Altered Phosphotransfer in an Activated Mutant of the *Saccharomyces cerevisiae* Two-Component Osmosensor Sln1p

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Received 14 September 2001/Accepted 19 December 2001

The *SLN1* two-component signaling pathway of *Saccharomyces cerevisiae* utilizes a multistep phosphorelay mechanism to control osmotic stress responses via the *HOG1* mitogen-activated protein kinase pathway and the transcription factor Skn7p. Sln1p consists of a sensor kinase module that undergoes histidine autophosphorylation and a receiver module that autokatalytically transfers the phosphoryl group from histidine to aspartate. The Sln1p aspartyl phosphate is then transferred to Ypd1p, which in turn transfers the phosphoryl group to a conserved aspartate on one of two response regulators, Ssk1p and Skn7p. Activated alleles of *SLN1* (*sln1*) were previously identified that appear to increase the level of phosphorylation of downstream targets Ssk1p and Skn7p. In principle, the phenotype of *sln1* alleles could arise from an increase in autophosphorylation or phosphotransfer activities or a decrease in an intrinsic or extrinsic dephosphorylation activity. Genetic analysis of the activated mutants has been unable to distinguish between these possibilities. In this report, we address this issue by analyzing phosphorelay and phosphohydrolysis reactions involving the Sln1p-associated receiver. The results are consistent with a model in which the activated phenotype of the *sln1* allele, *sln1*-22, arises from a shift in the phosphotransfer equilibrium from Sln1p to Ypd1p, rather than from impaired dephosphorylation of the system in response to osmotic stress.

The *SLN1* two-component signaling pathway of *Saccharomyces cerevisiae* is a multistep phosphorelay system that responds to changes in osmotic conditions via the *HOG1* mitogen-activated protein (MAP) kinase pathway and the transcription factor Skn7p (Fig. 1) (2, 3, 17, 22, 25). Histidine autophosphorylation of the Sln1p kinase domain (Sln1K) and phosphotransfer to a conserved aspartate within the Sln1p receiver domain (Sln1R) are followed by phosphotransfer to a histidine residue on the histidine-containing phosphotransfer (HPT) protein, Ypd1p (25, 34). From Ypd1p, the phosphoaspartyl group is transferred to an aspartate on one of two response regulators, Ssk1p and Skn7p (16, 25). The activities of Ssk1p and Skn7p depend on the phosphorylation state of the aspartate residue in the receiver domain. Dephospho-Ssk1p binds and activates the MEK kinases Ssk2p and Ssk22p, resulting in activation of the *HOG1* osmotic stress response pathway (17, 24). Skn7p is a transcriptional activator whose phosphorylation status dictates which set of target genes are activated (15, 16, 19, 18).

Sln1p is an osmosensor, but the nature of the stimulus and how it is sensed are unknown. Under nonstress conditions, Sln1p maintains Ssk1p and Skn7p in their phosphorylated forms and the Hog1 pathway is kept inactive. Activation of the *HOG1* pathway requires dephosphorylation of Ssk1p. Since Hog1p is phosphorylated within 1 to 3 min of a shift to a hyperosmotic environment (2), dephosphorylation of Ssk1p must be rapid. However, the intrinsic hydrolysis rate of the phosphorylated receiver domain of Ssk1p in vitro is very low, with an estimated half-life (*t*1/2) of 40 h (12). In vivo phosphorylation assays have shown that Ypd1p is dephosphorylated within 1 min of salt addition (25). Although no rate studies have been reported, Sln1p is also dephosphorylated under hypertonic conditions (21), suggesting that the dephosphorylation of Sln1p, Ypd1p, and Ssk1p in response to elevated osmolarity may be rapid and virtually simultaneous. In one mechanism for rapid, coordinate dephosphorylation of the pathway, Sln1p may actively participate in the dephosphorylation of Ssk1p by acting as an aspartyl phosphatase, as occurs in some bacterial systems (9, 27, 33). A second possible mechanism for rapid and coordinate dephosphorylation is a reversal in the directionality of the phosphorelay followed by hydrolysis of phospho-Sln1p receiver. However, this has not been observed in vitro (11).

Activation of Hog1p by Ssk1p is transient, which is due, at least in part, to the action of tyrosine and Ser/Thr-directed phosphatases whose expression is stimulated by the HOG1 pathway (35). The transient activation of Hog1p suggests that Ssk1p may be rephosphorylated after the cell establishes osmotic balance, thus eliminating the activating signal through the MAP kinase pathway (31). This implies that Sln1p elevates phosphorylation of its targets as the cytoplasmic osmolarity comes into balance with, or exceeds, external osmolarity, and this has led to a model in which Sln1p phosphorylation and phosphorelay activities are regulated positively and negatively in response to the osmotic balance of the cell (Fig. 1).

Previously, we identified activated alleles of *SLN1* (*sln1*) that cause elevated transcription of *SKN7*-dependent target genes and interfere with the cell's ability to grow in high salt (16, 36). Previous results have suggested that the activated phenotype may be due to an increase in phosphorylation of the Skn7p and Ssk1p response regulators (4). In principle, *sln1* mutations that lead to elevated phosphorylation of Skn7p or Ssk1p could occur by a variety of mechanisms. For example, increasing the histidine kinase activity of Sln1p or the rate of
mutations lead to activation, we have characterized the mutant points of regulation of the Sln1p phosphorelay system and how lysis (reaction 3). To gain a better understanding of possible with Ypd1p (reaction 2), and it can undergo irreversible hydrolysis (reaction 3). The in vitro results are consistent with the increased Skn7p activity observed in vivo when sln1* is present. To our knowledge, this is the first report of a eukaryotic two-component mutant with elevated phosphotransfer activity.

MATERIALS AND METHODS
Plasmids. pGEX-Sln1K (537-950), which encodes a glutathione S-transferase (GST) fusion with the Sln1p kinase domain (GST-Sln1K) (Fig. 3), was constructed by PCR amplification of an SLN1 DNA fragment corresponding to amino acids 537 to 950 by using oligonucleotides Oli195 (ATGACAGACGCA TGAATTCAACATTATGCTTCTTAG) and Oli176 (CTTTCTACTGCAGGGTTAATTCGTC). The product was digested with EcoRI and XhoI and ligated into pGEX-KG (7). pGEX-Sln1R (1070-1220) and pGEX-Sln1R* (1070-1220), which encode GST fusions with the Sln1p and Sln1P1148Sp receiver domains (GST-Sln1R and GST-Sln1R*), were constructed by PCR amplification of an SLN1 or sln1-22 fragment corresponding to amino acids 1070 to 1220 by using oligonucleotides Oli194 (ACATCAAGTAGGCAGAGTGATAGCTGGTTTTCTTGAAGTGTAG) and Oli341 (TTGTTCGAGGGTTAATTCGTC). The product was digested with EcoRI and dIII and ligated into pGEX-KG. pGEX-Skn7 was constructed of an SLN1 or SKN7 by using oligonucleotides Oli340 (GATGTGCTGGATTAAATTCGTC). The product was digested with EcoRI and ligated into pGEX-KG (49). The product was digested with EcoRI and HindIII and ligated into pGEX-KG. pGEX-Sln1KR (537-1220), which encodes a GST fusion with the Sln1p and Sln1P1148Sp receiver domains (GST-Sln1KR), was constructed by PCR amplification of an SLN1 DNA fragment corresponding to amino acids 537 to 1220 by using oligonucleotides Oli195 and Oli255 (GAGAAGCTTCGGACAAGAAACTC). The product was digested with BamHI and SalI and ligated into pGEX-KG. pGEX-Skn7 was constructed by PCR amplification of SKN7 by using oligonucleotides Oli340 (GATGTGCTGGATTAAATTCGTC). The product was digested with EcoRI and XhoI and ligated into pGEX-KG.

FIG. 3. Recombinant proteins used in this study. See Materials and Methods for details concerning construction. The region of Sln1p included in each construct is indicated by the amino acid numbers shown at top. The Ypd1p and Skn7p constructs contain the entire open reading frame of the gene. The predicted size of each protein is indicated on the right. Purified Ypd1p was a gift of Ann West (University of Oklahoma).

FIG. 1. The SLN1 signaling pathway in S. cerevisiae. In response to changes in osmotic balance, the Sln1p sensor-histidine kinase undergoes autophosphorylation followed by a series of reversible phosphorelay reactions involving the phosphotransfer protein Ypd1p and the response regulators Ssk1p and Skn7p. The dephospho form of Ssk1p activates the Hog1 MAP kinase pathway and genes controlling the osmotic response. Phospho-Skn7p is a transcription factor that regulates cell wall and cell cycle genes.

FIG. 2. Reactions involving the Sln1p-associated receiver domain. Sln1R undergoes reversible phosphotransfer reactions with Sln1K (reaction 1) and Ypd1p (reaction 2). Alternatively, the phosphoryl group can be lost from Sln1R-P by irreversible hydrolysis (reaction 3).
Growth and induction of bacterial strains expressing GST fusion proteins. GST fusion proteins were expressed in DH5α cells. With the exception ofGST-Sln1K(537–950) (see below), fusion proteins were expressed by growing l-1iters to an optical density at 600 nm of 0.6 to 1.0 in Luria-Bertani medium plus ampicillin (0.1 mg/ml) and inducing the cultures with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at the indicated time and temperature. GST-Sln1KR(537–1220) and GST-Skn7 were shifted to 16°C prior to induction and induced for 20 h. GST-Sln1R, GST-Sln1R*, and GST-Ypd1p cultures were induced for 2 h at 37°C. After induction, cells were harvested by centrifugation (5,000 × g, 20 min), resuspended in lysis buffer (10% glycerol, 50 mM Tris [pH 7.6], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% β-mercaptoethanol [βME], 1× protease inhibitor cocktail [PIC] [leupeptin, 0.05 mg/ml; pepstatin, 0.001 mg/ml; chymostatin, 0.001 mg/ml; aprotinin, 0.05 mg/ml [all from Sigma]]), and stored frozen at –80°C.

GST-Sln1K(537–950) was expressed by inoculating 6 liters of fermentor starting medium (containing, per liter, 5 g of ammonium sulfate, 1 g of tryptone, 5 g of yeast extract, 7 g of K2HPO4, 2 g of KH2PO4, 1 g of MgSO4, 7H2O, 100 mg of ampicillin, and 1 g of glucose) in a 10-liter fermentor with 1 liter of overnight starter culture. The partial oxygen pressure levels in the fermentor were maintained at 20% ± 5% with O2 gas, and the pH levels were maintained at pH 7.0 ± 0.2 with a 50% solution of ammonium hydroxide (unless otherwise noted, values are means ± standard deviations). Glucose levels were maintained at levels below 1 g/liter using fermentor feed medium (containing, per liter, 80 g of glucose, 40 g of yeast extract, and 3.2 g of MgSO4). The fermentor culture was grown at 37°C to an optical density at 600 nm of 10 and then shifted to 16°C for 1 h. Expression was induced with 0.2 mM IPTG, and the culture was incubated at 16°C for another 20 h. The cells were harvested by centrifugation (7,000 × g, 10 min), and the cell paste was stored frozen at –80°C. Before lysis, 10 ml of lysis buffer was added for each 5 g of frozen cell paste.

GSH-affinity purification of GST fusion proteins. Frozen bacterial cell pastes were thawed, 1 mM phenylmethylsulfonyl fluoride was added, and the cells were lysed by French press. The lysate was centrifuged for 10 min at 7,000 × g, and the supernatant was removed. Glutathione (GSH)-agarose beads were added as a 50% slurry to the supernatant (3 ml of beads/5 g of cell paste or 1-liter culture). Lysates and beads were incubated at 4°C for 60 min and then shifted to 16°C for 1 h. Eluted protein was separated from beads by transferring the elution reaction to a filtration unit and centrifuging briefly. Eluted GST-Sln1R and GST-Sln1R* were dialyzed into reaction buffer (50 mM Tris [pH 7.6], 50 mM KCl, 5 mM MgCl2, 0.1% βME, 50% glycerol). Bead-bound proteins were stored at –20°C.

GST-Sln1R, GST-Sln1R*, GST-Ypd1p, and GST-Skn7 were eluted from the GSH-agarose beads by incubation in GSH elution buffer (100 mM GSH, 50 mM Tris [pH 7.6], 50 mM KCl, 5 mM MgCl2, 0.1% βME) with shaking at room temperature for 1 h. Eluted protein was separated from beads by transferring the elution reaction to a filtration unit and centrifuging briefly. Eluted GST-Sln1R and GST-Sln1R* were dialyzed into reaction buffer (50 mM Tris [pH 7.6], 50 mM KCl, 5 mM MgCl2, 0.1% βME).

Sln1K, Sln1R, and Sln1R* were cleaved from the GSH-agarose beads with biotinylated thrombin (Novagen). A 50% slurry (1 ml) of bead-bound GST-Sln1KR with Ypd1p with Ypd1p with Ypd1p with Ypd1p in similar expression levels, stability, and purity (Fig. 4).

Preparation of radiolaabeled, phosphorylated intermediates for phosphorelay assays. Phosphorylated kinase domain was prepared by incubating the GSH-agarose beads containing GST-Sln1K with 10 μM [γ-32P]ATP (400 Ci/mM) for 15 min in storage buffer at room temperature. The beads were washed four times in reaction buffer to remove unincorporated label. Sln1K-P is chemically stable for >8 h at room temperature. The same protocol was used for labeling GST-Sln1KR. Labeling of Sln1R or Sln1R* was performed by incubating the receiver domain with GSH-agarose-bound, phosphorylated GST-Sln1K for 2 to 3 min. The phosphorylation reactions were transferred to filtration units (Novagen), and the phosphorylated receiver domain was separated from the bead-bound kinase by centrifugation. To obtain eventual labeling of Sln1R and Sln1R* (see Fig. 5), the reactions were driven by addition of a 10- to 15-fold molar excess of receiver domain. Typically, Sln1R phosphorylation levels were 75 to 85% that of the wild-type receiver. Phosphorylated receiver domain was used immediately, due to its short half-life (approximately 15 min). Phosphorylated Ypd1p was obtained by incubating phosphorylated, bead-bound GST-Sln1KR with Ypd1p for 2 or 3 min. The reaction was transferred to filtration units, and the labeled Ypd1p was removed by centrifugation. The lifetime of the phosphorylated Ypd1p is greater than 4 h at room temperature (13; T. Ault, unpublished data).

RESULTS

Purification of an activated Sln1 receiver domain mutant. Previously we reported the isolation of SLN1 mutants that increase SKN7-dependent transcription in vivo (4, 16, 36). The snl1-22-activating allele of SLN1 is a proline-to-serine (P1148S) substitution mutant four residues C-terminal to the phosphorylatable aspartate (D1144) in Sln1R (4). To determine how the SLN1 phosphorelay system is regulated and to identify which Sln1R-dependent reaction or reactions are affected by the snl1-22-activating mutation, we used an in vitro system to measure Sln1p kinase and phosphotransfer activity. Figure 3 shows schematically the recombinant proteins encoding Sln1K, Sln1R, Ypd1p, and Skn7p that were expressed as GST fusions in Escherichia coli. The expression and purification of Sln1R and Sln1R* (P1148S) were done in parallel and resulted in similar expression levels, stability, and purity (Fig. 4).

Hydrolysis of the Sln1R aspartyl phosphate is unchanged in the snl1* mutant. We began by comparing the dephosphorylation of 32P-Sln1R and 32P-Sln1R* (Fig. 2, reaction 3), reasoning that a change in the rate of dephosphorylation of Sln1R would ultimately have an effect on the phosphorylatable aspartate (Sklp) and Skn7p. To examine the intrinsic rate of hydrolysis of phospho-Sln1p and Sln1R*, each protein was 32P labeled by phosphotransfer from GSH-agarase-bound GST-Sln1K, separated from the kinase domain, and incubated at room temperature for the indicated times (Fig. 5). The half-lives of phospho-Sln1p and Sln1R* were essentially identical at 15 ± 1.8 min and 16 ± 1.4 min, respectively. Thus, the intrinsic stability of the Sln1R acyl phosphate is not affected by the Sln1R* mutation.

In some bacterial two-component systems the kinase domain exhibits a phosphatase-like activity that stimulates the normal rate of hydrolysis of the phospho-receiver (10). We examined whether this is also the case for Sln1p by adding GST-Sln1K to 32P-Sln1R or 32P-Sln1R* and measuring dephosphorylation as
a function of time. Under these conditions, there was very little reverse transfer of the phosphoryl group from Sln1R to Sln1K, but reactions involving Sln1R* resulted in a small, but reproducible, increase in phospho-Sln1K levels (Fig. 6A). However, the $t_{1/2}$s of phospho-Sln1R ($24.5 \pm 1.7$ min) and phospho-Sln1R* ($26.0 \pm 3.3$ min) were not significantly affected by the addition of Sln1K (Fig. 6). In fact, they were increased somewhat in the presence of the kinase domain. Addition of ATP did not stimulate the dephosphorylation of either receiver (data not shown). Thus, yeast Sln1p appears to lack a kinase domain-associated phosphatase activity capable of working in trans upon phospho-Sln1R. Since attempts to detect differences in receiver dephosphorylation were not successful, we examined phosphotransfer reactions involving Sln1R to determine whether phosphotransfer is altered by the sln1-22 mutation.

Phosphotransfer reactions involving the Sln1-associated receiver domains. Sln1R is involved in reversible phosphotransfer reactions with Sln1K (reaction 1) and Ypd1p (reaction 2). We examined these phosphotransfer reactions, beginning with phosphotransfer between Sln1K and either Sln1R or Sln1R*. Phosphotransfer between Sln1K and Sln1R was found to occur very rapidly, with more than half of the label transferred prior to the first time point at 5 min (Fig. 7). Phosphotransfer from Sln1K to Sln1R* was less efficient, with less than 20% of the label appearing on Sln1R* by 5 min. This was surprising given that $^{32}$P-Sln1R* was better than $^{32}$P-Sln1R at phosphorylating Sln1K (Fig. 6). In both cases, hydrolysis of the phospho-receiver caused the receiver signal to decay over time. In the case of Sln1R, for which phosphotransfer is rapid, the apparent $t_{1/2}$ for dephosphorylation is approximately 15 min as measured before (Fig. 5 and 6). In contrast, the level of phosphorylated Sln1R* is complicated by the occurrence of simultaneous phosphorylation and dephosphorylation.

We next examined phosphotransfer between Sln1R and Ypd1p. Figures 8A and D show the rates of spontaneous hydrolysis of the phosphorylated Sln1p receiver domains and Ypd1p, respectively. To investigate the phosphotransfer between Sln1R and Ypd1p, GST-Ypd1p was added to either $^{32}$P-Sln1R or $^{32}$P-Sln1R* (Fig. 8B). Steady-state distribution of the phosphoryl group occurred rapidly, reaching equilibrium by the first time point at 2.5 min. However, the distribution of

FIG. 5. Autohydrolysis of phosphorylated Sln1R and Sln1R*. Sln1R (5 µg) and Sln1R* (5 µg) were labeled as described in Materials and Methods, and the percent $^{32}$P-labeled Sln1 receiver remaining was determined as a function of time. Aliquots were removed at the indicated times and quenched by addition of 5× SDS loading buffer. Samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and exposed to film (A) or quantitated using a Packard Instant Imager (B). Quantitative data was fit to an exponential curve.

FIG. 6. Effect of GST-Sln1K on stability of phosphorylated wild-type and mutant Sln1p receiver domains. (A and B) $^{32}$P-Sln1R (0.2 µg) or $^{32}$P-Sln1R* (0.2 µg) was incubated with purified GST-Sln1K (0.4 µg) for the indicated times. This corresponds to a kinase/receiver molar ratio of approximately 1:2. (B) The percent phosphate remaining on Sln1R (closed circles) or Sln1R* (open circles) are plotted as a function of time. Reactions were processed as described in the legend to Fig. 5, and the data were fitted to an exponential curve. Fitting the Sln1R* data to a simple exponential decay results in a line that does not pass through 100%. This is likely due to the small, but significant, back reaction that occurs with Sln1K in reactions involving Sln1R*.
The osmosensitive phenotype of \( \text{sln1} \) mutants suggests that \( \text{sln1p} \) accumulates in a form that interferes with normal responsiveness to elevated osmolarity (4). This form was assumed to be the phospho form of \( \text{sln1p} \) since dephospho-\( \text{sln1p} \) is an important intermediate in activation of the HOG1 osmotic response pathway (17), whereas phospho-\( \text{sln1p} \) is required for activation of Skn7p (16). The increase in phospho-

Skn7p and decrease in phospho-Ssk1p could, in principle, arise from an increase in phosphotransfer or a decrease in the hydrolysis of one or more of the phospho intermediates in the pathway. Genetic analysis seems to favor the model in which dephosphorylation is affected (4, 36). However, we were unable to detect a difference in spontaneous hydrolysis of the aspartyl phosphate between \( \text{sln1r} \) and \( \text{sln1r}^* \) (Fig. 5). In some bacterial systems, the intrinsic hydrolysis rate of the aspartyl phosphate can be modulated by the cognate sensor kinase (9, 27, 33). However, addition of the \( \text{sln1k} \) domain had no effect on the rate of hydrolysis of either \( \text{sln1r} \) or \( \text{sln1r}^* \) (Fig. 6). The possibility remains that an independent phosphatase is involved in dephosphorylation of \( \text{sln1r} \), as is the case in Bacillus subtilis (23). However, attempts to identify aspartyl phosphatases that function on yeast response regulators have thus far been unsuccessful.

The \( \text{SLN1} \) phosphorelay system responds within minutes to an increase in osmolarity (2). This rapid response requires dephosphorylation of phospho-Ssk1p. In the presence of \( \text{YPD1p} \), phospho-Ssk1p has a \( t_{1/2} \) of 40 h in vitro (12). In the absence of \( \text{YPD1} \), the \( t_{1/2} \) is much shorter, approximately 13 min, leading to the suggestion that complex formation between \( \text{YPD1} \) and other components of the pathway controls phosphotransfer (11). Under the conditions tested, the \( \text{sln1}^* \) mutation affects both phosphotransfer from \( \text{Sln1K-P} \) and phosphotransfer to \( \text{YPD1} \), but the prevailing effect is to enhance \( \text{YPD1} \) phosphorylation. The increase in \( \text{YPD1} \) phosphorylation translates into a measurable increase in phosphorylation of Skn7p that is consistent with the reported elevation in \( \text{YPD1p-Skn7} \) signaling observed in vivo. Although it has not been tested directly, we presume, based on the osmosensitive phenotype of the \( \text{sln1-22} \) mutant (4), that the phosphorylation of Ssk1p is also elevated.

While the activated \( \text{sln1r}^* \) phosphotransfer to \( \text{YPD1} \) is consistent with the \( \text{sln1-22} \) phenotype, the impaired \( \text{Sln1K-Sln1r}^* \) phosphotransfer is not, underscoring the complexity of relating biochemical data to the in vivo phenotype. One explanation for the observation that the net effect of the \( \text{sln1}^* \) mutation was increased rather than decreased \( \text{YPD1p} \) phospho-

DISCUSSION

The \( \text{sln1}^* \) alleles were identified in a screen for increased transcription of a \( \text{SKN7} \)-dependent \( \text{lacZ} \) reporter gene (36). The osmosensitive phenotype of \( \text{sln1}^* \) mutants suggests that \( \text{sln1p} \) accumulates in a form that interferes with normal responsiveness to elevated osmolarity (4). This form was assumed to be the phospho form of \( \text{sln1p} \) since dephospho-\( \text{sln1p} \) is an important intermediate in activation of the HOG1 osmotic response pathway (17), whereas phospho-\( \text{sln1p} \) is required for activation of Skn7p (16). The increase in phospho-

FIG. 8. Phosphotransfer between \( \text{Sln1p} \)-associated receiver domains and \( \text{YPD1p} \). \( \text{Sln1R} \) and \( \text{Sln1R}^* \) were \( ^{32} \text{P} \)-labeled as described in Materials and Methods. \( ^{32} \text{P}-\text{Sln1R} \) or \( ^{32} \text{P}-\text{Sln1R}^* \) (2 \( \mu \text{g} \)) was incubated alone (A) or with 2 \( \mu \text{g} \) of GST-\( \text{YPD1p} \) (B) for 2.5, 5, 7.5, or 10 min. \( \text{Sln1R} \) (or \( \text{Sln1R}^* \)) and \( \text{YPD1p} \) were present in a 2.5:1 molar ratio. (C) GST-\( \text{Sln1R} \) or GST-\( \text{Sln1R}^* \) (2 \( \mu \text{g} \)) was incubated with \( ^{32} \text{P}-\text{YPD1p} \) (0.5 \( \mu \text{g} \)) (2:1 molar ratio) for 2.5, 5, 7.5, or 10 min. (D) \( ^{32} \text{P}-\text{YPD1p} \) was also incubated alone for 2.5, 5, 7.5, or 10 min. Samples were processed as described in the legend to Fig. 5.

FIG. 9. In vitro phosphotransfer reaction between \( \text{Sln1p} \)-associated receiver domains and \( \text{Sln1K} \), \( \text{YPD1p} \), and \( \text{SKN7p} \). \( ^{32} \text{P}-\text{Sln1R} \) (34 ng) (lanes 1 and 2) or \( ^{32} \text{P}-\text{Sln1R}^* \) (34 ng) (lanes 3 and 4) was incubated with buffer (lanes 1 and 3) or \( \text{YPD1p} \) (38 ng), \( \text{Sln1K} \) (91 ng), and GST-\( \text{SKN7p} \) (190 ng) (lanes 2 and 4) for 15 min at room temperature. The resulting molar ratio of \( \text{Sln1K} \) to \( \text{Sln1R} \) to \( \text{YPD1p} \) to GST-\( \text{SKN7p} \) is approximately 1:1:1:1. Samples were processed as described in the legend to Fig. 5.
phorylation is that the Sln1K-Sln1R phosphotransfer reaction is normally an intramolecular reaction, which is expected to be more efficient than the phosphotransfer between individual domains used in the in vitro system. In the intact molecule, there may be little difference in phosphotransfer to Sln1R versus Sln1R*. A second possible explanation is that the sln1* mutation changes the receiver domain such that it interacts better with Ypd1p while diminishing the interaction with the Sln1 kinase domain.

The SLN1 phosphorelay is a complex pathway consisting of multiple components. In the simplest two-component systems consisting of a kinase and a single terminal receiver domain, the equilibrium between kinase and receiver domains greatly favors the formation of the phospho-aspartate (30). The usual points of regulation are (i) stimulation of autokinase activity, thus making available additional phosphoryl groups for phosphotransfer, and (ii) stimulation of phospho-aspartate hydrolysis. Regulation of kinase activity and hydrolysis creates a sensitive regulatory circuit in which the ratio of kinase to phosphatase can be fine-tuned to create the desired level of signal flux through the pathway (26).

The more complex phosphorelay systems, on the other hand, are distinct in containing at least one receiver domain intermediate in addition to the terminal receiver. These systems introduce the potential for additional modes of regulation. Regulation of the KinA-Spo0F-Spo0B-Spo0A phosphorelay of Bacillus subtilis, for example, involves a family of extrinsic phosphatases that specifically dephosphorylate the intermediary receiver in Spo0F, in addition to Spo0E-mediated dephosphorylation of the terminal receiver in Spo0A (8). The yeast SLN1 phosphorelay also consists of one intermediary receiver domain and one terminal receiver domain. Unlike the Bacillus system, however, we find no evidence for regulation of pathway activity by stimulation of Sln1R phospho-aspartate hydrolysis.

How then is pathway activity regulated in the SLN1 phosphorelay? Despite the differences between the SLN1 phosphorelay and simpler two-component systems, our analysis shows that the equilibrium in the Sln1K to Sln1R phosphotransfer nonetheless lies in the direction of the receiver. In reactions involving wild-type receiver, phosphotransfer to the receiver is very efficient (Fig. 7). Furthermore, we find that the phospho-receiver appears to be an energetically favorable intermediate, since the equilibrium lies in direction of Sln1R even in the presence of Ypd1p (Fig. 8). Interestingly, we found that the Sln1R* receiver affects the equilibrium between phospho-Sln1R and phospho-Ypd1p, causing a relative increase in phospho-Ypd1p levels. Our results suggest the potential for natural regulation of pathway activity at this step.

Although structural information is not available for the Sln1p-associated receiver domain, it is possible to model Sln1R with SWISS-MODEL using the three-dimensional structure of the bacterial response regulator, CheY, as a template (29). The sln1-22 mutation is a Pro-to-Ser change of a highly conserved, helix-capping proline on the face of the receiver bearing the phosphorylated aspartate. This position corresponds to P61 of CheY, which is the structurally important γ-turn loop (5, 32). The receiver domain undergoes a conformational change once phosphorylated (1, 14), so it is possible that the loss of proline alters an equilibrium between naturally occurring conformations of the receiver domain. Interestingly, a proline-to-serine mutation in the B. subtilis response regulator Spo0A (P60S) corresponding to CheY P61 has also been reported (6, 20, 28). Genetic analysis suggests that these mutants are constitutively active, and are uncoupled from their requirement for phosphorylation via their native signaling pathway. Unlike Spo0A and many other prokaryotic receiver domains in which a DNA or protein binding activity is altered in response to phosphorylation, the only apparent function of the Sln1p receiver is to transfer a phosphoryl group to Ypd1p. In the case of Sln1p, phosphorylation might induce a conformation that facilitates phosphotransfer and the Pro-to-Ser mutation might mimic or predispose the receiver domain to this conformation. This would be consistent with our biochemical and genetic data.

In summary, we have demonstrated that the activated sln1-22 mutant has an increased phosphotransferase activity that leads to elevated levels of Ypd1p and Skn7p phosphorylation and provides an explanation for the activated phenotype of the mutant. Furthermore, our analysis of the sln1* mutant establishes a role for the Sln1R domain in regulating signal flux through the SLN1 system in response to changes in osmolarity. As an intermediate in a phosphorelay pathway, Sln1R is in a unique position to influence pathway activity. Other intermediate receiver domains in both prokaryotic and eukaryotic phosphorelay pathways may also exert this type of control. Further biochemical and structural analysis of sln1-22 and other activated SLN1 alleles may help to uncover the molecular basis of two-component signaling in yeast and other eukaryotes.

ACKNOWLEDGMENTS

We thank Ann West of the University of Oklahoma for providing purified Ypd1 protein. We also thank Peter Rubenstein and Madeline Shea for their helpful comments on the manuscript and Milinde Deshpande of the University of Iowa Center for Biocatalysis for assistance with bacterial fermentations.

This work was supported by awards to J.S.F and R.J.D. from the U.S. National Institutes of Health (R01 GM56719) and a predoctoral training grant T32AG (A.D.A.) from the National Institute on Aging.

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