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Yeast Skn7p functions in a eukaryotic two-component regulatory pathway

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Previous analysis of the amino acid sequence of Skn7p, the product of the yeast *SKN7* gene, revealed a potential 'receiver motif' homologous to that found in bacterial response regulators (signal-transducing effector proteins regulated by phosphorylation at a conserved aspartate residue corresponding to position D427 in Skn7p). We determined the effects of D427N and D427E mutations in Skn7p. The D427N substitution resulted in diminished activity in four independent *in vivo* assays of Skn7p function, while the D427E mutation enhanced Skn7p activity in these assays. Our results are consistent with predictions based on the bacterial two-component paradigm and provide experimental evidence that a receiver motif functions in regulating the activity of Skn7p in a eukaryote. Skn7p suppressed growth defects associated with a *pkc1Δ* mutation, raising the possibility that *PKC1* might play a role in regulating Skn7p. However, epistasis experiments indicate that Skn7p does not appear to function directly downstream of the *PKC1–MAP* kinase pathway. Rather, Skn7p may function in a two-component signal transduction pathway that acts in parallel with the *PKC1* cascade to regulate growth at the cell surface. We present evidence suggesting that Skn7p serves as a transcription factor in such a signaling pathway.

Key words: *PKC1*/transcription factor/two-component signal transduction pathway/yeast Skn7p

Introduction

Cells cope with a wide variety of environmental conditions by using numerous adaptive responses to appropriately tailor their physiologies. The study of such response systems has defined common signaling pathways that have been adapted and modified to function in a variety of specific contexts. In bacteria, the signal transduction pathways of many response systems utilize the basic signaling circuitry of the 'two-component regulatory systems'. Over 100 such systems have been cataloged in bacteria, and each involves at least two crucial protein components: a 'sensor' protein (often associated with the cytoplasmic membrane) that directs the activity of a cytoplasmic effector protein referred to as a 'response regulator' (Kofoid and Parkinson, 1988; Stock *et al.*, 1990; Parkinson and Kofoid, 1992).

Some of these two-component systems have been characterized in considerable detail, revealing that phosphorylation provides the biochemical mechanism of communication between each sensor and its cognate response regulator (Bourret *et al.*, 1991). The sensor component in each of these systems functions as an auto-phosphorylating histidine protein kinase whose activity is regulated in response to some specific stimulus. Communication between a sensor kinase and its cognate response regulator involves phosphotransfer, an event that results in phosphorylation of the response regulator at a specific highly conserved aspartate residue. Phosphorylation regulates the activity of the response regulator; mutations that alter the phosphorylation site result in a loss of this regulation (Sanders *et al.*, 1989, 1992; Bourret *et al.*, 1990; Jin *et al.*, 1990; Stewart *et al.*, 1990; Klose *et al.*, 1993).

Amino acid sequence comparisons have identified a conserved 'communication domain' in the large superfamily of response regulators (Volz, 1993); this ~120 amino acid segment is referred to as the 'receiver motif', and it spans the conserved aspartate phosphorylation site. In many two-component systems, the response regulator protein serves as a transcription factor whose activity is regulated by phosphorylation at the conserved aspartate position (Stock *et al.*, 1989b, 1990; Parkinson and Kofoid, 1992). In such systems, each response regulator is composed of two distinct functional domains: a receiver domain fused to a DNA binding domain.

Although there are many examples attesting to the utility of the two-component signaling paradigm in bacteria, only recently have there been indications that such systems might also operate in eukaryotes. Support for this idea arises primarily from modest sequence similarities between bacterial two-component proteins and potential counterparts in a variety of eukaryotes, including *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Neurospora crassa*, *Dictyostelium discoideum* (Alex and Simon, 1994; Swanson and Simon, 1994) and the plastid genome sequences of eukaryotic algae (Reith and Munholland, 1993). For example, recent work has identified: Skn7p, a potential response regulator homolog from *S.cerevisiae* (Brown *et al.*, 1993); Sln1p and Ssk1p, the sensor kinase and cognate response regulator components of an osmoregulation system in *S.cerevisiae* (Ota and Varshavsky, 1993; Maeda *et al.*, 1994); and Etr1p, a protein from *A.thaliana* that has segments corresponding to both bacterial sensor kinases and response regulators (Chang *et al.*, 1993). While such sequence similarities are intriguing, there has been little direct information about the functional roles proposed for the putative two-component motifs. In initiating the work described here, we sought to obtain experimental evidence that the receiver domain of Skn7p plays a functional role in regulating the activity of this protein. Such evidence would greatly strengthen the proposal that

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putative eukaryotic homologs of bacterial two-component signaling proteins participate in signaling pathways similar to the bacterial two-component systems.

Analysis of the deduced amino acid sequence of Skn7p indicated that it may be a eukaryotic homolog of bacterial response regulators and that it may function as a transcription factor in *S.cerevisiae* (Figure 1a). A segment in the N-terminal half of Skn7p appears identical to the highly conserved DNA binding domain of heat-shock transcription factors (Sorger and Pelham, 1988; Clos *et al.*, 1990; Schuetz *et al.*, 1991), and a glutamine-rich segment resembling a transcription activation domain (Carey, 1991) is located at the C-terminus. A distinct region of Skn7p appears to comprise an orthodox two-component receiver domain (Parkinson and Kofoid, 1992) that includes positions D384, D427, T455 and K477, corresponding to elements that are conserved in a large family of response regulators and thought to play key roles in signal transduction (Stock *et al.*, 1990; Volz, 1993). D427 corresponds to the conserved phosphorylation site in the response regulator family (Sanders *et al.*, 1989, 1992; Jin *et al.*, 1990). Here, we report that D427 plays a role in modulating Skn7p activity and provide evidence that this modulation affects the ability of Skn7p to mediate transcriptional activation.

Results

D427 mutations affect transcriptional activation mediated by Skn7p

We tested the idea that Skn7p serves as a transcription factor that is subject to regulation involving its receiver motif. Plasmids were constructed to direct expression of a hybrid fusion protein in which Skn7p was fused to the DNA binding domain of the yeast Gal4 protein (Figure 1b). We tested the *in vivo* ability of the Gal4−Skn7p fusion to activate transcription of a chromosomal *lacZ* reporter gene containing *GAL4* DNA binding sites (Song *et al.*, 1991). As shown in Figure 1b, expression of the Gal4p DNA binding domain alone failed to activate transcription of the reporter; however, the Gal4−Skn7p fusion protein caused significant expression of the reporter gene resulting in a level of β-galactosidase activity that was approximately five times the background level observed in the same host strain expressing the Gal4p fragment alone. This result demonstrates that Skn7p can activate transcription when it is bound to DNA by virtue of its attachment to the Gal4p DNA binding domain.

To test whether the two-component receiver domain of Skn7p could modulate its ability to activate transcription, we also quantified expression of the *lacZ* reporter gene directed by the Gal4−Skn7 fusion protein when the Skn7 segment carried a D427N or D427E mutation (see Materials and methods). The conservative asparagine substitution replaces the carboxyl group of Asp427 with an amino group which cannot be phosphorylated in two-component systems, a substitution predicted to result in a loss of signaling activity (Parkinson and Kofoid, 1992). The D427E mutation is also expected to eliminate phosphorylation of the receiver domain, but has the potential to force the receiver domain into a constitutively activated conformation in the absence of any phosphorylation (Klose *et al.*, 1993). The Skn7p D427E mutation enhanced the ability of the fusion protein to activate transcription, as indicated by significantly higher β-galactosidase activity: a 7-fold increase relative to that observed with the Gal4−Skn7p wild-type construct. The Skn7p D427N mutation reduced the ability of the fusion protein to activate transcription of the reporter gene by ~40% relative to that of the wild-type fusion protein. Although small, this difference was reproducible and exceeded the standard error associated with these measurements.

We confirmed the results described above by using another system to construct a hybrid fusion protein that drove expression of a different reporter gene. We constructed a fusion between Skn7p and the DNA binding domain of LexA and then determined the ability of this plasmid-encoded fusion to stimulate expression of a *HIS3* reporter gene. In this case, the level of expression of the reporter gene was assessed by determining the ability of cells to grow in the absence of histidine on minimal medium in the presence of 3-aminotriazole (3AT), an inhibitor of the *HIS3* gene product (Vojtek *et al.*, 1993). Our results (Figure 2) indicated that Skn7p enabled higher expression of the reporter than was observed with the starting LexA plasmid. Furthermore, the D427E mutation enhanced the ability of the LexA−Skn7p fusion to stimulate *HIS3* expression, while the D427N mutation reduced this ability.

D427 mutations affect the ability of Skn7p to suppress the growth defects of a kre9Δ mutant

In work reported previously (Brown *et al.*, 1993), we identified and cloned the yeast *SKN7* gene as a consequence of its ability to suppress the severe growth defect of a *S.cerevisiae kre9*Δ mutant strain; this mutation causes defective extracellular matrix assembly (Brown and Bussey, 1993). Here, we used this suppressor phenotype as an additional *in vivo* test for function of the Skn7p receiver domain. We assayed the abilities of wild-type Skn7p, Skn7p D427N and Skn7p D427E to suppress the growth defect of a *kre9*Δ*skn7*Δ strain (Figure 3) when expressed from single-copy plasmids. The Skn7p D427N mutant was unable to suppress the growth defect exhibited by the *kre9*Δ*skn7*Δ mutant. Indeed, there was no observable difference between the cells expressing Skn7p D427N and those carrying the vector only, indicating that under these conditions the D427N mutation diminishes Skn7p activity significantly. Control experiments, using immunoblotting to detect Skn7p, demonstrated that the D427N mutation did not appear to affect the expression or stability of Skn7p (data not shown).

Colonies of *kre9*Δ*skn7*Δ cells transformed with a single-copy plasmid directing expression of Skn7p D427E were found to grow noticeably and reproducibly faster than isogenic cells transformed with a plasmid encoding wild-type Skn7p (Figure 3). Thus, Skn7p D427E supported growth at a rate intermediate between the rate enabled by suppression of the *kre9*Δ defect by wild-type Skn7p and that exhibited by a wild-type *KRE9* strain. The enhanced suppressor activity exhibited by Skn7p D427E could indicate that this mutation causes hyperactivity of Skn7p, presumably through constitutive phosphorylation-independent activation of the protein.

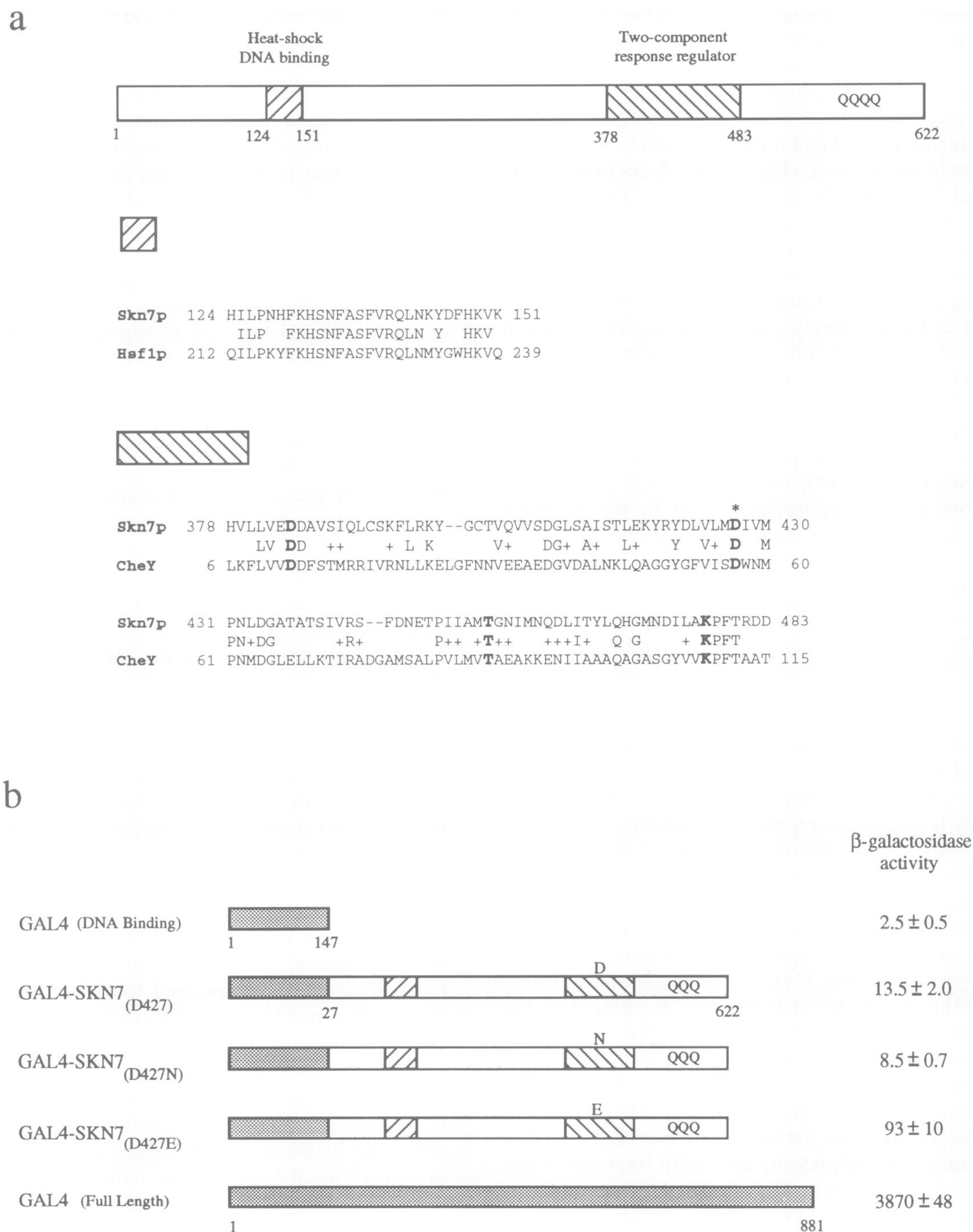

Fig. 1. Proposed functional domains of Skn7p. (**a**) Schematic representation of the domain organization of Skn7p (622 amino acids; 70 kDa) showing regions of the protein which are homologous to eukaryotic heat-shock transcription factors or prokaryotic two-component response regulators, and the approximate position of a glutamine-rich tract (QQQQ). Alignment of the predicted amino acid sequence of Skn7p (between residues 124 and 151) shown in single-letter code with the amino acid sequence of the DNA binding domain of the yeast heat-shock transcription factor, Hsf1p (Sorger and Pelham, 1988), (between residues 212 and 239). Sequence alignment of the orthodox receiver domain of Skn7p (residues 378–483) with CheY (Parkinson and Kofoid, 1992) (residues 6–115). Highly conserved amino acids found in receiver domains (positions D384, D427, T455 and K477 in Skn7p) are in bold, with an asterisk at D427 indicating the putative phosphorylation site, by analogy with CheY (Sanders *et al.*, 1989), NtrC (Sanders *et al.*, 1992) and VirG (Jin *et al.*, 1990). Similar amino acid residues (defined as K~R; L~I~M~V; E~Q~D~N; S~T~A~G) used in the alignments are indicated by a '+'. (**b**) Transcriptional regulation of Skn7p through its two-component receiver domain. Skn7p was tested for its ability to function as a transcriptional activator using a hybrid protein in which Skn7p (amino acid residues 27–622; open box) was fused to the DNA binding domain of the yeast Gal4 protein (Song *et al.*, 1991; residues 1–147; shaded box). This Gal4–Skn7 fusion protein was assayed *in vivo* for its ability to activate transcription of a *lacZ* reporter gene. Gal4–Skn7 hybrid fusion proteins containing mutations predicted to eliminate phosphorylation of D427 within the receiver domain and result in either reduced Skn7p function (D427N) or enhanced activity (D427E), were also tested *in vivo* for their effects on the transcription of *lacZ*. β-Galactosidase activity is expressed in Miller units (Miller, 1972).

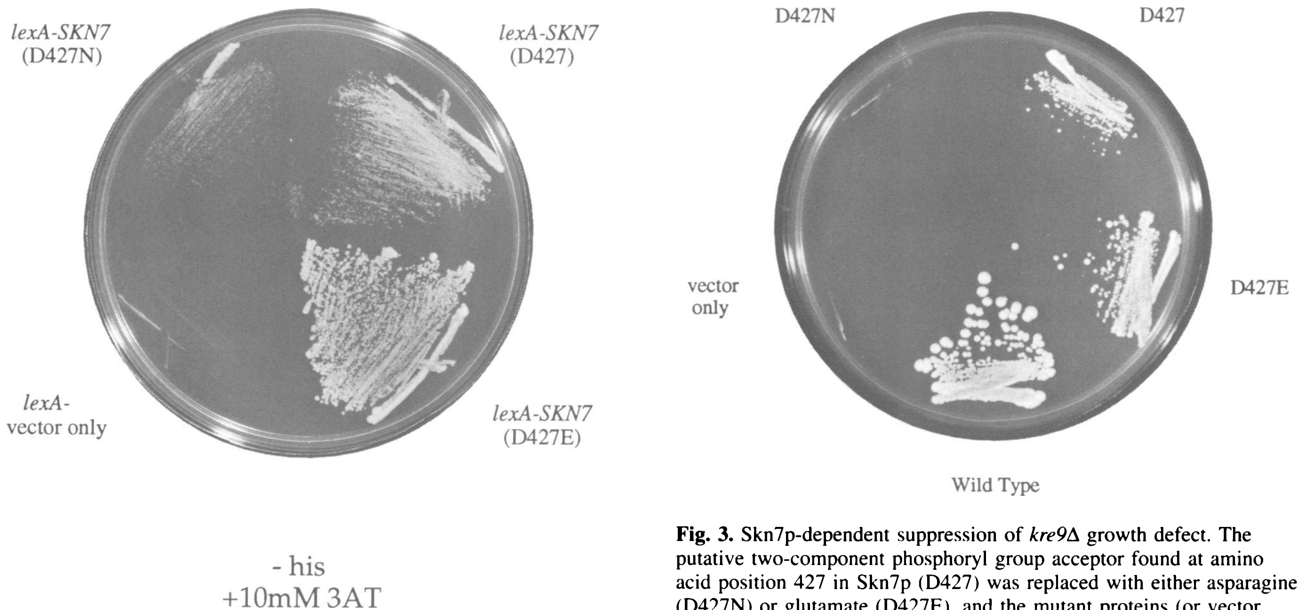

Fig. 2. Transcriptional activation of *HIS3* reporter by LexA−Skn7p fusions. Hybrid proteins containing the DNA binding domain of the *E.coli* LexA protein (amino acid residues 1–211) fused to Skn7p (residues 27–622) were tested for transcriptional activation of a *HIS3* reporter gene under the control of tandemly inserted *lexA* operators. Colonies transformed with plasmids directing the expression of either the LexA vector only (*lexA*-vector only), a LexA−Skn7p fusion containing a D427N mutation [*lexA−SKN7* (D427N)], a LexA−Skn7p wild-type protein [*lexA−SKN7* (D427)] or a LexA−Skn7p D427E mutant [*lexA−SKN7* (D427E)] were streaked onto minimal medium lacking histidine containing 10 mM 3AT (see Materials and methods). Under these conditions, growth rate is sensitive to the level of expression of the *HIS3* reporter gene.

Fig. 3. Skn7p-dependent suppression of *kre9*Δ growth defect. The putative two-component phosphoryl group acceptor found at amino acid position 427 in Skn7p (D427) was replaced with either asparagine (D427N) or glutamate (D427E), and the mutant proteins (or vector only) were assayed functionally for their ability to suppress the growth defect of a *kre9*Δ*skn7*Δ strain. A wild-type parental *KRE9 SKN7* strain (Wild Type) harboring a vector with no insert has been included for growth rate comparisons. Single-copy centromeric pRS316 plasmids containing fragments encoding either wild-type Skn7p, Skn7p D427N, Skn7p D427E or the vector with no insert, were transformed into *kre9*Δ*::HIS3 skn7*Δ*::TRP1* cells and uracil prototrophs selected following growth on casaminoacid medium (Sherman *et al.*, 1986) containing galactose at 30°C. Individual colonies were streaked onto casaminoacid medium containing glucose and incubated at 30°C for 4 days to test for Skn7p function. The *kre9* growth defect is most severe during growth on medium containing glucose as the carbon source, and can be partially restored on galactose medium in a manner that is not presently understood (J.Brown and H.Bussey, unpublished results; M.Ciriacy, personal communication).

Skn7p can suppress the growth defect caused by loss of PKC1

The yeast *PKC1* gene encodes a protein kinase C homolog that mediates a signal transduction cascade important for regulating growth and cell surface assembly in yeast (Paravicini *et al.*, 1992; Errede and Levin, 1993). Mutations in components of this protein kinase cascade lead to a lysis phenotype which can be prevented by the presence of osmotic support in the growth medium. We explored the possibility that *SKN7* could suppress the defects responsible for lysis in a *pkc1*Δ strain, as *SKN7* encodes a phosphorylated transcription factor (see below) first isolated as a multicopy suppressor of a mutation causing cell wall defects (Brown *et al.*, 1993). Figure 4 shows that overproduction of wild-type Skn7p, Skn7p D427N and Skn7p D427E from multicopy plasmids restored viability to a *pkc1*Δ mutant on media without osmotic support, while cells harboring the vector alone were not viable in the absence of osmotic support. Furthermore, when *pkc1*Δ cells were transformed with a low-copy centromere-based plasmid, the lysis defect was suppressed only in cells expressing the Skn7p D427E mutant protein, consistent with the D427E mutation causing hyperactivity of Skn7p.

The ability of Skn7p to suppress the *pkc1*Δ lysis defect provides independent support for Skn7p acting as a response regulator in a signal transduction pathway involved in regulating extracellular matrix assembly. To address whether Skn7p functioned downstream of *PKC1* in such a pathway, we constructed an *skn7*Δ*pkc1*Δ double mutant. Tetrad analysis of a diploid strain heterozygous for both mutations revealed that *skn7*Δ*pkc1*Δ double mutants were not viable even on media containing osmotic support, indicating that the effect of the double mutation is more severe than either single mutation. Overexpression of wild-type Skn7p, Skn7p D427N or Skn7p D427E failed to suppress *slk1*Δ (*bck1*Δ) mutations (Costigan *et al.*, 1992) and *mkk1*Δ*mkk2*Δ double mutations (Errede and Levin, 1993). In addition, no significant differences in the level of Skn7p phosphorylation were observed between *PKC1* and *pkc1*Δ strains for wild-type Skn7p, or the D427N and D427E mutants. These results indicate that Skn7p is unlikely to function downstream of the MAP kinase arm of the *PKC1* cascade, which includes Slk1p (Bck1p), Mkk1p/Mkk2p and Mpk1p as downstream components (Errede and Levin, 1993).

*Skn7p does not suppress growth defects caused by an sln1*Δ *mutation*

The recently identified *SLN1* gene is predicted to encode a yeast homolog of bacterial two-component sensory protein histidine kinases (Ota and Varshavsky, 1993), and is thought to direct the activity of a response regulator called Ssk1p (Maeda *et al.*, 1994). To explore the possibility that Skn7p may also serve as a response regulator of Sln1p, we constructed a deletion in the *SLN1* gene and asked whether overproduction of Skn7p could suppress

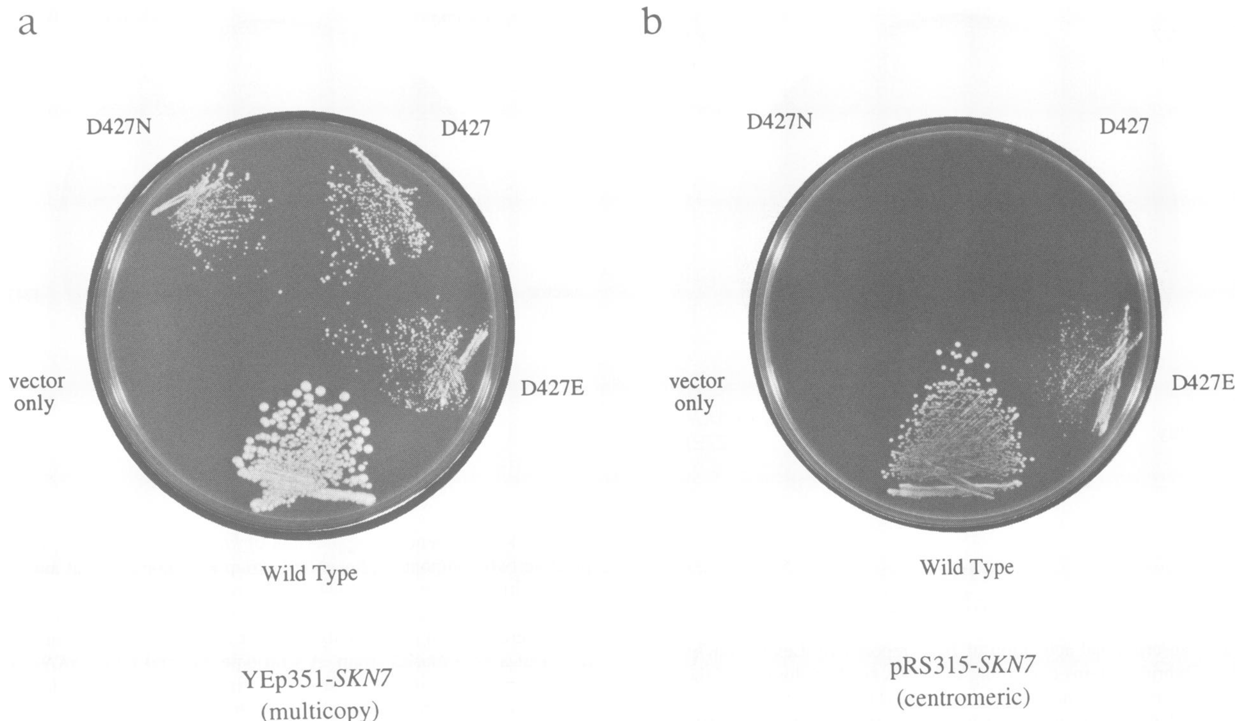

Fig. 4. Skn7p-dependent suppression of *pkc1*Δ osmotic lysis defect. (**a**) Yeast cells harboring a deletion in the *PKC1*-encoded protein kinase (Paravicini *et al.*, 1992) transformed with multicopy plasmids directing expression of wild-type Skn7p (D427), Skn7p D427N, Skn7p D427E or the YEp351 vector alone. Transformants were grown on selective media containing 1 M KCl prior to being streaked onto media without osmotic support to assess whether overproduction of Skn7p could suppress the lysis defect of *pkc1*Δ strains. (**b**) Wild-type Skn7p (D427) and the (D427N) and (D427E) substitution mutations tested for their ability to suppress *pkc1*Δ strains when present on low-copy centromere-based pRS315 plasmids. Parental *PKC1* strains (Wild Type) harboring either a YEp351 vector only, or a pRS315 vector only, have been included on appropriate plates for growth rate comparisons.

either of two growth defects associated with an *sln1*Δ mutation: (i) the lethality when growth is attempted on rich medium, or (ii) the slow growth on minimal medium. Overexpression of wild-type Skn7p, Skn7p D427N or Skn7p D427E from multicopy plasmids had no effect on either phenotype in our *sln1*Δ strain. While these experiments do not rigorously rule out the possibility that Sln1p and Skn7p interact, the inability of the constitutively active D427E mutant protein to affect the growth properties of the *sln1*Δ mutant suggests that Slnlp and Skn7p probably function in distinct signal transduction pathways.

In vivo phosphorylation of Skn7p

Phosphorylation of aspartate residues and the role of this modification in triggering conformational transitions in eukaryotic proteins have been described previously (Jencks, 1980; Tanford, 1984). Phospho-aspartate differs from the serine, threonine and tyrosine phospho-amino acids more commonly encountered in eukaryotic cells in that it readily hydrolyzes under mild conditions (Fujitaki and Smith, 1984; Martensen, 1984). We sought direct evidence for phospho-aspartate in Skn7p by immunoprecipitating Skn7p from [^{32}P]phosphate-labeled whole-cell lysates (Figure 5). The results indicated that Skn7p was phosphorylated, but no significant difference was detected in the level of [^{32}P]phosphate associated with wild-type Skn7p compared with the Skn7p D427N and Skn7p D427E mutants. Most of the labeled phosphate covalently attached to Skn7p survived boiling and was found to be acid-stable but base-labile (data not shown),

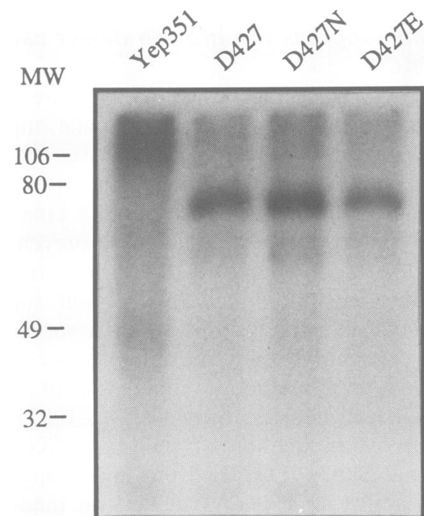

Fig. 5. *In vivo* phosphorylation of Skn7p. Autoradiograph of SDS−PAGE gel shows phosphorylation patterns of wild-type Skn7p, Skn7p D427N and Skn7p D427E. Epitope-tagged proteins were immunoprecipitated from [^{32}P]orthophosphate-labeled cell lysates derived from cultures of *skn7*Δ cells harboring multicopy YEp351 plasmids. Immunoprecipitation products were subject to SDS−PAGE under conditions expected to preserve phosphoaspartate prior to autoradiography. MW, molecular weight markers in kDa; Yep351, wild-type yeast harboring the YEp351 vector only; D427, D427N and D427E, epitope-tagged wild-type D427N or D427E Skn7p proteins, respectively.

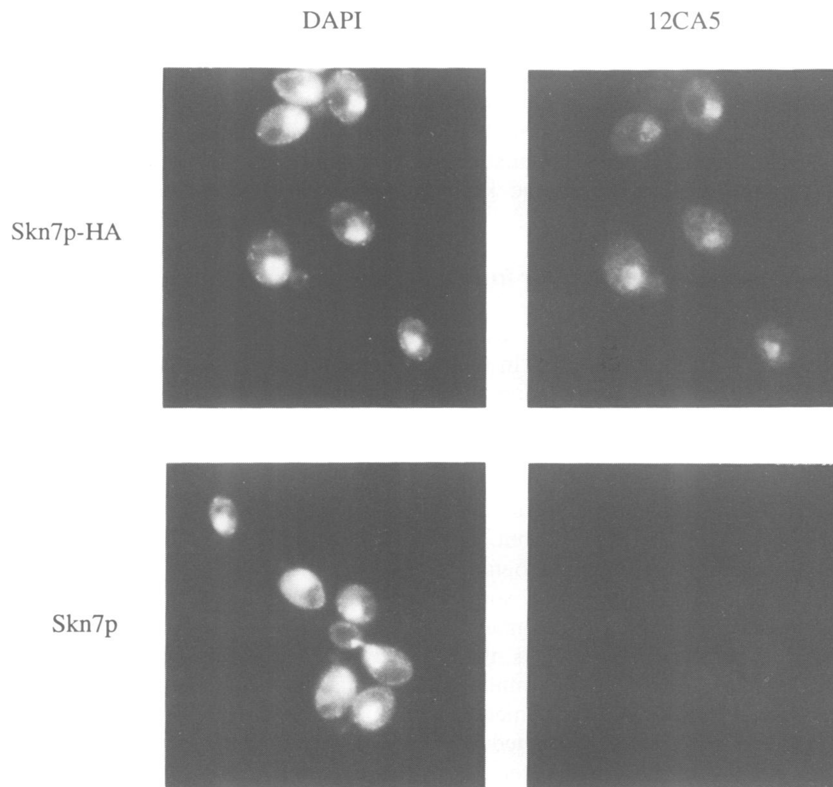

Fig. 6. Localization of Skn7p by immunofluorescence microscopy. Wild-type yeast strains harboring plasmids encoding either (upper panels) epitope-tagged Skn7p constructs, or (lower panels) native Skn7p were treated as described previously (Pringle *et al.*, 1991) and stained with DAPI and the 12CA5 mAb. Cells expressing epitope-tagged Skn7p (Skn7p–HA) viewed under excitation wavelengths for DAPI to visualize DNA (top left), or Texas Red (top right) to indicate the subcellular localization of Skn7p. (Bottom left) DAPI or (bottom right) Texas Red images of wild-type cells expressing untagged Skn7p. Wild-type (Skn7p) or epitope-tagged Skn7p (Skn7p–HA) used for localization studies were expressed from YEp351 multicopy plasmids. Images were obtained using a Zeiss Axioplan Microscope and represent ~2000-fold magnification.

consistent with phosphorylation of serine and/or threonine (Martensen, 1984). Skn7p contains 17 potential serine/threonine phosphorylation sites and a possible tyrosine phosphorylation site (Y100). Phosphorylation at one or more of these sites would complicate detection of a relatively unstable phospho-aspartyl residue. Our results raise the possibility that in addition to regulation involving the conserved aspartate at the receiver domain, Skn7p may be regulated by phosphorylation at other sites. *In vivo* phosphorylation of Skn7p was not affected noticeably by a *pkc1* deletion (data not shown).

Nuclear localization of Skn7p

In many bacterial systems, response regulators serve as transcriptional regulators (Stock *et al.*, 1989b; Parkinson and Kofoid, 1992). For Skn7p to function as a transcription factor in a eukaryotic two-component pathway, it must be capable of being targeted to the nucleus. To test whether Skn7p meets this requirement, we used indirect immunofluorescence microscopy to observe its subcellular localization (Figure 6). Skn7p was found to localize predominantly to the nucleus, as indicated by the co-localization of antibody staining with the DNA binding dye 4′,6-diamidino-2-phenyl-indole (DAPI; Figure 6 upper panels). Skn7p was found in the nucleus at all stages of the cell cycle (Figure 6 and data not shown), although some cytoplasmic staining was also observed in some cells (Figure 6 top right). Analysis of the Skn7p D427N and D427E mutants under similar conditions indicated that these mutant proteins also entered the nucleus (data not shown). This result suggests that the nuclear targeting of Skn7p is independent of the regulation involving D427. The nuclear and cytoplasmic localization pattern of Skn7p in cells demonstrates the potential of the protein to transduce cytoplasmic signals into the nucleus.

Discussion

Transmitter and receiver motifs are common in bacteria and enable interactions between specific protein pairs (sensor kinases and response regulators, respectively) to promote phosphotransfer reactions. These interactions comprise the central signaling events in a wide variety of sensory transduction systems. We and others have speculated that two-component signaling pathways may also operate in yeast and other eukaryotes (Brown *et al.*, 1993; Chang *et al.*, 1993; Ota and Varshavsky, 1993; Alex and Simon, 1994; Maeda *et al.*, 1994; Swanson and Simon, 1994). These proposals initially resulted from the apparent conservation of receiver and transmitter motifs in proteins encoded by genes isolated from eukaryotic organisms. The presence of these motifs in eukaryotes raises several questions about their fundamental functional roles in these systems. (i) Do these motifs play important roles in signal transduction or have the motifs been preserved primarily

as a mechanism for specific protein–protein contacts? (ii) Do the eukaryotic receiver and transmitter motifs participate in phosphotransfer reactions to accomplish signal transduction in the same basic way as their prokaryotic counterparts? This report describes the characterization of one eukaryotic response regulator, Skn7p, and the effects of mutations that alter the key regulatory site of its receiver motif.

Skn7p functions as a transcriptional regulator in a one-hybrid system

Three of our results suggest that Skn7p serves as a transcription factor: (i) Skn7p is located primarily in the yeast nucleus; (ii) when linked to the DNA binding domain of Gal4p or LexA, Skn7p activates *in vivo* transcription of reporter genes; and (iii) a segment of Skn7p appears identical to the highly conserved DNA binding domain of heat-shock transcription factors. In view of these results, it is reasonable to envisage Skn7p serving as a transcriptional activator by using its DNA binding motif to recognize specific promoter elements and then recruiting some additional component(s) of the transcription machinery. This recruitment would utilize the same contacts that allow the Gal4–Skn7p and LexA–Skn7p to stimulate transcription of reporter genes. In these experiments, recognition of the reporter gene promoter was directed by the DNA binding domain of the heterologous protein. It seems likely that the DNA binding motif in the N-terminal half of Skn7p would enable Skn7p to recognize and activate transcription of specific genes; however, we emphasize that we have yet to identify a specific recognition sequence. Thus, formally it remains possible that Skn7p participates in regulating the expression of genes recognized by a distinct DNA binding protein, not by Skn7p itself.

Regulation of Skn7p activity involves D427 of the receiver motif

Although we have yet to identify the downstream target gene(s) of Skn7p, we do have several ways of assaying the *in vivo* activity of this protein: (i) suppression of growth defects associated with a deletion of *KRE9*; (ii) suppression of the lysis phenotype that results from deletion of *PKC1*; (iii) activation of transcription of a *lacZ* reporter gene when Skn7p is fused to the Gal4p DNA binding domain; and (iv) activation of transcription of a *HIS3* reporter gene by Skn7p fused to the DNA binding domain of LexA. In each of these assays our results indicated that D427 of Skn7p plays an important role in regulating the activity of the protein: Skn7p D427N had diminished activity relative to wild-type Skn7p, and Skn7p D427E had enhanced activity. The correlation between the results for suppression of growth defects and those for activation of transcription suggests that these four assays are measuring a common activity of Skn7p. In interpreting these results within the framework of the two-component systems, diminished activity is expected for the D $\rightarrow$ N mutant if phosphorylation of Skn7p would result in its activation. The diminished activity of Skn7p D427N is consistent with the wild-type protein being partially activated under the growth conditions of our assays.

The hyperactivity observed with Skn7p D427E could result from either an enhanced phosphorylation level for the mutant protein or, alternatively, constitutive phosphorylation-independent activation of the protein. The former possibility appears unlikely in view of the effects of D $\rightarrow$ E substitutions in a variety of bacterial response regulators: for CheY (Bourret *et al.*, 1990), CheB (Stewart *et al.*, 1990), VirG (Pazour *et al.*, 1992), Spo0A (Green *et al.*, 1991) and NtrC (Klose *et al.*, 1993), analogous D $\rightarrow$ E substitution mutants were incapable of being phosphorylated. Interestingly, the NtrC D54E substitution was shown recently to cause activation of NtrC (Klose *et al.*, 1993) in a manner that mimicked the normal activation associated with phosphorylation of the aspartate residue at position 54 (corresponding to D427 in Skn7p). However, this activation was only observed *in vitro*: it did not cause a detectable *in vivo* phenotype. Our result with Skn7p D427E appears to represent the first system in which this substitution causes observable constitutive activity *in vivo*.

The effects of the D427E substitution can be interpreted in greater detail assuming the receiver domain of Skn7p adopts a 3-D structure similar to that of CheY (Stock *et al.*, 1989a; Volz and Matsumura, 1991). The additional aliphatic carbon present in the side chain of glutamate (compared with aspartate) may serve to alter the configuration of the 'acid pocket' of Skn7p formed in part by the carboxylate groups of D384, D427 and either E383 or D385. In particular, the interaction of the carboxyl group of D427 with the ε-amino group of a key conserved lysine residue (corresponding to K477 in Skn7p) might be affected by this substitution in a way that imparts an activated conformation to the receiver domain. Perturbation of this interaction has been implicated in the activated conformation caused by a D $\rightarrow$ K mutation in the acid pocket of CheY (Bourret *et al.*, 1990, 1993). Regardless of the details underlying the effects of substitutions at D427 of the Skn7p, two of our results strongly suggest that Skn7p activity is regulated in a manner similar to that observed with prokaryotic response regulators: (i) the Skn7p receiver domain clearly plays an important role in modulating the activity of Skn7p; and (ii) the D $\rightarrow$ N and D $\rightarrow$ E mutations at the predicted phosphorylation site of the receiver motif have predictable and opposite effects on Skn7p activity.

Signaling pathway(s) utilizing Skn7p

Our results indicate that Skn7p functions as a transcription factor that is subject to modulation by a two-component signal transduction pathway. We propose that this modulation enables it to regulate expression of specific genes, perhaps those involved in cell wall biosynthesis. Phosphorylation of Skn7p at D427 could potentially influence the ability of the protein to recognize/bind to its recognition sequence or could alter the ability of Skn7p to recruit other transcription factors to specific promoters. A mutation (D427N) expected to disrupt the two-component phosphotransfer circuitry results in a less active version of this protein. However, Skn7p can still operate in the absence of activation of its receiver domain; Skn7p D427N has some activity in each of our assays of Skn7p function. These results suggest that the proposed two-component pathway is not essential for Skn7p function, but rather serves to modulate the activity of the protein.

At present, the cognate sensor kinase protein which lies upstream of Skn7p and the nature of the stimulus to which it responds remain unknown. In yeast only one potential homolog of the family of bacterial sensor kinases has been identified to date: Sln1p, which appears to interact with Ssk1p in a two-component system that responds to changes in osmolarity (Ota and Varshavsky, 1993; Maeda *et al.*, 1994). Several results argue against any direct connection between Skn7p and Sln1p. For example, deletion of *SLN1* strongly impairs cell growth (Ota and Varshavsky, 1993), while deletion of *SKN7* has no observable phenotype (Brown *et al.*, 1993). Furthermore, overexpression of Skn7p (wild-type, D427N or D427E mutants) did not suppress the growth defects associated with an *sln1* null mutation. The simplest interpretation of these results is that Sln1p is not the cognate kinase of Skn7p. However, because the signaling circuitry involving these components may not be linear, our results cannot formally exclude the possibility of an interaction between Sln1p and Skn7p. An alternative explanation would suggest that in addition to Sln1p, another two-component histidine kinase homolog is present in yeast.

Skn7p may plausibly function by receiving extracellular signals through a two-component phosphorylation system following the bacterial paradigm, and then migrating into the nucleus where it acts as a transcription factor to control gene expression. Unfortunately, examination of the *in vivo* phosphorylation of Skn7p did not provide direct evidence for phosphorylation at position D427, but these data did suggest that control of Skn7p activity may be complex, involving a regulatory pathway(s) in addition to the proposed two-component pathway. Specifically, our results indicated the phosphorylation of Skn7p at serine and/or threonine. We have little information about this additional phosphorylation, its role in regulation and the nature of the kinase responsible. However, our results do suggest that the *PKC1–MAP* kinase cascade is not directly responsible for Skn7p phosphorylation, nor is this cascade likely to be involved in regulating Skn7p activity. Thus, the signaling pathway(s) involving Skn7p may act in concert with the *PKC1* protein kinase cascade to coordinate extracellular signals which regulate cell surface assembly.

Signal transduction circuits containing proteins with modular body plans are characteristic of bacterial two-component systems (Stock *et al.*, 1990; Parkinson and Kofoid, 1992). The eukaryotic homologs of two-component sensor proteins, Etr1p and Sln1p, appear to have conserved the modular domain organization shared by the prokaryotic sensor kinases (Chang *et al.*, 1993; Ota and Varshavsky, 1993; Alex and Simon, 1994). The response regulator proteins of two-component systems also exhibit modular domain organization in which phospho-accepting receiver domains have been coupled to, and therefore regulate, different adjunct domains that carry out a variety of different functions (Parkinson and Kofoid, 1992). The yeast Skn7 protein appears to have such a modular organization, resulting in the coupling of a two-component receiver motif to an element that stimulates transcription.

Materials and methods

Strains, plasmids and procedures

Yeast and bacterial strains were grown and maintained as described previously (Bussey *et al.*, 1982). Yeast transformations, genetic manipulations and gene disruptions followed established protocols (Ito *et al.*, 1983; Sherman *et al.*, 1986). Multicopy shuttle plasmids used for suppression experiments were derived from YEp351 or YEp352, and single-copy plasmids from pRS315 or pRS316. Plasmid pGBT9 for the construction of Gal4–Skn7p fusions (Song *et al.*, 1991) and yeast strain YBP2 (*UAS_{GAL}–GAL1–lacZ leu2 his3*) were kindly provided by S.Fields (SUNY at Stony Brook); plasmid pBTM116 (from S.Fields and P.Bartel) was used to construct LexA–Skn7p fusions (Vojtek *et al.*, 1993) which were tested in host strain L40 [*MATa his3 trp1 leu2 ade2 LYS2::$(lexAop)_4$–HIS3 URA3::$(lexop)_8$–lacZ GAL4*], kindly provided by S.Hollenberg (F.Hutchinson Cancer Research Center). The Gal4–Skn7p hybrid contained the DNA binding domain of yeast Gal4p fused in-frame to Skn7p (amino acids 27–622). The LexA–Skn7p hybrid contained the DNA binding domain of LexA (amino acids 1–211) fused in-frame to Skn7p (amino acids 27–622). Both of these fusions were constructed using the *Pst*I site near the 5′ end of *SKN7* and unique *Pst*I sites in vectors pGBT9 and pBTM116.

The *kre9Δskn7Δ* strain was constructed as described previously (Brown *et al.*, 1993) by crossing isogenic haploid strains of opposite mating type harboring either *kre9::HIS3* or *skn7::TRP1* disruptions in the SEY6210 genetic background. The resulting double-heterozygous diploid colonies were subject to sporulation and tetrad analysis to obtain a haploid *kre9Δskn7Δ* strain. A similar approach was used to obtain an *skn7Δpkc1Δ* double mutant using strain GPY1115 harboring a *pkc1::HIS3* deletion in the SEY6211 background (Paravicini *et al.*, 1992), kindly provided by G.Paravicini (Glaxo Institute, Geneva, Switzerland). An *sln1Δ::LEU2* fragment was created by PCR amplification of the *SLN1* coding sequences spanning codons 37–320, and replacement of the 386 bp *Sac*I–*Hin*dIII *SLN1* segment within the PCR fragment by a *LEU2* restriction fragment with compatible ends (Jones and Prakash, 1990). This *sln1Δ::LEU2* fragment was then introduced into a diploid SEY6210 strain (Brown and Bussey, 1993) by single-step gene replacement (Rothstein, 1983), and correct integrations confirmed by Southern analysis. An *sln1Δ::LEU2* heterozygous diploid strain was then either transformed with Yep352-based plasmids expressing Skn7p wild-type, Skn7p (D427N) or Skn7p (D427E), or transformed with the YEp352 vector only. Representative colonies were then subject to sporulation and tetrad dissection onto appropriate medium to assay haploid *sln1Δ::LEU2* spore progeny for *SKN7*-dependent growth suppression.

Site-directed mutagenesis was performed as described previously (Kunkel *et al.*, 1987) to change the codon for Asp427 in *SKN7* to either asparagine or glutamate [oligonucleotides D427N (GTTTTAATGAATATTGTTATG) and D427E (GATTTGGTATTAATGGAAATTGTTATG) were purchased from the Regional DNA Synthesis Centre, University of Calgary]. The presence of each mutation was confirmed by DNA sequencing (Sanger *et al.*, 1977). A DNA fragment (820 bp *Dra*III–*Esp*I) encoding the D427N or D427E substitution was used to replace the corresponding region of wild-type *SKN7* in each of the plasmids.

β-Galactosidase activities were determined using ONPG essentially as described by Dolan and Fields (1990), except that protein extracts derived from whole-cell lysates were used in place of permeabilized cells. Values reported are the averages of three experiments from at least two independent transformations and are expressed in Miller units (Miller, 1972).

Subcellular localization of Skn7p

Immunofluorescent localization of Skn7p made use of epitope-tagged Skn7p which we call Skn7p–HA. The epitope tag was introduced using synthetic complementary oligonucleotides with cohesive *Pst*I termini; the oligonucleotides were phosphorylated, annealed and then ligated into the unique *Pst*I restriction site located between codons 28 and 29 in *SKN7*, generating an in-frame insertion of nine amino acids, an epitope which is recognized by mAb 12CA5 (Babco). Constructs carrying a single copy of the inserted fragment in the correct orientation were identified by DNA sequencing. The Skn7p–HA fusion was as effective as wild-type Skn7p when tested for the ability to suppress the growth defect in *kre9Δskn7Δ* cells. Exponentially growing diploid yeast cells were fixed with 5% formaldehyde and treated for immunofluorescence microscopy using standard techniques as described (Pringle *et al.*, 1991).

In vivo phosphorylation of Skn7p

Immunoprecipitations (Harlowe and Lane, 1988) were performed using the 12CA5 mAb and epitope-tagged versions of wild-type or mutant Skn7p. *skn7Δ* cells harboring appropriate YEp351-based Skn7p plasmids were grown to mid-log phase in low phosphate medium (Haguenauer-Tsapis and Hinnen, 1984), and ~10^8 cells were labeled with 250 mCi of [^{32}P]orthophosphate for 15 min at 30°C. All subsequent procedures were

carried out at 4°C in an effort to avoid hydrolysis of phosphoaspartate. Cells were harvested by centrifugation, then lysed with glass beads in the presence of protease and phosphatase inhibitors. Immunoprecipitated complexes were disrupted by the addition of SDS-containing sample buffer prior to electrophoresis on SDS–polyacrylamide gels (10%) which were also run at 4°C. Gels were dried and subjected to autoradiography for ~10 h.

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