1p, and Ssk1p.

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This phosphorelay regulates a MAP kinase cascade that is composed of three tiers of protein kinases, namely SSK2 and SSK22 MAP kinase kinase kinases (MAPKKKs), PBS2 MAP kinase kinase (MAPKK), and HOG1 MAPK (Boguslawski, 1992; Brewster et al., 1993; Maeda et al., 1995).

Prokaryotic two-component signal transduction systems are composed of a sensor and a response-regulator (Bourret et al., 1991; Stock et al., 1991; Parkinson and Kofoid, 1992). A sensor molecule (e.g., the Escherichia coli osmosensor EnvZ) may have an extracellular input domain and a cytoplasmic histidine kinase domain. A typical response-regulator is a cytosolic protein containing a conserved receiver domain and a nonconserved DNA binding domain. Recognition of an environmental stimulus by a sensor molecule results either in the activation or inhibition of its histidine kinase domain and phosphorylation of a histidine residue within the kinase domain. This phosphate group is then transferred to an aspartate residue in the receiver domain of a cognate response-regulator molecule, resulting in the switching of its output function (in most prokaryotic cases, transcriptional activation). Because a complete signaling pathway is composed of only two molecules, they are called two-component systems. Although the two-component systems were initially identified in prokaryotes, they are now known to exist in eukaryotes including plants, fungi, and yeasts (Brown et al., 1993; Chang et al., 1993; Ota and Varshavsky, 1993; Maeda et al., 1994; Hua et al., 1995; Wilkinson et al., 1995; Alex et al., 1996).

The architecture of the yeast osmosensor Sln1p is unorthodox in the sense that it contains both a histidine kinase domain and a receiver domain within the same molecule. Thus, it may appear that Sln1p by itself is a complete two-component system with the two components fused into one. However, our previous studies demonstrated that Sln1p activity requires another receiver domain protein, termed Ssk1p (Maeda et al., 1994). Genetic analyses of various mutants in *SLN1* and *SSK1* are consistent with the following model. At normal osmolarity, the Sln1p histidine kinase is activated and phosphorylates an aspartate residue within the Ssk1p receiver domain. Phosphorylated Ssk1p is incapable of activating the SSK2 and SSK22 MAPKKKs and thus inhibits signaling via the HOG1 MAPK cascade. At high osmolarity, the Sln1p histidine kinase is inhibited, resulting in an accumulation of unphosphorylated Ssk1p, which then interacts with SSK2/SSK22 MAPKKKs to activate the HOG1 MAPK cascade. While the exact mechanism by which Ssk1p activates Ssk2p/Ssk22p is not known, the finding that Ssk1p interacts with the noncatalytic "inhibitory" domain of Ssk2p/Ssk22p suggests that Ssk1p activates Ssk2p/Ssk22p by blocking the inhibitory effect of their N-terminal sequences (Maeda et al., 1995).

In wild-type yeast cells, increased extracellular osmolarity rapidly induces the transcription of *GPD1* as well as genes that are necessary for general stress responses, such as *CTT1* (catalase T) and *HSP12* (small heat-shock

protein; Schüller et al., 1994; Hirayama et al., 1995; Varela et al., 1995). *pbs2*Δ or *hog1*Δ mutants lack these responses. The inability to activate the HOG pathway under hyper-osmotic conditions, as is the case in a *pbs2* mutant, is conditionally lethal because of the lack of the proper adaptive responses. However, continuous activation of the HOG pathway is lethal even in the absence of hyper-osmotic conditions. For example, the expression of constitutively active forms of Ssk2p and Ssk22p (by truncation of the N-terminal inhibitory sequences; *SSK2*ΔN and *SSK22*ΔN) is highly toxic to yeast cells (Maeda et al., 1995). Similarly, genetic disruption of the *SLN1* gene (*sln1*Δ) is lethal, because the ensuing accumulation of nonphosphorylated Ssk1p constitutively activates the HOG pathway (Maeda et al., 1994). As might be expected, inactivation of downstream genes (for example, *pbs2*Δ or *hog1*Δ) suppresses the lethal effects of *SSK2*ΔN and *sln1*Δ (Maeda et al., 1995).

Although it has been established that both Sln1p and Ssk1p are upstream regulators of the HOG1 MAPK cascade, their actual signaling role has been unclear. In particular, the function and the relationship of the two receiver domains (one in Sln1p and another in Ssk1p) has not been obvious. Here, we describe a multistep phosphorelay mechanism in which a phosphate is transferred sequentially from Sln1p to Ssk1p: first from a histidine in the Sln1p kinase domain to an aspartate in the Sln1p receiver domain, then to a histidine in a novel molecule we named Ypd1p, and finally to an aspartate in Ssk1p. The architectural similarity between Sln1p and several other "unorthodox" two-component proteins, e.g. Arabidopsis ethylene receptor ETR1 and Bordetella pertussis BvgS (Chang et al., 1993; Uhl and Miller, 1996), suggests that the multistep phosphorelay mechanism is universally utilized both in prokaryotes and eukaryotes.

Results

Isolation of *ypd1* Mutants

Disruption of *SLN1* is lethal owing to the constitutive activation of the HOG1 MAPK cascade, because mutations in any of the four downstream genes (*SSK1*, *SSK2*, *PBS2*, and *HOG1*) suppress the *sln1*Δ lethality by preventing the activation of HOG1 MAPK (it should be noted that although *SSK2* and *SSK22* have a redundant function, the activity of Ssk22p alone appears to be insufficient to transduce lethal levels of signal caused by *sln1*Δ; Maeda et al., 1994, 1995). Overexpression of the *PTP2* tyrosine phosphatase gene also rescues the *sln1*Δ lethality by dephosphorylating and inactivating the HOG1 MAPK (Maeda et al., 1994; S. M. W.-M and H. S., unpublished data). To identify other elements necessary for the Sln1p signaling, yeast mutants were isolated that had a similar phenotype to *sln1*Δ by screening for lethal mutations that could be suppressed by *PTP2* overexpression (Maeda et al., 1993, 1994). Resulting mutants consisted of two complementation groups, one of which was *sln1*, and another named *ypd1* (**Y**rosine **p**hosphatase **d**ependent; Maeda et al., 1993).

Genomic DNA clones containing the *YPD1* gene were isolated by complementation (Rose and Broach, 1991).

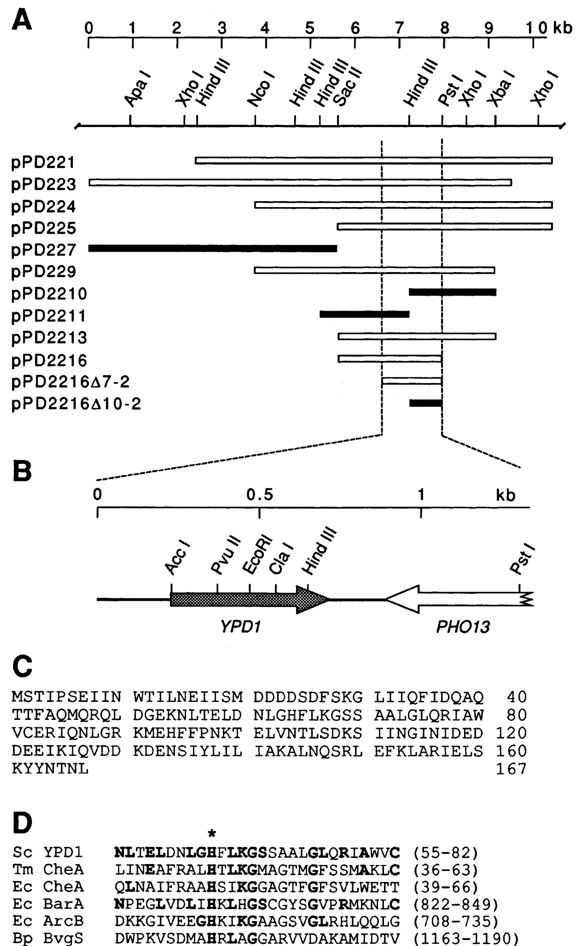

Figure 1. Cloning and Characterization of the Yeast *YPD1* Gene

(A) A restriction enzyme map of the region of the yeast genome that complements *ypd1* mutations is shown at the top. Open and closed bars indicate the DNA inserts in various genomic clones. pPD221 and 223 were isolated from a YEp13-based yeast genomic library by complementation of *ypd1* mutations. Other plasmids are derived from the two original clones by either restriction-fragment subcloning or by exonuclease digestion. Open bars indicate the *YPD1*-complementing clones, whereas closed bars indicate noncomplementing clones.

(B) *YPD1* is the only complete open reading frame within the 1.5 kb that was defined by deletion analysis. This segment also contains the 3′-half of the *PHO13* gene.

(C) Deduced amino acid sequence of Ypd1p.

(D) The amino acid sequences around known or predicted histidine-phosphorylation sites of several bacterial two-component proteins are aligned with the YPD1 sequence around His64. The histidine-phosphorylation site is indicated by asterisk. Sc, Saccharomyces cerevisiae; Tm, Thermotoga maritima; Ec, Escherichia coli; Bp, Bordetella pertussis.

The location of *YPD1* was then determined by generating a series of deletion clones and examining the capacity to complement *ypd1* mutants (Figure 1A). DNA sequencing of a 1.5 kb region that corresponds to the smallest complementing DNA revealed that this segment contained an open reading frame of 501 bp encoding a protein of 167 amino acids (Figures 1B and 1C). Disruptants of this open reading frame complement the *sln1* mutants but not the *ypd1* mutants, indicating that

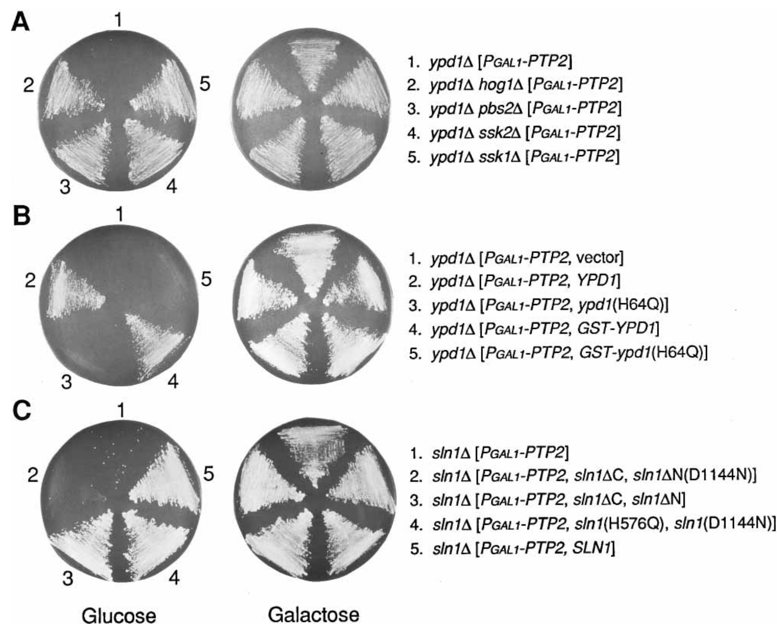

Figure 2. Phenotypes of *ypd1* and *sln1* Mutant Cells

P_{GAL1}–*PTP2* is a modified *PTP2* gene that is under the control of the inducible *GAL1* promoter. On glucose plates, *PTP2* expression is repressed, while on galactose plates, *PTP2* expression is induced. Only relevant genotypes are indicated. The plasmid-borne genes are in square brackets.

(A) The *ypd1*Δ mutant is lethal, but its lethality can be suppressed by the overexpression of *PTP2* on galactose plates (sector 1). The lethality of *ypd1*Δ is also suppressed when any one of *SSK1*, *SSK2*, *PBS2*, or *HOG1* is disrupted, as evidenced by their growth on glucose plates. Strains used: SW100, SW102, SW104, SW108, and FP30.

(B) The lethality of *ypd1*Δ is suppressed by the plasmid-borne *YPD1* gene but not by the His64→Gln mutant allele, *ypd1*(H64Q). Vector is pRS416 (Sikorski and Hieter, 1989). The *GST–YPD1* fusion gene, but not *GST–ypd1* (H64Q) mutant, also complements ypd1Δ. Strains used: SW100 carrying various plasmids.

(C) The *sln1*Δ defect can be complemented by combinations of two defective *sln1* alleles: *sln1*ΔC and *sln1*ΔN (sector 3) or *sln1*(H576Q) and *sln1*(D1144N) (sector 4). Strains used: TM181 carrying various plasmids.

it is the *YPD1* gene. *YPD1* is adjacent to *PHO13* and is located on chromosome IV. A sequence similarity search of the GenBank protein database identified a short segment of similarity between Ypd1p and the chemotactic CheA protein of the hyperthermophilic bacterium Thermotoga maritima (Figure 1D). Although the statistical significance of this similarity was questionable, it was later proven to be of functional significance.

The *ypd1*Δ phenotype is very similar to that of *sln1*Δ: namely, the lethality of *ypd1*Δ is suppressed either by overexpression of *PTP2* (e.g., using the strong *GAL1* promoter), or by any of the *ssk1*Δ, *ssk2*Δ, *pbs2*Δ, and *hog1*Δ mutations (Figure 2A). These results suggest that Ypd1p plays a role in the Sln1p-Ssk1p two-component osmosensing system.

Interaction of Ypd1p with the Receiver Domains of Sln1p and Ssk1p

To test whether Ypd1p interacts with either Sln1p or Ssk1p, two-hybrid tests were conducted. Although the DNA binding domain–YPD1 construct by itself had weak activity, when DNA binding domain–YPD1 was combined with activation domain–SSK1, a significant increase in β-galactosidase expression was observed (Table 1, experiment 1). This stimulation was dependent upon the presence of the Ssk1p receiver domain (amino acids 427–712) but not the N-terminal sequence (amino acids 1–427). DNA binding domain–YPD1 also interacted with activation domain–SLN1, and this interaction required Sln1p amino acids 1020–1220, which include the receiver domain (Table 1, experiment 2). No direct interaction between Ssk1p and Sln1p was observed (Table 1, experiment 3). These results indicate that Ypd1p interacts with the receiver domains of both Sln1p and Ssk1p and thus raise the possibility that Ypd1p functions as an intermediary between Sln1p and Ssk1p.

In this context, it appeared significant that the Ypd1p sequence around His64 has limited sequence similarity to the sequences around the histidine-phosphorylation site of bacterial two-component CheA proteins. Further inspection of this stretch of sequence revealed weak similarities to the C-terminal histidine-phosphorylation sites of several prokaryotic histidine kinases (see Figure 1D; Ishige et al., 1994; Uhl and Miller, 1996). To test if Ypd1p-His64 was essential for Ypd1p function, the ability of *ypd1*(H64Q) containing the His64→Gln mutation to complement *ypd1*Δ was examined. The *ypd1*(H64Q) plasmid was unable to render the *ypd1*Δ mutant cells independent of *PTP2* overexpression (Figure 2B), indicating that *ypd1*(H64Q) is nonfunctional and that His64 is essential for Ypd1p function.

Histidine Phosphorylation of Ypd1p In Vivo

To test if Ypd1p-His64 is a phosphorylation site in vivo, we constructed a *GST–YPD1* fusion gene that contains the entire Ypd1p coding sequence (amino acids 1–167). This *GST–YPD1* fusion gene functionally complemented the *ypd1*Δ mutation (Figure 2B). The *GST–YPD1* fusion construct was expressed in various yeast mutant cells, and the cells were pulse-labeled with [^{32}P]orthophosphate. Purified GST–YPD1 protein isolated from wild-type cells was phosphorylated (Figure 3A). This phosphate was alkali-resistant and acid-labile, suggesting that the phosphorylated amino acid was histidine (Fujitaki and Smith, 1984). In contrast, the GST–ypd1(H64Q) mutant protein was not phosphorylated (Figure 3A). The levels of expression of the GST–YPD1 fusion protein were comparable among the various transformed cells

Table 1. Two-Hybrid Analysis Demonstrating the Interactions of Ypd1p with the Receiver Domains of Both Ssk1p and Sln1p

Experiment	GAL4 DNA binding domain construct (pAS2)	GAL4 activation domain construct (pACTII)	β-galactosidase (Unit)[a]
1.	–[b]	–[b]	0.2
	–	SSK1(1–712)[c]	0.1
	–	SSK1(1–427)	0.2
	–	SSK1(427–712)	0.2
	YPD1(2–167)	–	20
	YPD1(2–167)	SSK1(1–712)	490
	YPD1(2–167)	SSK1(1–427)	15
	YPD1(2–167)	SSK1(427–712)	230
2.	–	SLN1(496–1220)	0.1
	–	SLN1(496–1019)	0.2
	YPD1(2–167)	–	17
	YPD1(2–167)	SLN1(496–1220)	75
	YPD1(2–167)	SLN1(496–1019)	7.3
3.	–	SLN1(496–1220)	0.3
	SSK1(1–712)	–	0.2
	SSK1(1–712)	SLN1(496–1220)	0.4

[a] Values are arbitrary units of β-galactosidase activity (see Experimental Procedures), averaged from three independent assays.
[b] Dash (–) indicates that the vector plasmid (either pAS2 or pACTII) without any insert was used.
[c] The numbers in parentheses indicate the positions of the protein included in the fusion constructs. The total sizes of Ypd1p, Ssk1p, and Sln1p are, respectively, 167, 712, and 1220.

used, as determined by anti-GST immunoblotting (Figure 3). Ypd1p phosphorylation is dependent upon Sln1p activity, as no phosphorylation was observed in *sln1*Δ *ssk1*Δ mutant cells (Figure 3B). In this mutant, the *ssk1*Δ mutation was included to suppress the lethality of the *sln1*Δ mutation; the *ssk1*Δ mutation alone had no significant effect on Ypd1p phosphorylation (Figure 3B).

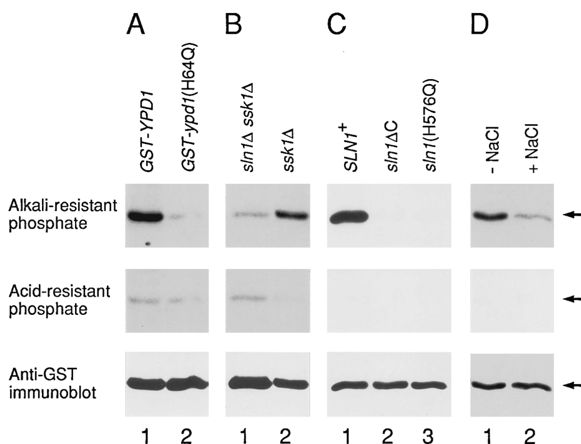

Figure 3. In Vivo Phosphorylation of Ypd1p

Yeast cells growing in phosphate-depleted media were pulse-labeled with [^{32}P]orthophosphate. GST–YPD1 recombinant molecule was purified using glutathione–Sepharose beads and applied to SDS–polyacrylamide gel electrophoresis. Proteins were transferred to nylon membrane and sequentially treated with alkali and acid. Autoradiograms were taken after each treatment. Finally, the filter was probed with anti-GST monoclonal antibody. Arrows indicate the position of GST–YPD1.

(A) TM141 (wild-type) cells transformed with either p426TEG1–YPD1 or p426TEG1–ypd1(H64Q) were tested.

(B) TM227b (*sln1*Δ *ssk1*Δ) and TM188 (*ssk1*Δ) transformed with p426TEG1–YPD1 were used.

(C) TM227b (*sln1*Δ *ssk1*Δ) transformed with p426TEG1–YPD1 plus one of the following plasmids were examined: pRS414–SLN1 (lane 1); pRS414–sln1ΔC (lane 2); or pRS414–sln1(H576Q) (lane 3).

(D) TM141 (wild-type) transformed with p426TEG1–YPD1 was used. Cells were harvested before (lane 1) or after (lane 2) a brief exposure (less than 1 min) to 0.4 M NaCl.

The following experiment using the *sln1*ΔC mutant, however, suggests that Ypd1p is not directly phosphorylated by the histidine kinase activity of Sln1p. The Sln1ΔC mutant protein contains Sln1p amino acids 1–1070 (thus containing the entire histidine kinase domain) but lacks the C-terminal receiver domain. Although *sln1*ΔC is functionally defective, it can complement another defective *sln1* mutant allele, *sln1*ΔN, that contains only the receiver domain (amino acids 1059–1220; see Figure 2C, sector 3). This complementation is dependent on the Asp1144 phosphorylation site of the Sln1p receiver domain, because the *sln1*ΔN(D1144N) mutant allele cannot complement *sln1*ΔC (see Figure 2C, sector 2). These complementation results suggest that the Sln1ΔC protein has a part of the Sln1p activities, including the histidine kinase activity. Indeed, a GST–SLN1 fusion protein that contains only the histidine kinase domain but not the receiver domain has histidine kinase activity in vitro (see below). Nevertheless, the *sln1*ΔC mutant could not support the histidine phosphorylation of Ypd1p (Figure 3C, lane 2), indicating that the Sln1p kinase domain alone is not sufficient to phosphorylate Ypd1p.

The *sln1*(D1144N; Asp1144→Asn) mutant, which lacks the putative aspartate phosphorylation site in the Sln1p receiver domain, also failed to phosphorylate Ypd1p in vivo (data not shown), further supporting the role of the Sln1p receiver domain in Ypd1p phosphorylation. A mutant *sln1* that lacks the histidine autophosphorylation site *sln1*(H576Q; His576→Gln) also failed to phosphorylate Ypd1p in vivo (Figure 3C, lane 3), indicating that phosphorylation of Sln1p-His576 is a prerequisite for the histidine phosphorylation of Ypd1p. These findings demonstrate that Ypd1p-His64 is phosphorylated in vivo and that this phosphorylation is dependent upon both

the Sln1p-His576 and Sln1p-Asp1144 phosphorylation sites.

Based on the phenotypes of *sln1* and *ssk1* mutants, we previously hypothesized that the Sln1p histidine kinase is active under normal osmotic conditions, while it is suppressed under hyper-osmotic conditions (Maeda et al., 1994). To test this prediction, we examined the phosphorylation state of GST–YPD1 after the addition of 0.4 M NaCl. The level of GST–YPD1 phosphorylation is much lower in the presence of 0.4 M NaCl than in control cells (Figure 3D), corroborating our prediction that the Sln1p histidine kinase activity is suppressed under hyper-osmotic conditions.

Phosphotransfer from Sln1p-His576 to Sln1p-Asp1144

To investigate more directly the origin of the phosphate that is transferred to Ypd1p-His64 and its relationship to the predicted phosphorylation of Ssk1p-Asp554, we performed phosphorylation reactions in vitro. For this purpose, we first tested whether a phosphate at Sln1p-His576 was transferred directly to Sln1p-Asp1144. The GST–SLN1–HisK fusion protein contains the Sln1p histidine kinase domain (amino acids 450–1070) but no transmembrane segments or receiver domain (Figure 4B). When purified GST–SLN1–HisK was incubated in the presence of [γ-^{32}P]ATP, ^{32}P was incorporated into the fusion protein (Figure 4A, lane 1). At 25°C, this autophosphorylation reaction was linear up to at least 45 min (data not shown). A slightly smaller protein, marked by an asterisk (*), was also phosphorylated and was probably a proteolytic product of GST–SLN1–HisK. In contrast, the GST–SLN1–HisK(H576Q) mutant protein did not incorporate ^{32}P under the same conditions (data not shown). Thus, the recombinant GST–SLN1–HisK protein has histidine kinase activity.

^{32}P-labeled GST–SLN1–HisK (which was bound to glutathione–Sepharose beads) was washed to eliminate [γ-^{32}P]ATP and then mixed with purified GST–SLN1–Rec, which contains the Sln1p receiver domain (amino acids 1059–1220) but not the kinase domain. In a 15 min reaction, nearly half of the ^{32}P was transferred from ^{32}P-GST–SLN1–HisK to GST–SLN1–Rec, and a corresponding decrease of radioactivity in GST–SLN1–HisK was observed (Figure 4A, lane 2). In contrast, when GST–SLN1–Rec(D1144N) was used, there was no transfer of ^{32}P (Figure 4A, lane 3). Alkali treatment removed all the ^{32}P from GST–SLN1–Rec but not from GST–SLN1–HisK protein (Figure 4A, lane 7), whereas acid treatment removed radioactivity from both recombinant proteins (data not shown). The pH sensitivities, as well as the effects of mutations, indicate that GST–SLN1–HisK contains a phosphohistidine residue whereas GST–SLN1–Rec contains a phosphoaspartate residue.

To eliminate the possibility that the phosphate at Sln1p-Asp1144 was derived from contaminating proteins (e.g., E. coli protein) rather than ^{32}P-GST–SLN1–HisK, additional experiments were carried out using the GST–SLN1(H576Q) construct. The GST–SLN1(H576Q) fusion protein contained both the histidine kinase and the receiver domains (amino acids 450–1220), but because of the His576→Gln mutation, no autophosphorylation occurred (Figure 4A, lane 4). In reactions that were identical to those indicated in lanes 2 and 3, except that GST–SLN1(H576Q) was used in place of GST–SLN1–HisK, neither GST–SLN1–Rec nor GST–SLN1(H576Q) was phosphorylated (Figure 4A, lanes 5 and 6). Thus, we conclude that the phosphate residue on Sln1p-His576 is transferred to Sln1p-Asp1144.

Figure 4. Phosphotransfer Reaction from Sln1p-His576 to Sln1p-Asp1144

(A) GST–SLN1–HisK or GST–SLN1(H576Q) protein was autophosphorylated in vitro and separated from [γ-^{32}P]ATP. After mixing with the indicated substrate proteins, the phosphotransfer reaction was carried out at 25°C for 15 min, and proteins were separated by SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to nylon membrane and exposed to X-ray film (lanes 1–6). The same membrane was then treated with 3M KOH (lane 7). The position of each protein is indicated on the left. Asterisk indicates a partial product of GST–SLN1–HisK.

(B) Schematic representation of the in vitro phosphotransfer reaction. At the top is the full-length Sln1p, and below are two GST fusion constructs. Arrows indicate the flow of the phosphate group. In the first step, the γ phosphate group from ATP is transferred to Sln1p-His576. In the second step, this phosphate group is transferred to Sln1p-Asp1144.

Phosphotransfer from Sln1p-Asp1144 to Ypd1p-His64

To determine the source of phosphate on Ypd1p-His64, we initially tested whether phosphate could be directly transferred from Sln1p-His576 to Ypd1p-His64 by incubating ^{32}P-autophosphorylated GST–SLN1–HisK with either GST–YPD1 or GST–YPD1(H64Q) (Figure 5A). No transfer of phosphate was detected with either protein (Figure 5A, lanes 2 and 3), indicating that phosphotransfer from Sln1p-His576 to Ypd1-His64 does not occur.

To test then whether the phosphate at Sln1p-Asp1144

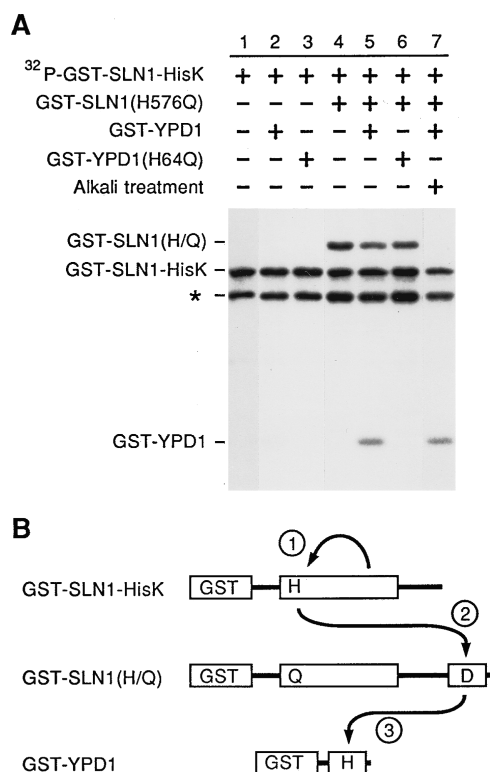

Figure 5. Phosphotransfer Reaction from Sln1p-Asp1144 to Ypd1p-His64

(A) Purified ^{32}P-GST–SLN1–HisK was mixed with indicated substrate proteins, and the phosphotransfer reaction was carried out at 25°C for 15 min. H/Q stands for the H576Q mutation.

(B) Schematic representation of the in vitro phosphotransfer reaction. In the third step of the phosphorelay reaction, a phosphate group is transferred from Sln1p-Asp1144 to Ypd1p-His64.

could be transferred to Ypd1p-His64, a [^{32}P-Asp-1144]Sln1p intermediate was prepared by incubating ^{32}P-GST–SLN1–HisK and GST–SLN1(H576Q) (Figure 5A, lane 4). The larger molecule, GST–SLN1(H576Q), was used as a phospho-acceptor instead of GST–SLN1–Rec, because the size similarity between GST–YPD1 and GST–SLN1–Rec did not allow for separation of these molecules by SDS–polyacrylamide gel electrophoresis analysis. Incubation of the three proteins, ^{32}P-GST–SLN1–HisK, GST–SLN1(H576Q), and GST–YPD1 resulted in the phosphorylation of GST–YPD1 with a corresponding decrease of radioactivity in GST–SLN1 (H576Q) (Figure 5A, lane 5). In a parallel reaction, GST–YPD1(H64Q) was not phosphorylated (Figure 5A, lane 6). Alkali treatment removed the phosphate from GST–SLN1(H576Q) (this protein is phosphorylated at Asp 1144) but not from the histidine-phosphorylated GST–SLN1–HisK or GST–YPD1 proteins (Figure 5A, lane 7). Acid treatment removed radioactivity from all three recombinant proteins (data not shown). Thus, the phosphate group on Ypd1p-His64 is derived from Sln1p-Asp1144.

Phosphotransfer from Ypd1p-His64 to Ssk1p-Asp554

To investigate the phosphorylation of Ssk1p-Asp554, ^{32}P-GST–YPD1 was generated by incubating ^{32}P-GST–SLN1–HisK, GST–SLN1(H576Q), and GST–YPD1 (Figure 6A, lane 1). Addition of GST–SSK1 fusion protein, which contains the entire Ssk1p coding sequence (amino acids 1–712), in the reaction resulted in GST–SSK1 phosphorylation (Figure 6, lane 2). Phosphorylation of GST–SSK1 did not occur if the reaction contained GST–YPD1(H64Q) in place of GST–YPD1 (Figure 6A, lane 3). Alkali and acid sensitivities of ^{32}P-GST–SSK1 (Figure 6, lanes 4–6) indicated that GST–SSK1 was phosphorylated on an aspartate residue. Furthermore, when ^{32}P-GST–SLN1–HisK was incubated with GST–SLN1–Rec, GST–YPD1, and GST–SSK1, the predicted intermediates ^{32}P-GST–SLN1–Rec and ^{32}P-GST–YPD1 comigrated, but ^{32}P-GST–SSK1 could be more clearly discerned (Figure 6C, lane 1). Incubation of GST–YPD1(H64Q) instead of GST–YPD1 blocked phosphorylation of GST–SSK1 (Figure 6C, lane 2), indicating that Ypd1p-His64 is essential for Ssk1p phosphorylation.

However, this result does not by itself demonstrate that the phosphate on Ssk1p-Asp554 is derived from Ypd1p-His64. For example, it is possible that either Sln1p-His576 or Sln1p-Asp1144 was the immediate donor of the phosphate, with the phosphorylated Ypd1p acting merely as a catalyst. To eliminate this possibility, ^{32}P-GST–YPD1 was prepared by an in vivo labeling reaction as shown in Figure 3 and incubated with either GST–SSK1–Rec or GST–SSK1–Rec(D554N). The GST–SSK1–Rec molecule contains only the Ssk1p receiver domain (amino acids 475–712). Under these conditions, GST–SSK1–Rec was phosphorylated but not GST–SSK1–Rec(D554N) (Figure 6D, lanes 2 and 3). Because this reaction does not contain SLN1 protein, it demonstrates that Ypd1p can directly donate its phosphate to Ssk1p. Furthermore, these results support the assignment of Ssk1p-Asp554 as the phosphorylation site based on sequence analysis (Maeda et al., 1994). Taken together, these results demonstrate that Ypd1p-His64 is the immediate phospho-donor of Ssk1p-Asp554.

Discussion

Phosphorelay Mechanism in the SLN1–YPD1–SSK1 Two-Component System

We demonstrate here that the yeast osmosensory two-component system is actually composed of three proteins, Sln1p, Ypd1p, and Ssk1p. We have further shown that a phosphate group is transferred sequentially by a phosphorelay mechanism from Sln1p-His576 to Sln1p-Asp1144, then to Ypd1p-His64, and finally to Ssk1p-Asp554. Mutations at Sln1p-His576, Sln1p-Asp1144, and Ypd1p-His64, individually, result in constitutive activation of the HOG1 MAPK cascade, which results in cell death. Although both the *sln1*(H576Q) and *sln1*(D1144N) mutations are lethal by themselves, they complement each other (Figure 2C; see also Maeda et al., 1994). This result implies that the phosphotransfer reaction from His576 to Asp1144 can occur between two Sln1p molecules, rather than intramolecularly. Dimerization and interallelic complementation of bacterial sensor histidine kinases have been well documented (Gegner and Dahlquist, 1991; Yang and Inouye, 1991; Ninfa et al., 1993).

Based on our findings, we propose that the multistep

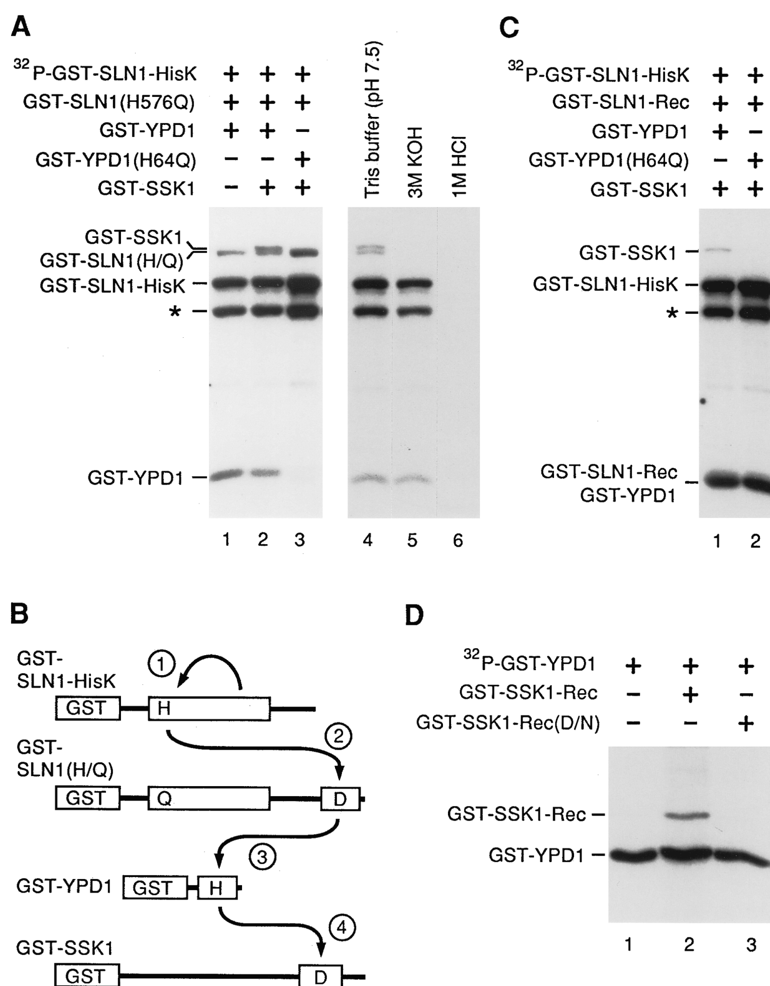

Figure 6. Phosphotransfer Reaction from Ypd1p-His64 to Ssk1p-Asp554

(A) Purified ^{32}P-GST–SLN1–HisK was mixed with indicated substrates, and phosphotransfer reaction was carried out at 25°C for 15 min (lanes 1–3). The same reaction as lane 2 was transferred to three separate nylon membranes, and each membrane was treated with either neutral buffer (lane 4), 3M KOH (lane 5), or 1M HCl (lane 6).

(B) Schematic representation of the in vitro phosphotransfer reaction. In the fourth step of the phosphorelay reaction, a phosphate group is transferred from Ypd1p-His64 to Ssk1p-Asp554.

(C) Purified ^{32}P-GST–SLN1–HisK was mixed with indicated substrate proteins, and the phosphotransfer reaction was carried out at 25°C for 15 min. Because ^{32}P-GST–SLN1–Rec and ^{32}P-GST–YPD1 have the same mobility, these cannot be separated.

(D) GST–YPD1 was labeled in vivo using [^{32}P]orthophosphate and purified using glutathione–Sepharose beads. ^{32}P-GST–YPD1 was mixed with indicated substrate, and the phosphotransfer reaction was carried out at 25°C for 15 min. H/Q stands for the H576Q mutation and D/N for D554N.

phosphorelay mechanism whereby a phosphate is transferred in the order histidine (H1) → aspartate (D1) → histidine (H2) → aspartate (D2) is a universal signal transduction apparatus utilized in both prokaryotic and eukaryotic cells (Figure 7A). In the case of the yeast osmosensor, Sln1p contains both the H1 and D1 sites, while Ypd1p contains an H2 site and Ssk1p contains a D2 site. The original phosphorelay concept was proposed for the Bacillus subtilis sporulation pathway, in which four phosphorylation sites are found in four different molecules: KinA (H1), Spo0F (D1), Spo0B (H2), and Spo0A (D2) (Burbulys et al., 1991). Although the precise phosphorylation site of Spo0B (either histidine or lysine) is not known, and the Spo0B amino acid sequence does not have any similarity to Ypd1p, the overall similarity of the Sln1p-Ypd1p-Ssk1p pathway and the Spo pathway is striking. Previously, the Spo phosphorelay had been considered as a unique case. Now, our results clearly demonstrate that a homologous phosphorelay mechanism also exists in eukaryotic cells. Furthermore, examination of structural and functional properties of several unorthodox two-component proteins from both prokaryotic and eukaryotic cells suggests that a similar phosphorelay may be more widely utilized.

Most prokaryotic two-component systems contain only two elements, a sensor histidine kinase and a response-regulator with a receiver domain. However, several unorthodox two-component systems are known that involve more than one receiver-domain protein. For example, bacterial BvgS and ArcB contain a histidine kinase domain and a receiver domain, but their functions require separate receiver domain proteins, BvgA and ArcA, respectively (Ishige et al., 1994; Uhl and Miller, 1996). Thus, both BvgS and ArcB are architecturally similar to Sln1p. However, unlike the yeast system, BvgS and ArcB seem to have a second histidine phosphorylation site at their C-terminus. Thus, conceptually, BvgS and ArcB can be considered as a fusion of Sln1p and Ypd1p. In the above notation, BvgS and ArcB contain the H1, D1, and H2 phosphorylation sites, and BvgA and ArcA contain the D2 phosphorylation sites (Figure 7A). Available data on BvgS–BvgA and ArcB–ArcA systems are consistent with the linear transmission of a phosphate in the order of H1-D1-H2-D2, although other interpretations cannot be excluded (Iuchi, 1993; Ishige et al., 1994; Tsuzuki et al., 1995; Uhl and Miller, 1996).

The phosphorelay model may also be applicable to other eukaryotic two-component systems. Both the plant ethylene receptor ETR1 and fungal Nik-1 are architecturally similar to Sln1p in the sense that they both have the H1 and D1 phosphorylation sites within the same molecule, but no obvious H2 sequence (Chang et al., 1993; Alex et al., 1996). Thus, it seems likely that the functioning of these molecules also utilizes a similar

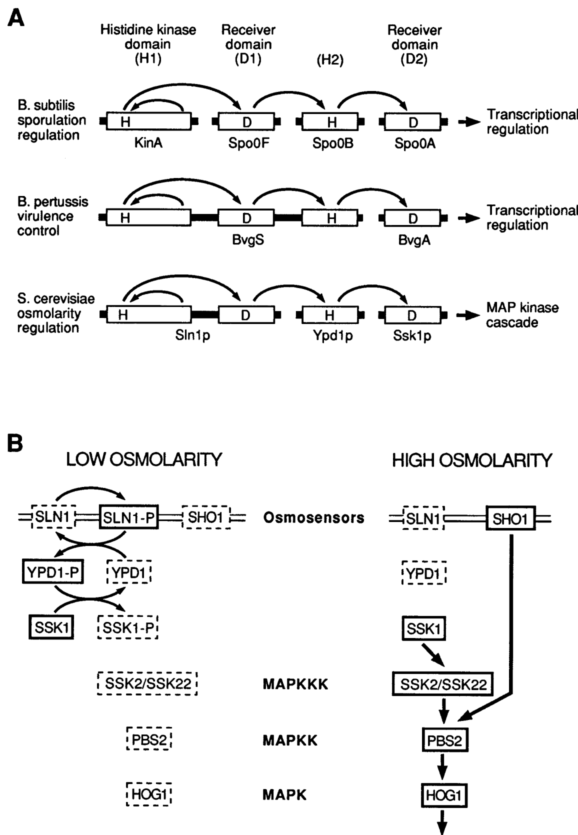

Figure 7. Models

(A) The proposed SLN1–YPD1–SSK1 phosphorelay reaction is compared with potentially homologous phosphotransfer reactions in bacteria (see text).

(B) A current model of the yeast HOG pathway is schematically shown. Inactive elements are indicated by dashed boxes. Arched arrows indicate phosphotransfer reactions, whereas straight arrows simply indicate signal flow. P indicates a phosphorylated form (not all phosphorylation events are shown).

phosphorelay mechanism involving additional protein or proteins with the H2 and D2 phosphorylation sites.

What is the Advantage of Multistep Phosphorelay Mechanism?

If the sole function of the multistep phosphorelay is to transfer phosphate from H1 to D2, there seems no advantage to having the extra components (D1 and H2), since phosphate can be directly transferred from H1 to an aspartate in receiver domain (D2), as is the case in numerous orthodox two-component systems. Furthermore, unlike cascades of enzymes (e.g., kinase cascades and protease cascades), the phosphorelay mechanism does not serve to amplify signals: if anything, the final yield is less than 100% of the initial histidine kinase reaction. Nevertheless, the evolutionary conservation of a similar phosphorelay mechanism between bacteria and eukaryotic cells implies that there must be a selective advantage of maintaining such a complex mechanism.

An obvious advantage of the phosphorelay mechanism is that it allows integration of multiple signals at the intermediate steps. For instance, in the Bacillus sporulation pathway, the receiver domain of Spo0F can accept phosphate from either KinA or KinB, thus integrating two distinct signals (Trach and Hoch, 1993). This type of signal integration, including cross-talk between distinct pathways, may be particularly important in prokaryotic cells in which many two-component systems coexist (Wanner, 1992). However, a search of the complete yeast genome DNA sequence revealed that Skn7p is the only other two-component protein present in the yeast genome besides Sln1p and Ssk1p, and no evidence exists that might suggest a functional interaction between the SKN7 and SLN1 pathways (Brown et al., 1994; Morgan et al., 1995). Nevertheless, it is possible that an unidentified signaling element exists that feeds signals into the HOG1 pathway through Ypd1p or Ssk1p. Such an upstream element may be another osmosensor or a sensor for distinct type of environmental stress.

Some receiver domains (e.g., CheY) can accept phosphate from low molecular mass metabolic intermediates, such as acetyl phosphate and carbamoyl phosphate (Lukat et al., 1992). A similar reaction may serve as a mechanism to monitor the metabolic state and to integrate such information with signals that originate from the extracellular space. In this context, it is worth noting that HOG pathway activation results in the synthesis of glycerol-3-phosphate dehydrogenase, which converts dihydroxyacetone phosphate to glycerol-3-phosphate. If glycerol-3-phosphate or a related metabolic intermediate donates its phosphate to either Sln1p-Asp1144, Ypd1p-His64, or Ssk1p-Asp554, it would serve as a negative feedback regulator of the HOG1 MAPK cascade.

Alternatively, the D1 and H2 phosphorylation sites may serve as extra regulatory points in complex two-component systems. Involvement of specific phosphatases is frequently observed in both simple and complex two-component systems. For example, in the Bacillus sporulation pathway, specific response-regulator aspartate phosphatases (RapA and RapB) dephosphorylate Spo0F (Perego et al., 1994). Dephosphorylation of the chemotactic response-regulator CheY is stimulated by CheZ (Hess et al., 1988). Yeast homologs of Rap or CheZ are not known, but often the histidine kinase domain of a transmembrane sensor has aspartate phosphatase activity (Keener and Kustu, 1988; Igo et al., 1989). Furthermore, the yeast protein phosphatases PP1, PP2A, and PP2C have potent histidine phosphatase activity (Kim et al., 1993). Thus, there may exist a phosphatase that negatively regulates the phosphate flow through the SLN1–YPD1–SSK1 phosphorelay.

Most bacterial two-component systems are self-contained signal transduction mechanisms, because the response-regulators themselves have effector functions such as transcriptional activation. In contrast, the yeast SLN1–YPD1–SSK1 phosphorelay is only the initial segment of a more extensive signaling pathway that regulates the HOG1 MAPK cascade (Maeda et al., 1995; Figure 7B). A similar phosphorelay regulatory mechanism for a MAPK cascade can be predicted for the plant ethylene response pathway, because a protein kinase of the Raf family (CTR1) has been found downstream of the ETR1 histidine kinase (Kieber et al., 1993). Furthermore, a mammalian MAPK cascade that is activated by

stress (including hyper-osmolarity) involves PBS2-like and HOG1-like kinases (Galcheva-Gargova et al., 1994; Han et al., 1994; Lin et al., 1995), suggesting a possibility that an SLN1-like phosphorelay signal transduction mechanism may also exist in animal cells. Thus, we propose that the multistep phosphorelay mechanism is a universal signal transduction apparatus that is utilized both in prokaryotic and eukaryotic cells.

Experimental Procedures

Materials

Buffer A is 50 mM Tris–HCl (pH 8.0), 15 mM EDTA, 15 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 2 mM dithiothreitol (DTT), 0.1% Triton–X100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml of leupeptin, and 5 μg/ml of pepstatin.

Buffer B is 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml of leupeptin, and 5 μg/ml of pepstatin. Buffer C is 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, and 2 mM DTT. SDS loading buffer is 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue, and 10% glycerol.

Yeast Strains

The *YPD1* and *SLN1* genes were disrupted using the *hisG-URA3-hisG* cassette (Alani et al., 1987). The *URA3* marker was then deleted by homologous recombination between the two *hisG* sequences.

The following yeast strains were used:

SW100 *MAT***a** *ura3 leu2 trp1 ypd1::hisG* [pGAL1-PTP2].
SW102 *MAT***a** *ura3 leu2 trp1 ypd1::hisG hog1::URA3* [pGAL1-PTP2].
SW104 *MAT***a** *ura3 leu2 trp1 ypd1::hisG pbs2::URA3* [pGAL1-PTP2].
SW108 *MAT***a** *ura3 leu2 trp1 ypd1::hisG ssk2::URA3* [pGAL1-PTP2].
FP30 *MAT***a** *ura3 leu2 trp1 ypd1::hisG ssk1::URA3* [pGAL1-PTP2].
TM141 *MAT***a** *ura3 leu2 trp1 his3.*
TM181 *MAT***a** *ura3 leu2 trp1 sln1::hisG* [pSSP25].
TM188 *MAT***a** *ura3 leu2 trp1 ssk1::LEU2.*
TM227b *MAT***a** *ura3 leu2 trp1 sln1::hisG ssk1::LEU2.*
Y190 *MAT***a** *ura3 leu2 trp1 his3 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3 cyh^r* (Harper et al., 1993).

Plasmids

pSSP25 (*P_{GAL1}-PTP2, URA3, ADE3, CEN3*) was as described (Maeda et al., 1993). The *P_{GAL1}–PTP2* fusion gene from pSSP25 was inserted in pRS414 (Sikorski and Hieter, 1989) to generate pGAL1–PTP2 (*P_{GAL1}–PTP2*, *TRP1, CEN6*). GST fusion genes for bacterial expression were constructed using the expression vector pGEX-4T-1 (Pharmacia) and polymerase chain reaction fragments amplified from either wild-type or site-directed mutant plasmid DNA. GST fusion genes for yeast expression were constructed using the expression vector p426TEG1 (*P_{TEF2}–GST*, *URA3*, 2 micron). p426TEG1 is a derivative of p426TEF (Mumberg et al., 1995) and contains the GST domain under the control of the yeast *TEF2* promoter (M. Takekawa and H. S., unpublished data).

Two-Hybrid Assay

The two-hybrid analysis was carried out essentially according to Durfee et al. (1993), using pACTII (Li et al., 1994) and pAS2 (Harper et al., 1993) as the activation domain plasmid and the DNA binding domain plasmid, respectively. The reading frame of each fusion construct was confirmed by DNA sequence determination of the vector–insert junction. Expression of the fusion proteins was confirmed by Western blotting analysis using the 12CA5 antihemagglutinin monoclonal antibody (Boehringer Mannheim). Assays for β-galactosidase activities were performed in triplicate according to Yocum et al. (1984). β-galactosidase activity (one unit) is defined as an increase of 0.001 OD_{420} in 1 min by extract prepared from 3×10^7 cells.

In Vivo Protein Labeling

Phosphate-depleted selective media were prepared according to Warner (1991). Fresh culture (10 ml) in a phosphate-depleted selective medium ($OD_{660} = 1$) was centrifuged and resuspended in 1 ml of phosphate-depleted media. After incubation at 30°C for 15 min, cultures were added with 0.75 mCi of [^{32}P]orthophosphate (9000 Ci/mmol) and further incubated at 30°C for 5 min. Cultures were then mixed with 0.1 ml of 4M NaCl, incubated at 30°C for 15 min, diluted with 10 ml of water, and incubated for an additional 5 min. Cells were harvested, washed once with ice-cold water, and resuspended in 0.4 ml of buffer A plus 10 μg/ml of pancreatic RNase. Cells were broken by vortexing with glass beads and centrifuged at 10,000 rpm for 10 min in a microcentrifuge. A 50 μl suspension of glutathione–Sepharose beads (Pharmacia) was added to each supernatant and incubated with gentle rotation at 4°C for 25 min. Beads were washed once with buffer A, four times with buffer A plus 150 mM NaCl, and finally suspended in 40 μl of 2 × SDS loading buffer. Samples were applied to SDS–polyacrylamide gel without boiling, and electrophoresis was carried out at 4°C to minimize heating of the samples. After electrophoresis, proteins were transferred to Immobilon-P nylon membrane (Millipore) by electroblotting. Filters were dried and exposed to X-ray film at −80°C with an intensifying screen. Chemical stabilities of phosphorylated proteins were determined by treating the nylon membranes with either neutral solution (50 mM Tris–HCl [pH 7.5]), alkali solution (3M KOH), or acid solution (1M HCl) for 2 hr at 25°C. Membranes were rinsed with water, dried, and exposed to film as above. Anti-GST immunoblotting was done using anti-GST monoclonal antibody (Pharmacia) and the ECL reagent (Amersham).

Expression and Purification of GST Fusion Proteins

E. coli DH5 cells transformed with various GST fusion constructs were grown in 1 l of Luria-Bertani broth with 100 μg/ml of ampicillin at 37°C. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM, and the cultures were shaken for an additional 4 hr at 25°C. Cells were harvested and resuspended in 20 ml of buffer B. Bacteria were lysed by sonication, and the lysates were clarified by centrifugation at 7,000 g for 10 min at 4°C. Aliquots of the supernatants were stored at −80°C. Freshly thawed supernatant (3 ml) was mixed with 0.5 ml of glutathione–Sepharose beads and incubated for 30 min at 4°C. The Sepharose beads were washed four times with buffer B and once with 50 mM Tris–HCl (pH 8.0), 2 mM DTT. GST fusion proteins were eluted with 10 mM glutathione in 50 mM Tris–HCl (pH 8.0), 2 mM DTT. Protein concentration was determined according to Bradford (1976).

In Vitro Protein Labeling

GST–SLN1–HisK (either wild-type or H576Q mutant) were expressed in E. coli DH5 and purified as described above, except that beads were washed twice in buffer C instead of elution buffer containing glutathione. Beads bound with approximately 10 μg of GST–SLN1–HisK were resuspended in 20 μl of buffer C plus 20 μM [γ-^{32}P]ATP (50 Ci/mmol) and incubated at 25°C for 45 min. Beads were then washed four times with buffer C. Phosphotransfer reactions were carried out at 25°C for 15 min in the presence of 3–5 μg of each recombinant protein. Reactions were stopped by the addition of 2 × SDS loading buffer. SDS–polyacrylamide gel electrophoresis, transferring of proteins to nylon membranes, and acid and alkali treatments were same as described for in vivo labeling.

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GenBank Accession Number

The nucleotide sequence of the *YPD1* gene is available under accession number U62016.