Activated Alleles of Yeast SLN1 Increase Mcm1-dependent Reporter Gene Expression and Diminish Signaling through the Hog1 Osmosensing Pathway*

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Two-component signal transduction systems involving histidine autophosphorylation and phosphotransfer to an aspartate residue on a receiver molecule have only recently been discovered in eukaryotes, although they are well studied in prokaryotes. The Sln1 protein of Saccharomyces cerevisiae is a two-component regulator involved in osmotolerance. Phosphorylation of Sln1p leads to inhibition of the Hog1p mitogen-activated protein kinase osmosensing pathway. We have discovered a second function of Sln1p by identifying recessive activated alleles (designated npr2) that regulate the essential transcription factor Mcm1. npr2 alleles cause a 5-fold increase in the activity of an Mcm1-dependent reporter, whereas deletion of SLN1 causes a 10-fold decrease in reporter activity and a corresponding decrease in expression of Mcm1-dependent genes. In addition to activating Mcm1p, npr2 mutants exhibit reduced phosphorylation of Hog1p and increased osmosensitivity suggesting that npr2 mutations shift the Sln1p equilibrium toward the phosphorylated state. Two npr2 mutations map to conserved residues in the receiver domain (P1148S and P1196L) and correspond to residues implicated in bacterial receivers to control receiver phosphorylation state. Thus, it appears that increased Sln1p phosphorylation both stimulates Mcm1p activity and diminishes signaling through the Hog1 osmosensing pathway.

Two-component regulators are a family of signal transduction molecules prevalent in prokaryotic organisms that play a major role in the adaptation of microorganisms to changes in the extracellular environment (1, 2). The response of cells to chemotactic agents, sporulation conditions, and changes in osmolarity, for example, are each governed by two-component regulators in which information about external conditions is converted via a sensor-associated kinase into high energy phosphoryl groups on histidine and aspartate side chains ultimately activating an appropriate set of genes. The "two-component" designation derives from the observation that in many cases the pertinent activities are divided between two polypeptides. One is designated the "sensor/kinase" and contains an extra-cellular domain (sensor) and a histidine autokinase activity (transmitter). The second is called the response regulator and is made up of a receiver domain containing a conserved aspartate residue and, in some cases, an output domain.

Two-component regulators have been recently identified in eukaryotes including Arabidopsis, Saccharomyces, Dictyostelium, and Neurospora (3). In most of these eukaryotic examples, the transmitter and the receiver domains are present in a single hybrid molecule. Examples of hybrid kinases are also known in prokaryotes, and in certain cases, the attached receiver domain is thought to regulate phosphotransfer to a cytoplasmic receiver containing an output domain (4, 5). The strength of the signal through a given pathway is determined by the stability of the phosphoryl aspartate whose susceptibility to hydrolysis depends on the tertiary or higher order structure of the receiver domain. Stability of the aspartyl phosphate may be regulated by "phosphatase" activities present either in the sensor/kinase, receiver domain, or in auxiliary factors (6, 7). Whether the role of these phosphatases is enzymatic or rather to trigger a conformational alteration that alters the exposure of the residue to water is unclear.

The yeast SLN1 gene is a member of the two-component family that encodes a hybrid kinase including sensor, histidine kinase, and receiver domains (8). Its role is to sense and respond to changes in osmolarity by regulating a MAP3 kinase pathway composed of Hog1p (MAP kinase), Pbs2p (MAP kinase kinase), and Ssk2p/Ssk22p (two MAP kinase kinases) (9). Sln1p phosphorylates a second two-component molecule, Ssk1p consisting of a receiver domain, via the phosphorelay intermediate, Ypd1p (10). Under conditions of increased osmolarity, the equilibrium between phosphorylated and unphosphorylated Sln1p shifts to favor the unphosphorylated form (10), and the resulting accumulation of unphosphorylated Ssk1p leads to activation of the Hog1 MAP kinase pathway (9). Phosphorylated Hog1p, in turn, activates a set of hyperosmotic response genes necessary for survival under increased external osmotic pressure (11). Also contributing to Hog1p phosphorylation in response to hyperosmolarity is Sho1p, a putative membrane-associated molecule that interacts with the Hog1p kinase Pbs2p via its Sre homology 3 domain (12).

Alleles of the SLN1 gene that increase expression of an Mcm1-dependent reporter were previously isolated in a screen for mutants that increased the activity of the CYC1-lacZ reporter gene under the control of a high affinity Mcm1 binding

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The abbreviations used are: MAP kinase, mitogen-activated protein kinase; P, Mcm1 binding site; P-lacZ, Mcm1-dependent reporter; kb, kilobase pair(s); UAS, upstream activation sequence; UASp, upstream activation sequence from the GAL1, 10 genes; HA, hemagglutinin; aa, amino acid.
site (referred to as a P site) (13). Deletion of the SLN1 gene causes a 10-fold reduction in Mcm1-dependent reporter gene expression without affecting Mcm1p levels, thus Snl1p is an activator of Mcm1p or of an Mcm1p auxiliary protein, and the so-called npr2 (negative regulator of P site) mutants are recessive activating alleles of SLN1 (13). Since deletion of the HOG1 gene has no effect on the activity of an Mcm1-dependent reporter, we suggested that Mcm1p is not directly regulated by Hog1p and that Snl1p has two separate regulatory functions (13). It is not clear, however, how Snl1p accomplishes both functions. Although the mechanism by which Snl1p regulates the Hog1 MAP kinase pathway involves phosphorylation and phosphotransfer, whether the same activities are required for Snl1p regulation of Mcm1p is an open question. In addition, although we have shown that Hog1p is not required for Snl1p activation of Mcm1p, other intermediates in the Snl1-Hog1 pathway have not been tested so the point at which the two "pathways" branch has not been defined.

Our present results indicate that Snl1p activation of Mcm1p uses regulatory mechanisms in common with Snl1p activation of the Hog1 pathway and indicates that signaling through the two pathways may depend on the same phosphorylation capabilities of Snl1p. The hypothesis that the npr2 mutations affect the Snl1p phosphorylation state is further supported by our finding in two instances that the amino acid changes responsible for the npr2 mutant phenotype map to positions likely to have an effect on the equilibria between phosphorylated and unphosphorylated Snl1p. Based on these results, we propose a model in which shifting the equilibrium of Snl1 to the phosphorylated state results in inactivation of the Hog1 pathway and activation of the Mcm1 pathway. Given the multiple roles of Mcm1p in growth control and cell physiology, this may represent an integrated mechanism by which cells respond to stress.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Yeast strains used in this work are listed in Table I. All strains were grown at 30 °C unless indicated otherwise. Diploids containing the sln1::LEU2 or sln1::TRP1 mutations were constructed by transformation with fragments isolated from plasmids pGY48 and pY115 as described previously (15). The SHO1 and SSK1 loci were disrupted by single step gene replacement (14) using, respectively, a Sall-NotI fragment from plasmid pDOS84 in which TRP1 is inserted between the two EcoRV sites of the SHO1 gene (gift of H. Saito) (12) and a PstI-XbaI fragment from the ssh1::LEU2 plasmid, pDSS14 (gift of H. Saito) (9). The presence of the disruption alleles was confirmed by Southern hybridization analysis.

The media were prepared as described by Sherman et al. (15). Plates for the detection of β-galactosidase contained 50 µg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside/ml and were prepared as described by arseneau et al. (16).

Plasmids—Plasmids used in this work are listed in Table II. The Mcm1-dependent reporter, P-lacZ, is carried on the previously described plasmid, pGY48 (17), a derivative of the 2-µm URA3 CYC1-lacZ reporter, pLG6702 (18). pHL589 consists of the entire SLN1 gene inserted into pRS314 (19). It was constructed by a recombination strategy using KpnI-digested pHL566 as the gapped recipient vector. pHL566 consists of a 2.9-kb EcoRI/KpnI fragment of SLN1 inserted into the EagI and KpnI sites of pRS314 (19). KpnI-digested pHL566 was cotransformed into yeast strain JF1455 with a 2.86-kb polymerase chain reaction fragment of SLN1 generated using oligonucleotides SLN 1588F 5'-ACTATGACAGGACACGGTCAGCAACCATATG-3' and SLN 3212R 5'-GCTCGGAATTAACCCTCACTAGGAACAAAAGCTGGT-ACCATTCAATTTGTGGTTCATGCTCTC-3' of FY251xGY32. Generation of a full-length functional pRS314 SLN1 clone (pHL589) was confirmed by restriction and sequence analysis, as well as by demonstrating the ability to support SLN1-dependent growth when JF1455 was sporulated. Construction of pHL582 containing a SLN1 gene lacking the receiver domain (deletion of aa 1078–1220) was done by cutting pHL589 with SphI and inserting a 5-kb EagI/KpnI fragment of SLN1 into the EagI and KpnI sites of pRS314 (19). KpnI-digested pHL566 was cotransformed into yeast strain JF1455 with a 2.86-kb polymerase chain reaction fragment of SLN1 generated using oligonucleotides SLN 1057F 5'-ACCATCCAAATTTGTGGTTCATGCTCTC-3' and SLN 1587R 5'-ACTATGACAGGACACGGTCAGCAACCATATG-3' of FY251xFY834 diploid.

TABLE I

| Strain name | Genotype |
|-------------|----------|
| JF1321      | MATα his4-197 lys2-1288 leu2 trp1Δ ura3-52; plasmid pGY48 |
| JF1356      | MATα npr2-1 his4-197 lys2-1288 leu2 ura3-52; plasmid pGY48 |
| JF1357      | MATα npr2-2 his4-197 lys2-1288 leu2 ura3-52; plasmid pGY48 |
| JF1358      | MATα npr2-3 his4-197 lys2-1288 leu2 ura3-52; plasmid pGY48 |
| JF1362      | hog1::TRP1 of JF1331 (Yu et al., 1995) |
| JF1433      | MATα npr2-1 ssh1::LEU2 his4-197 lys2-1286 trp1Δ ura3-52 leu2; plasmid pGY48 |
| JF1598      | sho1::TRP1 of JF1331 |
| JF1599      | sho1::TRP1 of JF32 |
| JF1600      | sho1::TRP1 of JF36 |
| JF1675      | npr2-2 of FY251 |
| JF1705      | sln1::LEU2 of FY251xFY834 diploid |
| JF1706      | MATα npr2-1 his4-197 leu2 trp1Δ ura3-52; plasmid pGY48 |
| JF1455      | MATα npr2-2 his4-197 leu2 trp1Δ ura3-52; plasmid pGY48 |
| FY251       | MATα his3::2000 leu2Δ1 ura3-52 trp1Δ3 leu2 from Fred Winston |
| FY834       | MATα his3::2000 leu2Δ1 ura3-52 trp1Δ3 leu2Δ202 from Fred Winston |

* Constructed by two-step transformation using the npr2-2 integrating plasmid, pWT5 and confirmed by direct sequence analysis of a polymerase chain reaction fragment encompassing the mutation.
with HA antibody. Following electrophoresis on 12% SDS-polyacrylamide gels and transfer to nitrocellulose, filters were bracketed for 15 min at 37 °C in 5% milk powder dissolved in 0.1% Tween/phosphate-buffered saline. Filters were subsequently incubated (6 h) with HA antibody (1:1000) (Babco) followed by secondary antibody anti-IgG horseradish peroxidase (1:2500) (Sigma). Immune complexes were detected by enhanced chemiluminescence according to protocols supplied by the manufacturer (Amersham Corp.).

**Gap Rescue and Reconstruction of nrp2 Mutations—**Three SLN1-gapped plasmids (A, B, and C) were generated by digesting pHL589 with EcoRI/Stu(I) (A), StuIIClai (B), or BstEI/HindIII (C). Gap rescue (23) was performed by transforming gap A, B, or C plasmids into mutant yeast strains harboring the nrp2-1 (JF1356), nrp2-2 (JF1357), or nrp2-3 (JF1358) mutations. The presence of the mutation on a rescued gap plasmid was assessed by the blue color of colonies on 5-bromo-4-chloro-3-indolyl β-D-galactosidase plates. Plasmids were rescued from yeast (24) and subjected to sequence analysis. The sequence of both early log phase (107/ml) and serially diluted 1:10 in YPD or YPD

**Dilution Plating for Osmosensitivity Testing—**Cells were cultured to early log phase (7 × 106/ml) and serially diluted 1:10 in YPD or YPD + 0.9 m NaCl. Two μl of the appropriate dilutions were spotted onto YPD and YPD + 0.9 m NaCl plates. Plates were incubated at 30 °C for up to 3 days.

**Protein Extracts and Western Analysis—**Cells were cultured to early log phase (7 × 106/ml) in YPD at which time solid NaCl was added to 0.9 m. 10-ml aliquots were taken at indicated times following the addition of salt. The t = 0 time point was taken prior to the addition of salt. Cells were pelleted at 4 °C at low speed and frozen in dry ice prior to storage at −70 °C. Cell pellets were washed once with 5 ml of cold protease inhibitor mixture (50 μg/ml leupeptin, 25 μg/ml aprotinin, 1 μg/ml pepstatin, 20 μg/ml chymostatin, 2 μM phenylmethylsulfonyl fluoride in water). Extracts were processed for antiphosphotyrosine immunoblotting as essentially described by Maeda et al. (9). Proteins were resolved by electrophoresis on 10% SDS acrylamide gels (6% stacking gel) and electroblotted to 0.45 μm nitrocellulose (Protran, Schleicher & Schuell). The nitrocellulose filter was blocked overnight at 4 °C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) + 4% bovine serum albumin, washed twice for 10 min in TBST, incubated for 2 h at 4 °C with RC20 recombinant antiphosphotyrosine antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) diluted 1:2500 in TBST + 4% bovine serum albumin, and washed twice with TBST. Immune complexes were visualized by enhanced chemiluminescence (Amersham Corp.).

**RESULTS**

**nrp2 Mutations Cause an Osmosensitive Phenotype—**Activation of the Hog1 MAP kinase pathway depends on the phosphorylase capacities of the Sn1p hybrid kinase (9). Mutation of either the phosphatase histidine or the phosphorylated aspartate confers a phenotype equivalent to the null, indicating the requirement of these residues for signaling (9). Activation of Mcm1-dependent transcription may likewise involve Sn1p phosphorylation and phosphotransfer to downstream molecules. Alternatively, activation of Mcm1p may be independent of the phosphorylation state of Sn1p. Assessment of Mcm1p activity in the null or in phosphorylation defective mutants is difficult since the SN1 deletion mutation causes lethality on rich media and extremely slow growth on minimal media in some genetic backgrounds (13) and unconditional lethality in other genetic backgrounds (9). To circumvent these difficulties, we tested the involvement of Sn1p phospho-intermediates in Mcm1 signaling by examining the effect of the nrp2 mutations on the ability of cells to respond to osmotic stress. If the stimulating effect of nrp2 mutations on Mcm1-dependent transcription is due to a shift in the Sn1p equilibrium to the phosphorylated state, for example, the resulting decrease in unphosphorylated Sn1p might limit the amount of unphosphorylated Ssk1p available to trigger a normal osmotic response. To test this idea, we examined the osmotic sensitivity of our nrp2 mutants. To render the cell completely dependent on Sn1p signaling for its response to hyperosmolality (12), it was necessary to first delete the SPO1 gene which encodes a second osmosensor. The SPO1 gene was deleted by one-step disruption in nrp2-1 and nrp2-3 strains. The osmotolerance of iso-genic nrp2-1 and nrp2-1 sho1-Δ strains and isogenic sho1-Δ and wild type strains was compared by spotting serial dilutions of cells on 0.9 m NaCl plates or on unsupplemented YPD media. SHO1 deletion exacerbated the effect (Fig. 1). The enhanced osmosensitivity of the double nrp2 sho1-Δ mutant is consistent with nrp2 mutations shifting the Sn1p equilibrium to favor the phosphorylated form, thus diminishing or inhibiting the normal phosphorylating effect of Ssk1p available to trigger a normal osmotic response. To test this idea, we examined the osmotic sensitivity of our nrp2 mutants. To render the cell completely dependent on Sn1p signaling for its response to hyperosmolality (12), it was necessary to first delete the SPO1 gene which encodes a second osmosensor. The SPO1 gene was deleted by one-step disruption in nrp2-1 and nrp2-3 strains. The osmotolerance of iso-genic nrp2-1 and nrp2-1 sho1-Δ strains and isogenic sho1-Δ and wild type strains was compared by spotting serial dilutions of cells on 0.9 m NaCl plates or on unsupplemented YPD media. SHO1 deletion exacerbated the effect (Fig. 1). To examine in more detail the basis for the osmosensitivity of nrp2 mutants, we examined the tyrone phosphorylation of Hog1p in the nrp2 sho1-Δ double mutant and sho1 and nrp2 single mutants. Strains were grown in rich media, and NaCl was added to 0.4 m at time 0. Aliquots were taken over time for immediate processing, denaturing gel electrophoresis, and immunoblot analysis with antiphosphotyrosine antibody. As expected, a 50-kDa band corresponding to Hog1p was apparent in wild type strains at the earliest point assayed (5 min) after addition of salt (Fig. 2). Likewise, in sho1 deletion and nrp2 single mutants, Hog1p phosphorylation was rapidly induced and persisted for 15–20 min. In contrast, in the nrp2 sho1 double mutants, Hog1p phosphorylation was not detectable (Fig. 2). Taken together, these studies indicate that Mcm1p-depend-
were performed as described under “Experimental Procedures.” The stream of the aspartate residue in the Sln1p receiver domain leading to changes in conserved proline residues just downstream of the histidine kinase do-

**Fig. 1.** nrp2 mutants are sensitive to 0.9 M NaCl in a *sho1Δ* background. Cells of the designated genotype were diluted serially in YPD + 0.9 M NaCl and spotted onto YPD + 0.9 M NaCl plates (A) or diluted in YPD and spotted onto YPD plates (B). Sln1p receiver domains were functional. We tested whether the two half-modules, CheY, for which a crystallographic structure is available. As described in detail below (see “Discussion”), the alignment of Sln1p and CheY receiver domains shows that the nrp2-2 and nrp2-3 mutations map to positions that are likely to affect Sln1p function and are consistent with changes in Sln1p phosphorylation state.

The *Sln1* Pathways Branch Prior to *Ssk1p*—Since phosphorylated Sln1p inhibits the activity of the Hog1 pathway but appears to stimulate the activity of the Mcm1 pathway, it seemed possible that phosphorylated and unphosphorylated Ssk1p might likewise play opposing roles in regulating the two pathways. We tested the importance of Ssk1p in Sln1-Mcm1 signaling by measuring the effect of an Ssk1 deletion on the activity of the Mcm1-dependent reporter, *P*-lacZ (*P* = Mcm1 binding site). Unlike the Sln1 deletion which causes constitutive activation of the Hog1 pathway, and perhaps, as a result, lethality, *Ssk1* deletion is not lethal. In the absence of Ssk1p, the Hog1 pathway fails to be activated by the Sln1 pathway but is under normal regulation via Sho1-Pbs2p interactions. If the presence of Ssk1p is required for signaling to Mcm1p, deletion of *Ssk1* would be expected to prevent the activation of the Mcm1-dependent reporter by nrp2 mutations. To examine the involvement of Ssk1p in Sln1-Mcm1 signaling, we deleted the *Ssk1* gene by one-step transformation of wild type and nrp2-1 mutant strains. We found that the activity of our reporter was virtually unchanged as a result of *Ssk1* deletion in either wild type or nrp2 backgrounds (Table III).

**The *Sln1* C-terminal Receiver Domain Has an Essential Role in Signal Transmission**—The absence of an effect of the Ssk1p-receptor on Sln1-Mcm1 signaling suggested the possibility that Sln1p itself interacts with downstream non-two-component signaling molecules. In this case, the receiver domain of the Sln1p hybrid kinase may be an important signaling intermediate rather than an inhibitory domain as postulated for other hybrid kinases. We examined this possibility by deleting the receiver domain from Sln1p and separately expressing it under the control of an inducible promoter. Before evaluating the effect of expressing the transmitter in the absence of the receiver, it was necessary to establish that the two separated domains were functional. We tested whether the two half-modules could function in trans by simultaneously introducing plasmid pHL592 carrying a receiverless Sln1p (aa 1–1077) and plasmid pBG95 carrying the galactose-inducible receiver (aa 1037–1220, end) into a diploid strain (JF1705) heterozy-

FIG. 3. Domain organization of the Sln1 protein showing the locations of the nrp2 mutations. The two presumed transmembrane spanning regions are indicated by black boxes; the transmitter and receiver domains are gray and striped boxes, respectively. H represents the phosphorylated histidine in the transmitter domain of Sln1 (aa 576), and D represents the phosphorylated aspartate in the receiver domain (aa 1144). Partial sequence of the *Sln1* receiver domain is aligned with the sequence of the bacterial chemotaxis two-component receiver module, CheY. Numbers above the alignment refer to CheY amino acids. Positions of *Sln1* receptor mutants, nrp2-2 and nrp2-3 are shown below the alignment.
gous for a LEU2 marked sln1-Δ allele. The sln1-Δ heterozygote was then sporulated and dissected on media prepared with galactose instead of glucose. The appearance of both Leu+ (sln1-Δ) and Leu− (SLN1+) viable spores at equal frequencies (Table IV) and indistinguishable growth rates suggests that the two plasmids fully complemented the sln1-Δ mutant phenotype. Next, we asked whether either domain alone was able to complement the sln1-Δ lethality. To this end we compared the frequency of plasmid loss in Leu+ (sln1-Δ) and Leu- (SLN1+) spore colonies. After 8 h of growth in non-selective media, 40% of the cells in a culture from a Leu− colony had lost either the plasmid carrying the N-terminal (marked with TRP1) or the C-terminal (marked with HIS3) SLN1 portion. In contrast, no His− or Trp− segregants were present in a culture from a Leu− (sln1-Δ) colony (Table V). This result indicates that both the transmitter kinase and receiver domains are required for Sl1 function. Neither the C terminus nor the N terminus alone is sufficient for complementation. Further evidence that the receiver domain is essential for viability comes from the failure of a sln1Δ::LEU2 strain carrying the UASΔ receiver and transmitter plasmids to survive when shifted from galactose to glucose media (data not shown).

**Table III**

| Straina | Relative β-galactosidase activityb | Y-lacZ |
|---------|-----------------------------------|-------|
| Wild type | 100 | |
| sln1-Δ | 116 | |
| npr2-1 | 408.5 | |
| npr2-1 sln1-Δ | 688 | |

*a Strains were as follows: wild type (JF1331); npr2-1 (JF1359); sln1-Δ (JF1331); npr2-1 sln1-Δ (JF1359).†

*b Activities are the averages of at least four measurements. Standard deviations are less than 25% of the average. Average activities were normalized to wild type.

**Table IV**

| Transmitter plasmid*a | Receiver plasmid*a | None | None | Vector | UASΔ receiver | SLN1 ΔR | Vector | UASΔ receiver |
|-----------------------|-------------------|------|------|--------|--------------|---------|--------|--------------|
|                       | Leu+  b            | Leu- | Leu+  c           | Leu-  | Leu+  b          | Leu-  | Leu+  d          | Leu-      |
|                       | 0                  | 12   | 0                  | 8     | 0               | 11    | 19                 | 18         |

*a Transmitter plasmids were SLN1 ΔR (pHL592) or the parental vector pRS314 (vector). Receiver plasmids were UASΔ receiver (pBG95) or the parental vector, pBS146 (vector).†

*b A total of six tetrads from the untransformed JF1705 (sln1Δ::LEU2/SLN1+) were dissected onto YPD plates.

**Discussion**

Sl1p Phosphorylation Controls Two Pathways—The phosphotransfer capabilities of Sl1p have been shown to be important for regulation of the Hog1 MAP kinase osmotic response pathway, and it is clear that hyperosmotic conditions promote the accumulation of unphosphorylated Sl1p (9). We previously showed that Sl1p separately regulates the activity of Mcm1-dependent genes and suggested that this function of Sl1p might involve the phosphorylated form of Sl1p (13). To provide evidence for this assertion and to determine whether activation of the Mcm1 pathway, like activation of the Hog1 pathway, involves the phosphotransfer capabilities of Sl1p, we investigated the effect of npr2 mutations on the capacity of the cell to respond to osmotic stress. The osmotic phenotype of npr2 mutants suggests that activation of the Mcm1 pathway by the npr2 mutations decreases the unphosphorylated Ssk1p that is available to stimulate the Hog1 pathway. The absence of Hog1p phosphorylation in the npr2 strains is consistent with this interpretation. These results demonstrate that phosphorylation of Sl1p has at least two functions: one is to turn off the Hog1 pathway through the action of the receiver, Ssk1p, and the other is to activate Mcm1p. This is accomplished in an Ssk1p-independent manner. These results do not distinguish whether Mcm1p activation is dependent on the phosphotransfer capabilities of Sl1p or if it simply depends on histidine phosphorylation of Sl1p. For example, Sl1p phosphorylation may be required to induce a conformational change which in turn mediates a particular protein-protein interaction. However, the location of the npr2-2 and npr2-3 mutations in the receiver domain at positions likely to affect the stability of the aspartyl phosphate suggests that Sl1p-H to Sl1p-D phosphotransfer as well as the histidine autokinase activities of Sl1p are important for Mcm1p activation. In vitro phosphorylation and phosphotransfer experiments are underway that will further test this idea.

npr2 Mutations Are Localized in Both the Transmitter Kinase and Receiver Domains of Sl1p—Two of three npr2 alleles are changes in proline residues in positions highly conserved between other two-component receivers. In CheY, these residues are postulated to be critical for the conformation of the CheY receptor. The npr2-2 mutation is a P→S mutation at residue
1148 of Sln1p. This position, four amino acids downstream of the phosphoryl aspartate residue, corresponds to P61 of CheY. This residue is part of a structurally important 5-turn loop. The proximity of this base pair to the phosphorylation domain, as well as its solvent accessibility and high degree of conservation, has led to the suggestion that this loop region of response regulators is likely to be important for recognition by kinases (25).

The nrp2-3 mutation is a P→L mutation at residue 1196 of Sln1p. This position corresponds to the highly conserved P110 of CheY which makes a cis-peptide bond with the absolutely conserved Lys-109 residue to form a rigid 5-turn loop. The side chain of Lys-109 interacts with Asp-57 in the acidic pocket in the structure of unphosphorylated CheY and phosphorylation is expected to perturb the Lys-109→Asp-57 interaction, initiating the switch to the active conformation. The β5→α5 loop also contributes to the solvent accessible face that interacts alternately with CheA and the flagellar motor proteins (27). Interestingly, the K109R mutation in CheY blocks the interaction of P-CheY with the phosphatase, CheZ, and therefore results in substantial decreases in dephosphorylation rates as well as decreased phosphorylation rates (25, 28).

In contrast to nrp2-2 and nrp2-3, the nrp2-1 mutation is not located in the receiver domain. nrp2-1 is a T→I mutation located in the linker region between the histidine kinase domain and the transmembrane domains. The position of this mutation is reminiscent of activating mutations in bvgS, a hybrid two-component regulator involved in virulence from Bordetella pertussis and narX, an Escherichia coli sensor involved in regulation of anaerobic respiratory genes. Constitutive mutations in these proteins have been mapped to a flexible linker region between the transmembrane domains and the histidine kinase domain (29, 30). This type of mutation may trigger signal transmission in a signal-independent fashion by mutational simulation of an appropriate conformational state. Alternatively, in the case of Sln1p, phosphorylation of the affected threonine may normally modulate the activity of the histidine kinase.

An explanation of the activating phenotype of nrp2 mutations we have identified must account for the fact that they are recessive. An increase in receiver phosphorylation can occur by either increased rate of phosphorylation or a decreased rate of dephosphorylation. We do not believe that the nrp2 mutations increase kinase activity because it is not apparent how an increase in kinase activity could be recessive (13). The recessiveness of the nrp2 mutations is more consistent with a defect in a postulated Sln1p-associated aspartyl-directed phosphatase-like activity. Perhaps the receiver domain mutations nrp2-2 and nrp2-3 cause decreased sensitivity to a phosphatase. These mutations lie within the exposed γ and β5→α5 loops which are postulated to define a surface involved in protein-protein interactions. On the other hand, since many bacterial transmitter kinases possess inherent phosphatase activity toward their respective receivers, the nrp2-1 mutant may be defective in a putative Sln1p transmitter kinase-associated phosphatase activity (7, 31, 32). Since Sln1p, like other two-component regulators appears to function as a dimer (9), functional interactions with a phosphatase may be provided in trans and thus account for the recessiveness of the nrp2 activating phenotype.

Two Possible Roles of the Sln1p Receiver Domain Regulatory Versus Signaling—Since SSK1 has no apparent role in signaling to Mcm1p, we considered a possible role for the Sln1p receiver domain in signaling to the Mcm1p branch of the pathway. The role of the attached receiver domain in hybrid kinases is not clear. In the case of VirA and ArcB and others, it appears to have at least in part an inhibitory role, since deletion of the domain leads to increased signaling (4, 5). Thus the attached receiver domain appears to prevent phosphotransfer to the cytoplasmic response regulator and to downstream targets until signal-mediated histidine autophosphorylation and internal phosphotransfer leads to a change in conformation that allows phosphotransfer to a second substrate. An alternate, but not necessarily mutually exclusive, role for the attached receiver is in signal transmission. Phosphorylation of the attached receiver may be required for subsequent phosphorylation of the cytoplasmic receiver or other downstream molecules. The observation that mutation of the Sln1p aspartate target from aspartate to asparagine (D1144N) has a phenotype equivalent to the Sln1 null (9) indicates that Asp-1144 is important but

### Table V

| Phenotype of starting colony | Plasmid loss events | Total colonies examined |
|-----------------------------|---------------------|-------------------------|
| Leu+                        | 0                   | 182                     |
| Leu−                        | 107                 | 270                     |

a One Leu+ and one Leu− spore colony were chosen from the segregants of JF1705 carrying plasmids pBG95 (UASr receiver) and pH592 (pRS SLN1ΔA) for this analysis. Each colony was cultured in YPD medium overnight and plated on YPD medium for single colonies. Single colonies were replica-plated to SC-His and SC-Trp plates.

b Single colonies that failed to grow on SC-His plates or on SC-Trp plates are presumed to stem from individual cells in which the receiver domain (HIS3) or transmitter domain (TRP1) plasmids have been lost.

### Table VI

| Genomic SLN1 | Plasmid | Growth condition | β-gal. activity (S.D.) | (n) |
|--------------|---------|-----------------|------------------------|-----|
| SLN1+        | Vector  | Glucose         | 215.0 (33)             | (4) |
| SLN1+        | UASr receiver | Glucose | 187.4 (17)         | (7) |
| nrp2-2       | Vector  | Glucose         | 658.9 (91)            | (8)*|
| nrp2-2       | UASr receiver | Glucose | 629.4 (89)       | (8)*|
| SLN1+        | Vector  | Galactose       | 115.5 (25.9)          | (4) |
| SLN1+        | UASr receiver | Galactose | 104.7 (19.6) | (7) |
| nrp2-2       | Vector  | Galactose       | 437.5 (66)            | (8)*|
| nrp2-2       | UASr receiver | Galactose | 259.0 (38.4)      | (8)*|

a The nrp2-2 strain (JF1675) is an isogenic derivative of the SLN1+ strain, FY251, created by two-step transformation using plasmid pWT5. Each strain carries the P-lacZ reporter, pGY48.
b Vector, pSV146; UASr receiver, pBG95.
c Cells were cultured overnight in SC raffinose-Ura His to maintain selection for the P-lacZ reporter and the receiver domain plasmid and then subcultured into SC galactose-Ura His or SC glucose-Ura His media and incubated for 5 h at 30 °C.
d β-Galactosidase activity is expressed in Miller units as the average of (n) measurements. S.D., standard deviation of the mean is shown in parentheses.
e Ten trials were performed; however, the high and low values were not included in calculating the average or the standard deviation.
does not distinguish between the two models. If the role of Sln1p Asp-1144 is in inhibiting phosphotransfer to a second molecule, removal of that domain might be expected to relieve the inhibition and promote phosphorylation of Ssk1p. Hyper-phosphorylation of Ssk1p might inhibit the activation of the Hog1 pathway in response to salt but would not be lethal. Hence the requirement for the Sln1p receiver domain to support viability in a sln1-Δ mutant argues that the Sln1p receiver domain must have a role in signal transmission. The signaling role of the Sln1p receiver is consistent with conclusions from a recent study by Saito and colleagues (10) in which an in vitro system was used to demonstrate transfer of the histidine phosphate from the transmitter kinase to the receiver aspartate followed by transfer to the histidine of a phosphorelay intermediate, Ypd1p, and finally to the aspartate of Ssk1p (10).

In conclusion, our analysis of the npr2 alleles of SLN1 has shown that phosphorylation of Sln1p has two effects in the cell, inhibition of the Hog1 MAP kinase pathway and activation of Mcm1-dependent transcription. It is interesting to speculate that the linkage of Mcm1p activation to the Hog1 osmosensing pathway through Sln1p may indicate that the cell must regulate the activity of Mcm1p in response to changes in environmental osmolarity. Since Mcm1p is suspected or known to be involved in regulating a large number of different physiological processes including cell cycle, cell wall structure, metabolism, and cell type (33–37), regulation of Mcm1p activity may coordinate the physiology of the cell under hyperosmotic conditions.

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