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A cytoplasmic coiled-coil domain is required for histidine kinase activity of the yeast osmosensor, SLN1

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Summary

The yeast histidine kinase, Sln1p, is a plasma membrane-associated osmosensor that regulates the activity of the osmotic stress MAP kinase pathway. Changes in the osmotic environment of the cell influence the autokinase activity of the cytoplasmic kinase domain of Sln1p. Neither the nature of the stimulus, the mechanism by which the osmotic signal is transduced nor the manner in which the kinase is regulated is currently clear. We have identified several mutations located in the linker region of the Sln1 kinase (just upstream of the kinase domain) that cause hyperactivity of the Sln1 kinase. This region of histidine kinases is largely uncharacterized, but its location between the transmembrane domains and the cytoplasmic kinase domain suggests that it may have a potential role in signal transduction. In this study, we have investigated the Sln1 linker region in order to understand its function in signal transduction and regulation of Sln1 kinase activity. Our results indicate that the linker region forms a coiled-coil structure and suggest a mechanism by which alterations induced by osmotic stress influence kinase activity by altering the alignment of the phospho-accepting histidine with respect to the catalytic domain of the kinase.

Introduction

Recent characterization of the osmotic response pathway in *Saccharomyces cerevisiae* has uncovered a complex pathway consisting of a prototypical MAP kinase pathway regulated by a two-component phosphorelay system (Posas *et al.*, 1996). Two-component signal transduction pathways were first characterized in bacteria and have since been identified in fungi, slime moulds and plants (Swanson and Simon, 1994; Loomis *et al.*, 1997; Mizuno, 1998). In *S. cerevisiae*, there is a single two-component-type phosphorelay pathway in which the sensor kinase, Sln1p, working as a dimer, autophosphorylates a conserved histidine in response to changes in the osmotic environment (Posas *et al.*, 1996). The phosphoryl group is transferred *in trans* to a conserved aspartate in the Sln1p receiver domain, then to a histidine in the phosphorelay molecule, Ypd1p, and finally to an aspartate on the receiver domains of the response regulators, Ssk1p and Skn7p (Posas *et al.*, 1996; Li *et al.*, 1998). The HOG1 MAP kinase pathway is repressed under normal growth conditions by Sln1p-dependent phosphorylation of the Ssk1p response regulator. Hypertonic conditions reduce Sln1p kinase activity, allowing Ssk1p to accumulate in the dephospho form to activate the HOG1 pathway (Posas *et al.*, 1996). In contrast, hypotonic conditions stimulate Sln1p kinase and apparently cause increased phosphorylation of the Ssk1p and Skn7p response regulators (Tao *et al.*, 1999). Mutants lacking Sln1p or the phosphorelay protein, Ypd1p, are inviable or very sick

(depending on the strain background) because of the accumulation of Ssk1p in the dephospho form and toxic constitutive activation of the Hog1p MAP kinase (Ota and Varshavsky, 1993; Maeda *et al.*, 1994). Mutations in *SSK1* or *HOG1* or overexpression of the Hog1p phosphatase, *PTP2*, rescue the lethality of *sln1Δ* or *ypd1Δ* mutants by reducing the level of phosphorylated Hog1p (Maeda *et al.*, 1994).

The Sln1 histidine kinase of yeast is a hybrid kinase consisting of both kinase and receiver domains (Ota and Varshavsky, 1993). A pair of transmembrane domains (TM1 and TM2) at the N-terminus are important for association with the plasma membrane (Ostrander and Gorman, 1999; C. L. Malone and R. J. Deschenes, unpublished data). Downstream of TM2 is the so-called 'linker' region, the function of which is not well characterized. Immediately after the linker is the kinase domain, which can be subdivided into several homology blocks common to all histidine kinases (Ota and Varshavsky, 1993). The H box contains the phospho-accepting histidine. The N, G1, G2 and F boxes located downstream of the H box are involved in ATP binding and autophosphorylation (Dutta and Inouye, 2000). Autophosphorylation of sensor kinases typically occurs through a bimolecular transphosphorylation reaction (Yang and Inouye, 1991; Uhl and Miller, 1994; Surette *et al.*, 1996). In some cases, such as EnvZ and CheA, dimerization domains have been identified (Park *et al.*, 1998; Bilwes *et al.*, 1999; Tomomori *et al.*, 1999). Unlike many eukaryotic receptors that are induced to dimerize by ligand binding (Heldin, 1995; Hunter, 2000), sensor kinases pre-exist in the dimeric form, and the signalling mechanism for this type of receptor is proposed to involve a conformational change within the dimer (Milligan and Koshland, 1988; Kim, 1994; Cochran and Kim, 1996). Genetic analysis is consistent with Sln1p functioning as a dimer, as mutations of the phosphorylated histidine (H576Q) or the receiver domain aspartate (D1144N) are individually lethal, but the heterozygote, *sln1-H576Q/sln1-D1144N*, is viable (Maeda *et al.*, 1994), implying that Sln1p kinase autophosphorylation occurs *in trans*.

Recent determination of the structure of the H box-containing portion of the histidine kinase domain of the *Escherichia coli* osmosensor, EnvZ, revealed a dimer consisting of two antiparallel helices from each of two EnvZ monomers (Tomomori *et al.*, 1999). This four-helix bundle mediates both inter- and intramolecular interactions. Alignment of the corresponding region of Sln1p with this region of EnvZ shows sequence conservation not only through the H box, but also throughout helix 1 and, to a lesser extent, Helix 2, suggesting that the H box region in Sln1p dimers may assume a four-helix bundle structure similar to EnvZ (Fig. 1). Although there is no direct structural information for sequences upstream of Helix 1, secondary structure analysis of this region of EnvZ and histidine kinases with related topology suggest the presence of two amphipathic helices separated by a loop region (Park and Inouye, 1997; Butler and Falke, 1998). In EnvZ, this so-called 'HAMP domain' extends from the second transmembrane domain to within five residues of Helix 1 (amino acids 180–229). In Sln1p, the HAMP domain is separated from helix 1 by 25 amino acids, thus accounting for the gap in the alignment shown in Fig. 1.

A mutation just upstream of the Helix 1 homology in Sln1p (T550I) causes a kinase active (constitutive) phenotype (Fassler *et al.*, 1997). We refer to this and similar mutations in *SLN1* as *sln1** mutations to emphasize their activated phenotype. Mutations in the linker region of other histidine kinases likewise have activating phenotypes (Kalman and Gunsalus, 1990; Collins *et al.*, 1992; Miller *et al.*, 1992; Tawa and Stewart, 1994). The isolation of linker mutants with altered activities has led to the suggestion that the linker plays a role in signal transduction or kinase regulation and is not simply a means of connecting the transmembrane domains to the kinase domain. Using the LEARNCOIL algorithm (Singh *et al.*, 1998) designed to detect coiled-coils motifs in histidine kinases, a putative coiled-coil was identified just upstream and overlapping with Helix 1 and just downstream and overlapping with the second helix of the HAMP motif (Fig. 1, shaded box) in both Sln1p and EnvZ. The T550I *sln1*

activating mutation maps within the LEARNCOIL predicted coiled-coil region (Fassler *et al.*, 1997). The analysis of *sln1** and other kinase active mutants is expected to provide insight into the mechanism by which the Sln1p osmosensor senses and responds to changes in the osmotic environment. The goal of the current study was to assess the importance of the Helix1/2 similarity and predicted upstream coiled-coil region in Sln1p kinase activity and signal transduction. Our results confirm the presence of a coiled-coil in the Sln1p linker and demonstrate its requirement for Sln1p dimerization and kinase regulation. Our data lead us to reject a model for osmotic control of kinase activity that involves changes in coiled-coil interaction strength and to suggest an alternative model in which changes in coiled-coil rotation modulate accessibility of the phospho-accepting histidine.

Results

The T550I activating mutation may reside within a coiled-coil region of Sln1

The T550I mutation in *SLN1* causes an activated phenotype that shifts the equilibrium between phospho Sln1p and dephospho Sln1p under ordinary growth conditions, so that the phosphorylated form of Sln1p is favoured (Fassler *et al.*, 1997). To investigate the importance of the isoleucine substitution in the regulation of the Sln1p kinase, additional mutations were generated at this position, as described in detail in *Experimental procedures*. In addition to the isoleucine mutation that was reisolated in this screen, substitution of T550 with leucine and phenylalanine also activated the reporter several-fold, whereas substitution of T550 with aspartate did not increase reporter activity compared with wild type (Fig. 2A). In a separate screen (W. Tao and J. S. Fassler, unpublished data), the nearby E543V mutation was also identified as a *sln1** mutation.

Although the linker domain has not been characterized structurally in any histidine kinase, the existence, position and register of a coiled-coil region is predicted to be a general feature of this domain of histidine kinases (Berger and Singh, 1997; Singh *et al.*, 1998). The LEARNCOIL algorithm for predicting histidine kinase-type coiled-coils (Singh *et al.*, 1998) identifies a possible coiled-coil in the linker of Sln1p overlapping helix 1 and terminating just before a conserved proline within the H block consensus (Fig. 1). In the predicted coiled-coil, the phospho-accepting histidine occupies position **f** of the coil heptad, where positions **a** and **d** represent the hydrophobic core of the coil that mediates interaction (Fig. 2B) (Singh *et al.*, 1998). T550 and E543, two positions in which mutations are known to cause a *sln1** phenotype, are predicted to occupy the **a** position in the hydrophobic core of the coiled-coil (Fig. 2B). This suggests the possibility that the effect of activating mutations such as T550 to I, L and F and E543 to V might be to increase the hydrophobicity of the coiled-coil interface, causing changes in the coil that mimic the response to osmotic stress.

The predicted Sln1 coiled-coil is essential for viability and kinase activity

Standard coil detection algorithms such as NEWCOILS (Lupas *et al.*, 1991) and PAIRCOIL (Berger, 1995) that are trained against dimeric, parallel coils from α -helical proteins such as myosin do not efficiently recognize the unusual subclass of coiled-coils present in histidine kinases (Singh *et al.*, 1998). A high frequency of charged residues in the core **a** and **d** positions is apparent in the predicted Sln1p coil and in the equivalent region of EnvZ, portions of which are represented in Fig. 2B. As the presence of charged residues at core positions of the coil is predicted to lead to destabilization of coil-mediated interactions (Tripet *et al.*, 2000), it was important to assess the functional importance of the putative coiled-coil region in *sln1p*. Two deletions were created in an otherwise intact *sln1p* gene. Deletion C1 (Δ 527–540) and deletion C2 (Δ 544–568) are both upstream of the H box (H576), and neither affects Sln1 protein levels detectably (data not shown). The deletion constructs were introduced into a *sln1* Δ strain (RJY1428) whose viability was maintained with a *URA3*-based plasmid overexpressing the *PTP2* gene (Maeda

et al., 1994). The $\Delta C1$ construct was found to support viability; however, $\Delta C2$ was inviable (Fig. 3A). As the viability of *sln1* mutants depends on the ability to phosphorylate pathway intermediates, these results imply that the region covered by the C2 deletion is critical for kinase activity. To test this assumption, kinase activity was compared in constructs containing or lacking the predicted coiled-coil domain. The recombinant Sln1p kinase fragment used in this and other (below) *in vitro* assays included sequences between amino acids 537 and 947. This fragment contains neither the transmembrane domains nor the receiver domain. The 537–947 kinase fragment is referred to as Sln1K. The Sln1K and the Sln1K Δ CC (570–947) lacking the coiled-coil domain were purified from bacteria as glutathione *S*-transferase (GST) fusions; however, the GST moiety was cleaved using thrombin before kinase activity assays. Reactions containing equivalent amounts of each kinase were incubated with [γ -P³²]-ATP and sampled over time. Figure 3B shows that only Sln1K, the form of Sln1p kinase containing the predicted coiled-coil, has detectable kinase activity.

The predicted Sln1 coiled-coil is required for Sln1 association

One possible role for the predicted coiled-coil region of the Sln1p linker is to mediate Sln1p dimerization. We used a co-immunoprecipitation assay to determine whether cytoplasmic derivatives of sln1p do in fact associate. As we had shown previously that the *sln1p* receiver domain does not interact (W. Tao and J. S. Fassler, unpublished data), the *SLN1* constructs used in this assay included only the sequences (amino acids 356–1077) between the second transmembrane domain and the receiver domain. One construct was tagged with the myc epitope and was under the control of the *GAL1* upstream activating sequence (UAS) (Fig. 4, lanes 1 and 2); the second construct was tagged with the HA epitope and was expressed under the control of the constitutive *ADHI* UAS (Fig. 4, lane 3). The two constructs were co-expressed in the *SLN1* wild-type strain, FY251. The presence of Sln1-HA in α -myc immunoprecipitates (Fig. 4, lanes 4 and 5) demonstrates an interaction between Sln1p monomers mediated by the kinase and/or linker domains.

Having established that the cytoplasmic Sln1p molecules do interact, two-hybrid assays were used to localize the interaction and evaluate the possibility that the coiled-coil region in the linker mediates the interaction (Fig. 5A). A comparison of the two-hybrid interaction was made between DNA-binding domain (DBD) and activation domain (AD) fusions to *SLN1* (amino acids 356–1077) containing the coiled-coil region and comparable constructs lacking both C1 and C2 sequences in the coiled-coil region (amino acids 356–1077; Δ 527–568). Expression of all fusions was roughly equivalent (Fig. 5B), but only the coil-containing constructs could be shown to interact. The absence of an interaction between constructs lacking the predicted coiled-coil region (Fig. 5A) indicates that the coiled-coil is needed for Sln1p interaction.

The C/EBP coiled-coil restores viability to the *sln1* Δ C2 mutant

If the function of the C2 region was simply to form a coiled-coil, unrelated sequences known to mediate a coiled-coil interaction might restore viability to the *sln1* Δ C2 mutant. The coiled-coil region of the mammalian transcription factor, C/EBP, was ligated into the C2 deletion junction in an otherwise full-length and functional *SLN1* gene, such that the register of the coil formerly present in the C2 sequences was preserved (Bustos and Schleif, 1993). Each construct (see *Experimental procedures*) contains six copies of the myc epitope at the 3' end of the *SLN1* open reading frame (ORF) to allow inspection of protein levels. To determine whether the *sln1*-C/EBP chimera is capable of supporting viability, the construct was introduced into the *sln1* Δ strain (RJY1428) carrying the *URA3 PTP2* overexpression plasmid (Maeda *et al.*, 1994), and growth was assayed on 5-FOA media as before. Figure 6A shows that the C/EBP sequences are able to substitute functionally for the sequences removed in the *sln1* Δ C2 mutant, thus suggesting that the importance of the C2 region of the Sln1 linker domain lies in its ability

to form a coiled-coil structure. (Results with *sln1-C/EBP* + X constructs in Figs 6B and C are described below.)

Substitution of the CC region with the C/EBP zipper causes salt sensitivity but no increase in in vitro kinase levels

Whether the C2 region is a simple zipper or a more sophisticated regulatory region was tested by assaying other *SLN1* phenotypes. As the C/EBP zipper has a more hydrophobic interface than the native Sln1p coil, we anticipated that the substitution mutant might exhibit hyperactive phenotypes like the *sln1** mutants. *sln1** mutants are osmosensitive in the absence of Sho1p, an osmosensor that functions independently of Sln1p (Fassler *et al.*, 1997). Osmosensitivity is taken to reflect impaired ability of the mutant Sln1p kinase to respond to salt because of either increased kinase or phosphorelay activity or the inability to turn off the kinase. The accumulation of phospho-Ssk1p prevents cells from activating the Hog1p MAP kinase pathway and inducing the genes needed for osmotic adaptation. A full-length *sln1-C/EBP* construct was expressed in the $\Delta sln1 \Delta sho1$ double mutant (RJY1525), and cultures were tested for osmosensitivity on media containing 0.9 M NaCl. Like the *sln1** mutants, strains expressing *sln1-C/EBP* grew less well than wild type on high-salt medium (Fig. 7).

To determine whether the basis for the *sln1** and *sln1-C/EBP* osmosensitive phenotype is increased kinase activity, we performed autokinase assays *in vitro*. Figure 8A shows the rate and extent of autophosphorylation by Sln1K*, which contains the T550I activating mutation in the coiled-coil region, and Fig. 8B shows autophosphorylation by Sln1-C/EBP kinase, in which the coiled-coil region has been replaced by the C/EBP zipper. Both mutant kinases are roughly comparable with Sln1K. In the representative experiments shown here, Sln1K* phosphorylation peaked at 3264 c.p.m. incorporated versus 3196 for wild-type kinase, and Sln1-C/EBP kinase phosphorylation peaked at twice the level of wild type (1375 versus 650). Sln1-C/EBP kinase activity fluctuated between one- and twofold of wild-type kinase activity depending on the amount of glycerol in the reaction. The lack of change in Sln1K* and the modest change in Sln1-C/EBP kinase activities are unlikely to explain fully the osmosensitive phenotypes of the *sln1** and *sln1-C/EBP* mutants. Alternative explanations for the phenotype of these mutants including elevated phosphorelay activity and the inability to turn off kinase activity in response to osmotic stress will be considered in the *Discussion*.

The activated phenotypes of *sln1 mutants and the C/EBP chimera are not a reflection of interaction strength**

A possible mechanism to account for the hyperactive phenotypes of *sln1** mutants and the *sln1-C/EBP* chimera is an increase in the strength of the coiled-coil-mediated interaction. If, as suggested by the unusual features of histidine kinase-type coiled-coils, the coiled-coil interaction between Sln1p monomers is normally weak in order to allow kinase regulation by osmotic stress, an increase in interaction strength might interfere with the osmotic response. This was tested using the two-hybrid assay. Figure 9 is a representative experiment showing that there is no detectable effect of the *sln1** mutation or C/EBP substitution on interaction strength. If the interaction between kinase monomers is not increased, what then accounts for the changes in pathway activity?

Sln1 kinase activity is sensitive to coil orientation

We explored the possibility that the *sln1** and C/EBP alterations might cause other types of changes in the coiled-coil interaction. For example, the coils might take on a different orientation with respect to one another, thereby changing the solvent accessibility of the phospho-accepting histidines (Fig. 2B). To evaluate the sensitivity of the Sln1p kinase to the precise position of each residue within the heptad, we used the Sln1-C/EBP chimera, in which the coiled-coil register is unambiguous, to construct kinase mutants in which one, two, three

or four amino acids were inserted at the interface between C/EBP and the downstream Sln1 sequence to create Sln1-C/EBP+1 (T), Sln1-C/EBP+2 (KT), Sln1-C/EBP+3 (AKT) and Sln1-C/EBP+4 (EAKT). These additions are presumed to have the effect of rotating the register of the predicted coil. Each construct was introduced into a *sln1*Δ strain (RJY1428) and assayed for viability. Strains expressing *sln1-C/EBP* and *sln1-C/EBP+3* were viable, whereas *sln1-C/EBP+1*, +2 and +4 were inviable (Fig. 6B). The inviability in the *sln1-C/EBP+1*, +2 and +4 mutants is not a consequence of protein instability, as α-myc Western blots show that Sln1 protein levels in the mutants are comparable with wild type (Fig. 6C).

The expected correlation between viability and kinase levels was also investigated. GST fusion proteins corresponding to the C/EBP+1 and C/EBP +3 kinase domains were purified and assayed *in vitro* for autokinase activity (Fig. 8B). Like Sln1KΔCC, which lacks the coiled-coil (Fig. 3), the Sln1-C/EBP+1 kinase exhibited very low *in vitro* autophosphorylation activity (Fig. 8B), thus accounting for its failure to sustain viability. Interestingly, the autokinase activity of the Sln1-C/EBP+3 kinase was higher than that of Sln1-C/EBP+1 kinase but substantially lower than that of Sln1-C/EBP kinase (Fig. 8B). This low level of autophosphorylation activity is apparently sufficient to sustain viability.

Discussion

Cellular responsiveness to signals from the environment depends on the conversion of extracellular cues into intracellular events. The mechanism for signal transduction across the membrane to the cytoplasm is proposed to involve conformational changes; however, the precise nature of the changes and how such changes result in altered function have been difficult to decipher. Various models have been proposed for ligand-induced movement in transmembrane receptors. For bacterial receptors, there is abundant experimental support for a subtle piston-like movement of a signalling helix towards the cytoplasm (Chervitz and Falke, 1996; Ottemann *et al.*, 1999); reviewed by Falke and Hazelbauer, 2001). Rotational movement may also play a role (Cochran and Kim, 1996). However, transducing the signal across the membrane is just the first step. Subsequently, the conformational change must be propagated through the linker to the catalytic domain. The structural basis for this step is not known.

In many histidine kinases and in the structurally related chemoreceptors, the linker is predicted to consist of helical elements named 'HAMP motifs' (Aravind and Ponting, 1999; Williams and Stewart, 1999), followed by the structurally characterized four-helix bundle (Kim *et al.*, 1999; Tomomori *et al.*, 1999), which includes the H box in the histidine kinases. The importance of the linker region in regulating signal transduction is well documented in the prokaryotic literature (reviewed by Singh *et al.*, 1998; Williams and Stewart, 1999). In one model for linker domain function, the HAMP motifs are proposed to mediate alternative interactions with the membrane and with one another to generate changes in conformation that transduce the signal to the kinase domain (Williams and Stewart, 1999). The present work concerns a region of the linker downstream of the HAMP motifs and upstream and overlapping helix 1 of the four-helix bundle, and proposes another potential mechanism for linker-mediated regulation of signal transduction. These models are not mutually exclusive, and both may be relevant to the control of kinase activity in response to environmental signals.

The Sln1p linker encodes a coiled-coil

Our attention was drawn to the linker domain of the Sln1p histidine kinase by a set of activating mutations that cluster there. Based on computational predictions of secondary structure, the linker region of histidine kinases had previously been postulated to form a coiled-coil. However, the unusual features of the computationally predicted coiled-coils in the linker domains of histidine kinases left open the possibility that the resemblance to coiled-coils might be fortuitous and that other features unrelated to this aspect of the predicted secondary structure

might account for the functional importance of this region. Several experimental lines of evidence support the computational hypothesis that the coiled-coil nature of these sequences is an important feature of this region of histidine kinases. First, mutations could be isolated that increase both the activity of the pathway and the hydrophobicity of the predicted hydrophobic core of the coil. Mutations such as T550D that did not increase hydrophobicity of the coil also did not affect pathway activity. Secondly, we demonstrate that the native *SLN1* sequences encoding the putative coil could be replaced with a heterologous leucine zipper. Finally, we demonstrate (discussed below) that these sequences are required for Sln1p interaction.

The Sln1 coiled-coil is required for dimer formation and normal kinase activity

Recent studies (Ostrander and Gorman, 1999) have shown that constructs lacking the extracellular domain (ECD) of Sln1p cause constitutive phosphorylation of Hog1p, implying that the domain is required for normal kinase activity. As normal activity could be restored by the addition of the C/EBP leucine zipper cassette, and mutations known to prevent dimerization of the leucine zipper reduced the activity of the Sln1p kinase, it was postulated that dimerization is mediated by the extracellular domain (Ostrander and Gorman, 1999). However, the viability of the Δ ECD construct (in spite of the elevated Hog1p phosphorylation) suggests that kinase activity and dimerization are not fully abolished in the mutant. Our studies of *SLN1* coiled-coil mutants indicate that the linker domain also makes an important contribution to dimerization and kinase activity.

Our analysis of *SLN1* linker domain deletion mutants revealed that the sequences deleted in the Δ C2 mutant are important for kinase activity. The inviability of *sln1* mutants stems from the accumulation of dephospho-Ssk1 and the resulting activation of the Hog1p MAP kinase pathway. As the 20-amino-acid deletion in the Δ C2 mutant causes inviability, the construct is presumed to reduce kinase and/or phosphotransfer activity below the threshold required to repress the MAP kinase cascade effectively. This experiment also suggests that the sequences encoding the predicted four-helix bundle (567–627), which are retained in the Δ C2 construct (Δ 544–568), are not sufficient for Sln1p dimerization. Interestingly, the Δ C1 deletion was viable. This may imply that the portion of the coiled-coil distal to the H box is less important than the proximal portion that appears to be sufficient to carry out the vital function of the region. As the C1 sequences overlap the second helix of the HAMP motif, these results imply that the HAMP helices are distinct from the C2 coiled-coil.

The C/EBP substitution is viable but not osmotolerant

Interestingly, *sln1-C/EBP*, although viable, is not wild type with respect to pathway activity. The osmosensitive phenotype of this mutant suggests that phospho-Ssk1p levels in the mutant may be increased relative to wild type. Thus, the C/EBP leucine zipper is sufficient for some but not all aspects of *SLN1* function. This suggests that the unusual features of the coiled-coil in *SLN1* and other histidine kinases may be a specialized adaptation for regulating kinase activity.

Alterations in the coiled-coil orientation may regulate SLN1 pathway activity

Although a substantial body of work in bacterial systems indicates that dimerization is necessary but not sufficient for histidine kinase activation (Yang and Inouye, 1991; Uhl and Miller, 1994; Surette *et al.*, 1996), we nonetheless tested the possibility that changes in osmotic environment might modulate interaction strength between cytoplasmic Sln1p monomers. This idea is attractive because the hydrophobic core of the coiled-coil region of native Sln1p contains a total of four hydrophobic residues, whereas the *sln1** mutations T550I and E543V each create one additional hydrophobic interaction and might therefore be expected to mediate a somewhat stronger interaction. However, this was not the case. There were no detectable differences in

kinase–kinase interactions despite alterations in the hydrophobicity of the coiled-coil in *SLN1* +, *sln1** or *sln1-C/EBP* constructs. Thus, as proposed for prokaryotic histidine kinases, conformational changes may play an important role in regulating Sln1p activation.

Despite insights from the recently determined individual structures of two EnvZ kinase subdomains (Tanaka *et al.*, 1998; Tomomori *et al.*, 1999) (the substrate phosphotransfer domain, A, whose dimerization yields a four-helix bundle including the phosphorylatable histidine, and the catalytic domain, B), the spatial relationship of these domains in a histidine kinase active centre remains unknown. Efforts to reconstruct a functional monomeric kinase by fusing two A and one B domains in tandem have led to an elegant model of kinase domain organization, in which the ATP binding site of domain B of one subunit is situated adjacent to the phosphorylatable histidine residue on Helix 1 of domain A of the other subunit in a homodimer (Qin *et al.*, 2000, Fig. 7). Using this model as a starting point, we propose that regulation of Sln1p kinase activity might involve changes in the relative orientation of the phosphorylatable histidine with respect to the catalytic domain (Fig. 10). Although a prototypical coiled-coil may not accommodate flexibility in the register of the coiled-coil, the special nature of histidine kinase coiled-coils, including the weak ‘hydrophobic’ character of the **a–d** core that holds the conserved histidine in the exposed **f** position in native Sln1p, might allow sufficient range of motion to allow changes in the absolute location of the **f** position within the coil. Alternatively, the histidine kinase coiled-coil could be designed to allow shifts in coil register, such that a different set of residues occupies the **a** and **d** positions of the heptad repeat under low versus high osmotic conditions. In this model, the T550I and E543V mutants would favour the coil register that leads to pathway activation.

We tested the idea that the relationship between the coiled-coil domain and the phosphorylatable histidine is important by introducing small insertions into the coil region of the Sln1-C/EBP chimera. We chose the C/EBP chimera for these experiments because the robust hydrophobic interface in this coil creates a situation in which the coiled-coil register is unambiguous. The effect of modification to the C/EBP coiled-coil could therefore be predicted with confidence. The position of the insertion was designed to maintain the C/EBP **a–d** interface. However, the phosphorylatable histidine should rotate with respect to the coil as a result of the insertion. The results of this analysis indicate that kinase activity is sensitive to insertion in the heptad repeat of the coil. Of the constructs we tested (+1, +2, +3 and +4), only +3 was viable. The 3-amino-acid insertion is presumed to move the histidine from the exposed **f** position to the adjacent **b** position, which is also reasonably exposed (Fig. 2B). Interestingly a +4 insertion is inviable, although it is expected to move the histidine to **c**, a position that appears superficially to resemble **b** in the extent to which it is solvent exposed. The difference between the +3 and +4 kinases may reflect the relative accessibility of the **b** and **c** positions to the catalytic domain.

Implications of the coiled-coil domain for Sln1 structure and function

Although the sequences upstream of Helix 1 (amino acids 223–235) appear to be disordered in the EnvZ structure (Tomomori *et al.*, 1999), this may result from the loss of secondary structure elements that reside further upstream. Our data suggest that sequences upstream of Helix 1 are also helical, and we have tentatively depicted them as continuous with the upstream half of Helix 1 (Fig. 10). The predicted Helix 1 of Sln1p and EnvZ (and approximately two-thirds of all known histidine kinases) contain a proline residue five residues distal to the phosphorylatable histidine (Grebe and Stock, 1999; Tomomori *et al.*, 1999). The placement of this proline inside Helix 1 coincides with a disordered region in the EnvZ helices and may create a kink in two of the four helices in the four-helix bundle, as shown in Fig. 10. In this case, changes in the orientation of the linker helix could allow rotation of the phosphorylatable histidine without disturbing the remainder of the four-helix bundle. We propose that increasing

or decreasing pressure on the membrane as the result of changing osmotic conditions causes a twist that propagates through the linker and influences the alignment of the phospho-accepting histidine in the H box located in Helix 1 and the catalytic part of the kinase domain, and that the alignment is more favourable under high kinase activity conditions such as hypo-osmotic stress or the *sln1** mutant than it is under low kinase activity conditions such as hyperosmotic stress.

Although kinase levels are reduced relative to wild type in the Δ CC mutant (Fig. 3), the *sln1* and *sln1-C/EBP* mutants that improve the hydrophobic interface do not appear to elevate kinase levels, as measured by our *in vitro* kinase assay (Fig. 8). This suggests that the role of the coiled-coil domain in kinase activity may not be to increase or decrease autophosphorylation, but rather to regulate some other aspect of Sln1p activity such as phosphotransfer or dephosphorylation. Most histidine kinases are bifunctional, possessing phosphatase activity (typically directed against the aspartyl phosphate of the response regulator) in addition to kinase activity (Egger *et al.*, 1997); the ratio of the two activities dictates flux through the pathway. Thus, pathway activity can be modulated by regulation of the kinase or the phosphatase. Interestingly, it has been proposed that the spatial arrangement of domain A relative to domain B of EnvZ may be altered under different osmotic conditions to favour one activity over the other (Zhu *et al.*, 2000). Although no Sln1p-associated phosphatase activity has yet been identified using the Sln1p-associated receiver domain as substrate (A. D. Ault *et al.*, in preparation), studies involving the Ssk1p and Skn7p response regulators have not yet been conducted. A second possible mechanism accounting for the activated phenotypes is a change in phosphorelay efficiency. Recent biochemical analysis of another *sln1** mutant, *sln1 (p114)* mutant (A. D. Ault *et al.*, in preparation) has revealed that its activated phenotype is at least partially attributable to changes in the efficiency of the Sln1p to Ypd1p phosphorelay step.

Many details of linker region regulation of kinase activity remain to be defined. Structural analysis of several histidine kinase domains is needed to test the structural predictions of the coiled-coil hypothesis fully. To date, no structures exist that include the region N-terminal to the kinase domain. In the absence of structural information, we have used functional analysis of coiled-coil mutants and chimeras to demonstrate the involvement of a coiled-coil domain in Sln1p signalling. Further analysis of the *in vivo* and *in vitro* characteristics of *SLN1* mutants and chimeras is expected to provide additional mechanistic detail.

Experimental procedures

Media, growth conditions and genetic methods

Media were prepared as described by Sherman *et al.* (1986) and included synthetic complete medium (SC) lacking one or more specific amino acids (e.g. SC-leucine), rich medium (YPD) and synthetic dextrose (SD). Plates for the detection of β -galactosidase activity contained 50 mg ml⁻¹ Xgal unless noted otherwise and were prepared as described by Larson *et al.* (1983). Yeast strains were grown at 30°C unless noted otherwise. Yeast transformation was performed by a modified LiOAc method (Ito *et al.*, 1983; Gietz *et al.*, 1995)

Yeast strains

All yeast strains (Table 1) used in this work are from our laboratory collections or were constructed for this study. Strains are isogenic or congeneric with the wild-type strain S288C as noted (Winston *et al.*, 1995).

Plasmids

All plasmids used in this work are from our collections or were constructed for this study (Table 2).

Two-hybrid constructs—The two-hybrid vector plasmids pAS1-CYH2 and pACTII contain the *GAL4* DNA-binding domain (amino acids 1–147) and *GAL4* activation domain (amino acids 768–881) driven by the *ADHI* promoter respectively (Durfee *et al.*, 1993; Harper *et al.*, 1993). Vector pAS1-CYH2 contains the yeast *TRP1* marker, and pACTII contains the *LEU2* marker. Both plasmids have one copy of the haemagglutinin (HA) epitope tag inserted behind the *GAL4* sequence and preceding the poly cloning sites.

The cytoplasmic *SLN1* kinase construct was amplified from *SLN1* plasmid pGY111 (Yu *et al.*, 1995) using synthetic primers SLN1060F (5'-CTA GCA CAC TGG ATC CTG CAA CCA ATT GTA CG-3') and SLN3231R (5'-CTC AGT CTC GAG TTA TCA GCG GCC GCC TGT GGG GAT GTT TCT ACT TG-3') and digested with *Bam*HI and *Xho*I. The 2.2 kb *Bam*HI–*Xho*I *SLN1* kinase fragment was cloned in *Bam*HI–*Sal*I-digested pAS-CYH2 vector and *Bam*HI–*Xho*I-digested pACTII vector respectively. The junctions of the insertion and the entire *SLN1* sequence in both plasmids were verified by DNA sequence analysis.

Full-length *Sln1* and *sln1-C/EBP* constructs—pCLM994 was constructed by cloning the *Sac*II to *Eco*RV fragment from pHL597 into the *Sac*II–*Eco*RV-digested pRS313 (Sikorski and Hieter, 1989). pHL597 is a full-length *SLN1* clone with six copies of the myc epitope added in frame by insertion into a newly created *Not*I site at the end of the ORF. The *sln1ΔC1* plasmid, pCLM1001, was constructed by polymerase chain reaction (PCR) amplification of *SLN1* sequences from pHL597 using synthetic oligonucleotide primers SLN718F (5'-GTA TAT AAT AGT CAA GGC-3') and SLN1578R (5'-TCA TCG CTT CTA GAA TTC TGT TAA ATC GGA AAG TTC ATC-3'), followed by digestion with *Stu*I and *Xba*I and cloning into pCLM994 also digested by *Stu*I and *Xba*I. Amino acids 527–540 are deleted in this construct. The *sln1ΔC2* plasmid, pCLM1002, was constructed in two steps. First, the product of PCR amplification of *SLN1* sequences from pHL597 using primers SLN1705F (5'-TCA TCG CTT CTA GAA GTC TTT ATT GCC AAT ATT TCG CAC G-3') and SLN2080R (5'-GGT GAA CTT TAG TGC ATT GGA-3') was digested with *Xba*I and *Cla*I and ligated into pCLM994 (*Xba*I, *Cla*I) to generate the intermediate plasmid pCLM996. pCLM996 was then digested with *Cla*I and ligated to the *Cla*I fragment from pCLM994. Amino acids 544–568 are deleted in this plasmid. The *sln1-C/EBP* plasmid, pCLM1021, was constructed by digesting pCLM1002 with *Xba*I, treating with calf intestinal phosphatase (CIP) and co-transforming with the PCR product generated using primers SLN1574F (5'-CAG AAA CCT TCA ATA CTA TGA CAG ACG CAT TAG ACC AAC ATT ATG CTC TTC GCA ACG TGG AGA CGC-3') and SLN1705R (5'-ATA CCA TTT AAA GGC GTT CTC AAT TCG TGC GAA ATA TTG GCA ATA AAG ACG CTC AGC TGT TCC ACC-3') with pGB008 (Landschulz *et al.*, 1988; Bustos and Schleif, 1993) as template. The *sln1-C/EBP+X* plasmids, pCLM1033 (+1), pCLM1034 (+2), pCLM1035 (+3) and pCLM1036 (+4), were constructed by digesting pCLM1002 with *Xba*I, treating with CIP and co-transforming with the PCR amplification product using pGB008 (Landschulz *et al.*, 1988; Bustos and Schleif, 1993) as template, forward primer SLN1574F and one of the following reverse primers: (+1) SLN1704R (5'-ATA CCA TTT AAA GGC GTT CTC AAT TCG TGC GAA ATA TTG GCA ATA AAG ACG GTG CTC AGC TGT TCC ACC-3'); (+2) SLN1701R (5'-ATA CCA TTT AAA GGC GTT CTC AAT TCG TGC GAA ATA TTG GCA ATA AAG ACG GTT TTG CTC AGC TGT TCC ACC-3'); (+3) SLN1698R (5'-ATA CCA TTT AAA GGC GTT CTC AAT TCG TGC GAA ATA TTG GCA ATA AAG ACG GTT TTT GCG CTC AGC TGT TCC ACC-3'); and (+4) SLN1695R (5'-ATA CCA TTT AAA GGC GTT CTC AAT TCG TGC GAA ATA TTG GCA ATA AAG ACG GTT TTT GCT TCG CTC AGC TGT TCC ACC-3').

In vitro constructs—pHL581 and pADA847 are Sln1K^{537–947} and Sln1K^{570–947} in pGEX-KG respectively. *SLN1* DNA corresponding to amino acids 537–947 or 570–947 was amplified using primers SLN1609F (5'-ATG ACA GAC GCA TGA ATT CAA CAT TAT GCT CTT CTA G-3') or SLN1711F (5'-TTT CTA GAC GGA TCC ATT GCC AAT ATT TCG CAC G-3') and SLN2827R (5'-CTT TCT ACT CTC GAG GAT TAA ATT CGT C-3'). PCR products were digested with *EcoRI* or *XbaI* and *XhoI* for ligation into pGEX-KG (Guan and Dixon, 1991).

pCLM1032, pCLM1037 and pCLM1038 are Sln1-C/EBP+0, +3 and +1, respectively, in pGEX-KG. They were constructed using the pCLM1021 and pCLM1033 (+1) and pCLM1035 (+3) plasmids as templates in PCR reactions with primer SLN1612F (5'-ACG CAT GGA ATT CAA CAT TAT GCT CTT CGC-3') and SLN2080R (above). PCR products were digested with *EcoRI* and *ClaI* (pCLM1021) or *BpII* and *ClaI* (pCLM1033 and pCLM1035) and the fragments cloned into pGEX-SlnK^{537–947}.

Sln1 T550 mutagenesis

To introduce amino acid substitutions at amino acid 550 of Sln1, the degenerate primer SLN1704R [5'-GGT TTT TGC TTC ATT TGC GGC CTC TGC CTC AAT CTT GGC TGC TTC CAG CTG TTT (T,G,A)(T,A,C)(C,G) CCT CGC CCT AAC TCT TTC-3'], corresponding to the reverse complement of Sln1 amino acids 544–568, was synthesized. This primer was designed to introduce 18 types of codons, encoding 14 different amino acids. SLN1704R and SLN1551F (5'-GCG CAA CTG GAT CCC TGA TGA ACT TTC CG-3') were used to amplify degenerate PCR products from an *SLN1* plasmid template under normal PCR conditions. These 154 bp degenerate PCR products containing substitutions at amino acid 550 were co-transformed with *XbaI*-linearized pWT1165 (*SLN1*, *HIS3*, *CEN*) into JF2057 (*sln1Δ*, *ssk1Δ*) carrying the P-*lacZ* reporter (Yu and Fassler, 1993). The *XbaI* site in *SLN1* is situated close to T550, and recombination events between the corresponding sequences in the PCR product and the plasmid repair the gap in the plasmid. Transformants were selected on plates lacking histidine and screened for an increase in the normal pale blue colour of this strain on plates containing 50 μgml⁻¹ Xgal. Six out of 110 transformants screened exhibited a blue colour on Xgal media and at least a twofold increase in β-galactosidase activity indicating reporter activation. *SLN1* plasmids in these transformants were isolated and subjected to sequence analysis. Four were T550F substitutions, one was T550L, and one was T550I. The T550D mutant was isolated in this screen by analysis of transformants whose colour appeared to be whiter than the pale blue of the starting strain. Of four white transformants analysed, only one (T550D) had a mutation.

Predicting coiled-coil motif in Sln1p using LEARNCOIL algorithm

The LEARNCOIL program and the histidine kinase probability table (Singh *et al.*, 1998) are available on the web at <http://nightingale.lcs.mit.edu/cgi-bin/learncoil>.

Two-hybrid interaction assays

Two-hybrid constructs carrying the *GAL4* DNA-binding domain and *GAL4* activation domain fusions were co-transformed into the two-hybrid test strain pJ69-4A (James *et al.*, 1996). Transformants were grown in selective media to log phase. Cells were washed twice with synthetic media before resuspension at a concentration of 2 × 10⁸ cells ml⁻¹. Tenfold dilutions (2 × 10⁷, 2 × 10⁶ and 2 × 10⁵) were spotted (2 μl) on test and control plates incubated at 30° C. Complete plates were SD + uracil, methionine, adenine and histidine; 3 amino-triazole (3-AT) plates were SD + uracil, methionine, adenine plus 1.0 mM 3-AT.

Immunoprecipitation assay

Yeast strain FY251 carrying myc-tagged and HA-tagged fusion constructs were grown to $OD_{600} = 0.5$ in selective media with 2% raffinose. Galactose (4%) was added to half the culture to induce protein expression from the P_{GAL} -driven myc-tagged construct. Cells were collected by centrifugation, resuspended in 200 μ l of lysis buffer [25 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF) and protease inhibitors] and broken by glass bead lysis. Cell debris was removed by microcentrifugation. Anti-myc antibody (5 μ l) was added to 100 μ l of extract and incubated on a rocker at 4°C for 1–2 h. An aliquot of 25 μ l (50% slurry) of washed (in lysis buffer) anti-IgG agarose (Sigma A-6531; goat anti-mouse) was added to the mixture and incubated at 4°C for 1–2 h. Agarose beads were washed three times with 1 ml of PBS buffer, collected by microcentrifugation and resuspended in 25 μ l of 2.5 $\times$ SDS loading buffer. Samples were boiled for 2 min, and proteins were separated by polyacrylamide (10%) gel electrophoresis. Proteins were transferred to nitrocellulose membrane, probed with anti-HA antibody (1:1000) and visualized using anti-mouse horseradish peroxidase (HRP) and ECL chemiluminescence (Amersham).

Growth and induction of bacterial strains expressing GST fusion proteins

GST fusion proteins were expressed in DH5 α cells. One litre cultures were grown to an OD_{600} of 0.6–1.0 in LB media and induced with 0.2 mM IPTG at 16°C for 20 h. After induction, cells were harvested by centrifugation (5000 g for 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, 1 $\times$ PIC (0.05 mg ml⁻¹ leupeptin, 0.001 mg ml⁻¹ pepstatin, 0.001 mg ml⁻¹ chymostatin, 1 $\times$ aprotinin (Sigma)] and stored frozen at -80°C.

Glutathione (GSH) affinity purification of GST fusion proteins

Frozen bacterial cells were thawed and lysed by French press in the presence of 1 mM PMSF (added immediately before lysis). The lysate was cleared by centrifugation. GSH-agarose beads were added as a 50% slurry to the supernatant. (3 ml of beads per 5 g of cell paste or 1 l of culture), and the mixture was incubated at 4°C for 1 h. Beads were pelleted and washed four times in lysis buffer, followed by four washes with storage buffer (50 mM Tris, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 0.1% β -mercaptoethanol, 50% glycerol) and stored at -20°C.

To cleave Sln1 kinase from GSH-agarose beads (Novagen), a 50% slurry (1ml) of bead-bound protein was washed four times in thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$) before the addition of 5 units of biotinylated thrombin. Cleavage reactions were incubated at 25°C for 4–6 h with streptavidin beads (25 μ l) added to the reaction during the final 30 min. Proteins were quantified by Bio-Rad protein assay and Coomassie staining of SDS–polyacrylamide gels.

Autokinase assay

Sln1 kinase domains (380 nM) were incubated with 10 μ M [γ -³²P]-ATP (400 Ci mM⁻¹) for 15 min in kinase reaction buffer (50 mM Tris, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 0.1% β -mercaptoethanol, 50% glycerol) at room temperature. Beads were washed four times with reaction buffer to remove unincorporated label. Aliquots were quenched in 5 $\times$ SDS loading buffer. Samples were resolved by SDS–PAGE and the gels analysed using a Packard Instant Imager.

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Fig. 1.

A. Diagram of the domain organization of the Sln1 protein. Raised rectangles denote, in order, two transmembrane domains, the H box that includes H576 (H), an ATP-binding domain including N, G1, F and G2 boxes and the receiver domain including the phospho-accepting aspartate residue (D) at amino acid 1144. The region between the two transmembrane domains is referred to as the extracellular domain (ECD). The region between the second transmembrane domain and the kinase is called the linker. Sln1 has a P-type linker, which includes two predicted amphipathic helices known as the HAMP motif (Aravind and Ponting, 1999; Williams and Stewart, 1999).

B. SLN1 sequences surrounding the H box are aligned with the corresponding sequence of the bacterial histidine kinase, *EnvZ*. Alignment of Sln1 with *EnvZ* and other histidine kinases (not shown) was generated using PSI-BLAST (Tomomori *et al.*, 1999). Vertical lines between the two sequences reflect sequence identity or conservation. A gap in the alignment reflects the presence of a 25-amino-acid insertion in Sln1p relative to EnvZ located between helix 1 and the HAMP motif. The region predicted by the LEARNCOIL algorithm to form a coiled-coil is shaded. The positions of the *SLN1* activating mutations T550I and E543V as well as helix 1 and helix 2 of *EnvZ* are indicated.

Fig. 2.

A. Expression of the *SLN1*-dependent P-*lacZ* reporter in the presence of various *SLN1* alleles. The *sln1Δ ssk1Δ* strain, JF2057, was transformed with each *SLN1* plasmid [pWT1165 (wild type), pWT1171 (*sln1 T550I*), pWT1172 (*sln1 T550L*), pWT1169 (*sln1 T550F*), pWT1170 (*sln1 T550D*) and pWT1193 (*sln1 E543V*)] and β -galactosidase activity measured. Values are averages of four measurements made on three or more independent transformants and are expressed relative to the *SLN1*⁺ allele, which is normalized to 100. [SD], standard deviation.

B. Helical wheel diagram showing the position of H576 relative to the hydrophobic core (consisting of **a** and **d** residues) within the predicted parallel coiled-coil. The sequence of a portion of the predicted coiled-coil is given showing the helical position of each residue. T550 and E543 both fall into the **a** position of the predicted coil.

Fig. 3.

A *SLN1* coiled-coil deletion mutant affects viability and kinase activity.

A. Viability assay. *SLN1* plasmids [pCLM994 (*SLN1*), pCLM1001 ($\Delta C1$ ($\Delta 527-540$) or pCLM1002 ($\Delta C2$ ($\Delta 544-568$)] were introduced into the *sln1* Δ strain, RJY1428, the viability of which was maintained by the presence of a *URA3*-based high-copy plasmid carrying the *PTP2* gene. Overexpression of the Ptp2p phosphatase rescues the lethality of *sln1* Δ mutations by lowering phospho-Hog1p levels. Upon introduction of an *SLN1* construct able to support viability, high levels of *PTP2* are no longer required, and the strain survives on media containing 5-FOA, a chemical that inhibits the growth of Ura⁺ cells and therefore selects for cells that have lost the *URA3 PTP2* plasmid (right). Growth on SC-Ura-His medium (control) is shown on the left. Each patch in a row represents individual transformants.

B. The predicted coiled-coil region is required for autophosphorylation *in vitro*. Reactions containing 2 μ g of each kinase, Sln1K⁵³⁷⁻⁹⁴⁷ or Sln1K Δ cc⁵⁷⁰⁻⁹⁴⁷, were incubated with [γ -³²P]-ATP and quenched in SDS loading buffer at the indicated times. Gels were exposed to film after SDS-PAGE.

Fig. 4.

Co-immunoprecipitation assay showing Sln1 kinase interaction. Cells (FY251) carrying $P_{ADH}HA-SlnK$ (pWT1119) and $P_{GAL}Sln1K-myc$ (amino acids 356–1077; pWT1224) were grown under inducing conditions (4% galactose, lanes 2 and 5) or non-inducing conditions (raffinose, lanes 1, 3 and 4). Equal amounts of yeast extracts were used in an α -myc immunoprecipitation reaction and loaded on the gel. α -HA antibody was used for detection (lanes 4 and 5). As controls, 1/100 of the yeast extracts used in immunoprecipitation were loaded directly on the gel and probed with α -myc or α -HA antibody as indicated (lanes 1–3).

Fig. 5.

Two-hybrid interaction assay showing that the predicted coiled-coil is required for Sln1 interaction.

A. Tenfold dilutions of the two-hybrid reporter strain, PJ69-4A (James *et al.*, 1996), carrying different pairs of Gal4 DNA-binding domain (DBD) and Gal4 activation domain (AD) plasmids as listed, were assayed for interaction by spotting on plates lacking histidine, leucine and tryptophan and containing 1.0 mM 3-amino-triazole (3-AT). Growth on this media reflects interaction strength. Growth on medium containing histidine (control) is shown on the right as a control. Vector, DBD (pAS1-CYH2) or AD (pACTII) plasmid with no insert; +CC, DBD or AD plasmids with Sln1³⁵⁶⁻¹⁰⁷⁷ inserts; ΔCC, DBD-Sln1³⁵⁶⁻¹⁰⁷⁷ or AD-Sln1³⁵⁶⁻¹⁰⁷⁷ plasmids with deletions of the coiled-coil region (Δ527-568).

B. α-HA Western analysis of levels of expression of the AD and DBD fusions in the two-hybrid strain. Lane 1 contains AD vector and DBD-ΔCC; lane 2 contains AD-ΔCC and DBD vector; lane 3 contains AD vector and DBD-Sln1³⁵⁶⁻¹⁰⁷⁷, and lane 4 contains AD-Sln1³⁵⁶⁻¹⁰⁷⁷ and DBD vector. The mobility of the kinase fusions is indicated on the right.

Fig. 6.

Viability of *SLN1-C/EBP* chimeras depends on the register of the *SLN1-C/EBP* fusion.

A. *SLN1* plasmids (6× myc epitope tagged) were introduced into the *sln1Δ* strain, RJY1428, the viability of which was maintained by the presence of a *URA3*-based high-copy plasmid carrying the *PTP2* gene. The ability of each *SLN1* plasmid to complement the inviability of the *sln1Δ* mutation was assessed on plates containing 5-FOA, which selects against the *URA3 PTP2* plasmid.

B. C/EBP sequences (RNVETQQKVLELTSDNDRLRKRVEQLS) were substituted for residues 542–568 (LEERVARTKQLEAAKIEAANEAKT) preserving the coil register of native Sln1p. *SLN1-C/EBP+1*, *SLN1-C/EBP+2*, *SLN1-C/EBP+3* and *SLN1-C/EBP+4* were created by insertion of T (+1), KT (+2), AKT (+3) and EAKT (+4) immediately after the C/EBP sequence.

C. Sln1p and Sln1-C/EBP protein levels are equivalent, as shown by an α-myc Western blot of equivalent amounts of extract prepared from strains carrying the following *SLN1* alleles: lane 1, *SLN1*; lane 2, *sln1-C/EBP*; lane 3, *sln1-C/EBP+1*; lane 4, *sln1-C/EBP+2*; lane 5, *sln1-C/EBP+3*; lane 6, *sln1-C/EBP+4*.

Fig. 7.

Effect of *SLN1-C/EBP* chimeras on osmotic stress tolerance of *sho1Δ* strains. *sho1Δ* strains carrying *SLN1* plasmids as indicated were grown in synthetic media to log phase. Tenfold dilutions were spotted on SC-His plates with 0 (control, right) or 0.9 M NaCl (left) added. Plates were scanned after 48–72 h incubation at 30°C. *sho1Δ* strain, RJY1525. *SLN1* plasmids: pCLM994 (*SLN1*), pWT1171 (*sln1**), pCLM1021 (*sln1-C/EBP*) and pCLM1035 (*sln1-C/EBP* +3).

Fig. 8.

In vitro autophosphorylation assay comparing the activity of Sln1, Sln1-C/EBP and Sln1-C/EBP+X kinases. Kinases were isolated and purified as GST fusion proteins, and the GST moiety was cleaved by thrombin treatment before use in the autokinase assays. Kinases were incubated with [γ - 32 P]-ATP for the indicated times before loading onto an SDS-polyacrylamide gel.

A. 2 μ g of Sln1 K⁵³⁷⁻⁹⁴⁷ and Sln1 K*.

B. 2 μ g of Sln1 K⁵³⁷⁻⁹⁴⁷, Sln1-C/EBP, Sln1-C/EBP+1 and Sln1-C/EBP+3 kinase

Fig. 9.

Effect of the *sln1** and *SLN1*-C/EBP coiled-coil mutants on the Sln1 kinase interaction. Dilutions of the two-hybrid reporter strain, PJ69-4A (James *et al.*, 1996), carrying matched pairs of Gal4 DNA-binding domain (DBD) and Gal4 activation domain (AD) plasmids, as listed on the right, were assayed for interaction by spotting on plates lacking histidine, leucine and tryptophan and containing 0.5 mM 3-amino-triazole (3-AT). Growth on medium containing histidine is shown on the right as a control. *SLN1*, DBD and AD plasmids with Sln1 (amino acids 356–1077) insert; *sln1**, DBD and AD plasmids with *SLN1* (amino acids 356–1077 with the T550I substitution) insert; *sln1-C/EBP*, DBD and AD plasmids with *SLN1* (amino acids 356–1077 with amino acids 542–568 substituted with equivalent length coiled-coil region of C/EBP) insert. Δ CC, DBD and AD plasmids with *SLN1* (amino acids 356–1077 with a deletion of the coiled-coil region, amino acids 527–568) insert is shown as a control.

Fig. 10.

Model for histidine kinase signalling. The portion of Sln1 between the second transmembrane domain and the receiver domain is shown. The HAMP motifs within the linker domain are shown as a grey helix upstream of the black coiled-coil domain that is the focus of this paper. A model is proposed in which a coiled-coil immediately upstream and including the H box histidine can rotate to regulate Sln1p activity. The coiled-coil region extends into a region known as Helix 1 shown by nuclear magnetic resonance (NMR) structural analysis of a portion of EnvZ to be a four-helix bundle dimerization domain (Tomomori *et al.*, 1999) and predicted to form the same structure in Sln1p. Phosphorylation of histidine kinases is believed to occur *in trans* between monomers of a dimer; thus, the ATP-binding domains are positioned near the phosphorylatable histidine of the opposite half of the dimer as proposed previously (Qin *et al.*, 2000). The EnvZ NMR structure shows a kink in the α -helix at a proline residue five residues C-terminal to the H box histidine and immediately downstream of the coiled-coil motif that is conserved in many histidine kinases including Sln1p. It is possible that the remainder of the four-helix bundle anchors the catalytic domain, leaving the coiled-coil domain N-terminal to the proline kink free to rotate with respect to the catalytic domain.

Table 1

Yeast strains.

Strain	Relevant phenotype	Notes
FY251	<i>MATa his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63</i>	Winston <i>et al.</i> (1995)
PI69-4A	<i>MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4-Δ gal80-Δ LYS2::GAL1-HIS3 MET2::GAL7-lacZ GAL2-ADE2 (at ADE2)</i>	PI69-4A (James <i>et al.</i> , 1996)
RIY1428	<i>MATa shi1Δ::LEU2 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 with pRS-PTP2 URA3</i>	
RIY1525	<i>MATa shi1Δ::LEU2 sho1Δ::TRP1 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 with pRS-PTP2 URA3</i>	
JF2057	<i>MATa his3-Δ200 lys2Δ202 ura3-52 shi1Δ::TRP1 ssk1Δ::LEU2</i>	

Table 2

Plasmids used in this study.

Strain	Description	Vector, references
pWT1113	<i>SLN1</i> kinase (356–1070) in pAS1-CYH2	pAS1-CYH2 (Durfee <i>et al.</i> , 1993)
pWT1114	<i>sln1-21</i> kinase (356–1070) in pAS1-CYH2	pAS1-CYH2 (Durfee <i>et al.</i> , 1993)
pWT1119	<i>SLN1</i> kinase (356–1070) in pACTII	pACTII (Durfee <i>et al.</i> , 1993)
pWT1120	<i>sln1-21</i> kinase (356–1070) in pACTII	pACTII (Durfee <i>et al.</i> , 1993)
pCLM1093	<i>SLN1</i> (356–1077; Δ527–568) in pACTII	pACTII (Durfee <i>et al.</i> , 1993)
pCLM1094	<i>SLN1</i> (356–1077; Δ527–568) in pAS1-CYH2	pAS1-CYH2 (Durfee <i>et al.</i> , 1993)
pWT1224	UAS _{GAL} <i>SLN1</i> Kinase(aa 356–1077) with 6× myc epitope tag; <i>CEN TRP1</i>	pRS314 (Sikorski and Hieter, 1989)
pWT1165	<i>SLN1</i> HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pWT1169	<i>sln1-T550I</i> HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pWT1170	<i>sln1-T550D</i> HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pWT1171	<i>sln1-T550I</i> HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pWT1172	<i>sln1-T550L</i> HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pWT1193	<i>sln1-E543V</i> HIS3, <i>CEN</i>	Modified pRS314 (<i>ClaI</i> site destroyed)
pWT1166	<i>sln1-21</i> HIS3, <i>CEN</i>	Modified pRS313 (<i>ClaI</i> site destroyed)
pCLM994	<i>SLN1</i> -6 × myc HIS3, <i>CEN</i>	pRS313 (Sikorski and Hieter, 1989)
pCLM1001	<i>sln1 ΔC1</i> (Δ527–540) HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1002	<i>sln1 ΔC2</i> (Δ544–568) HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1021	<i>sln1-C/EBP</i> HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1033	<i>sln1-C/EBP+1</i> HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1034	<i>sln1-C/EBP+2</i> HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1035	<i>sln1-C/EBP+3</i> HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pCLM1036	<i>sln1-C/EBP+4</i> HIS3, <i>CEN</i> note: <i>SLN1</i> is myc tagged	pRS313 (Sikorski and Hieter, 1989)
pHL581	GST- <i>Sln1</i> kinase (537–947) = <i>Sln1K</i>	pGEX-KG (Guan and Dixon, 1991)
pADA881	GST- <i>Sln1</i> kinase (537–947) T550I = <i>Sln1K*</i>	pGEX-KG (Guan and Dixon, 1991)
pADA847	GST- <i>Sln1</i> kinase (570–947) = <i>Sln1KΔCC</i>	pGEX-KG (Guan and Dixon, 1991)

Strain	Description	Vector, references
pM1032	GST-sln1-C/EBP (C/EBP followed by Sln1570-947)	PGEX-KG (Guan and Dixon, 1991)
pCLM1038	GST-sln1-C/EBP+1 (C/EBP+1 followed by Sln1570-947)	pGEX-KG (Guan and Dixon, 1991)
pCLM1037	GST-sln1-C/EBP+3 (C/EBP+3 followed by Sln1570-947)	pGEX-KG (Guan and Dixon, 1991)