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The eukaryotic response regulator Skn7p regulates calcineurin signaling through stabilization of Crz1p

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To survive ionic, pH and pheromone stress, the yeast *Saccharomyces cerevisiae* activates signaling through the Ca^{2+}-activated phosphatase calcineurin to the transcription factor Crz1p/Tcn1p. We show that the overexpression of *SKN7*, a response-regulator transcription factor, activates transcription from a calcineurin/Crz1p-dependent response element (CDRE). Ca^{2+}-induced, calcineurin/Crz1p-dependent activation of several genes is reduced in *skn7* mutants. Skn7p modulates CDRE-dependent transcription by affecting Crz1p protein levels. Specifically, the rate of Crz1p turnover is increased in *skn7* mutants. Calcineurin, but not its phosphatase activity, is required for Skn7p-mediated Crz1p stabilization. Skn7p binds to both calcineurin and Crz1p *in vitro*, and we suggest that this interaction is required for Skn7p regulation of Crz1p. The DNA-binding and internal coiled-coil domains, but not the response-regulator phosphorylation of Skn7p, are necessary for Crz1p-dependent transcriptional activation and Crz1p stabilization by Skn7 *in vivo*. The DNA-binding domain of Skn7p is also required for binding to Crz1p and calcineurin *in vitro*. Thus, we propose that Skn7p protects Crz1p from degradation by binding to it and calcineurin through its DNA-binding domain.

Keywords: calcineurin/Crz1/response regulator/*Saccharomyces cerevisiae*/Skn7

Introduction

Many different physiological conditions elicit changes in intracellular Ca^{2+} concentration. Calcineurin (PP2B), a Ca^{2+}- and calmodulin-dependent Ser/Thr phosphatase, is conserved from yeast to higher eukaryotes. Calcineurin regulates a large variety of processes in mammalian cells including cardiac hypertrophy (Molkentin *et al*., 1998), cardiac valve and skeletal muscle development (Chin *et al*., 1998; de la Pompa *et al*., 1998; Ranger *et al*., 1998), and neutrophil chemotaxis (Hendey and Maxfield, 1993; Lawson and Maxfield, 1995). Calcineurin-mediated regulation of the immune system has been particularly well studied in part by the use of FK506 and cyclosporin A, immunosuppressant drugs that inhibit the catalytic activity of calcineurin (J.Liu *et al*., 1991). Specifically, calcineurin regulates T-cell activation by dephosphorylating the transcription factor NF-AT (Clipstone and Crabtree, 1992; O'Keefe *et al*., 1992), which permits it to enter the nucleus and initiate transcription (Jain *et al*., 1993; Northrop *et al*., 1993).

In the budding yeast *Saccharomyces cerevisiae*, calcineurin allows the cell to adapt to environmental stress. Mutants lacking the genes encoding either the calcineurin catalytic subunits *CNA1* and *CNA2* (Cyert *et al*., 1991; Y.Liu *et al*., 1991), or the regulatory subunit *CNB1* (Kuno *et al*., 1991; Cyert and Thorner, 1992) are viable under normal growth conditions, but die in the presence of high concentrations of different ions including Mn^{2+}, Na^+, Li^+ and OH^- (Nakamura *et al*., 1993; Mendoza *et al*., 1994; Farcasanu *et al*., 1995; Pozos *et al*., 1996), as well as in the presence of the cationic aminoglycoside hygromycin B (Withee *et al*., 1998). Calcineurin mutants also lose viability during prolonged exposure to mating pheromone (Moser *et al*., 1996; Withee *et al*., 1997). These calcineurin mutant phenotypes are rescued by overexpression of the zinc finger transcription factor Crz1p/Tcn1p (Matheos *et al*., 1997; Stathopoulos and Cyert, 1997). Calcineurin directly dephosphorylates Crz1p, which then translocates to the nucleus after Ca^{2+} stimulation. Crz1p binds directly to a 24 bp DNA element from the β1-3 glucan synthase *FKS2* promoter, the calcineurin-dependent response element (CDRE) (Stathopoulos and Cyert, 1997; Stathopoulos-Gerontides *et al*., 1999). In addition to *FKS2*, calcineurin and Crz1p (Matheos *et al*., 1997; Stathopoulos and Cyert, 1997) activate the transcription of numerous genes including *PMR2*, a Na^+ ATPase (Rudolph *et al*., 1989; Haro *et al*., 1991), and *PMC1* and *PMR1*, both Ca^{2+} ATPases (Rudolph *et al*., 1989; Cunningham and Fink, 1994, 1996; Mendoza *et al*., 1994; Mazur *et al*., 1995). To identify other molecules that might regulate calcineurin signaling, we screened for genes which, when overexpressed, activate calcineurin/Crz1p-dependent transcription. In this way we isolated Skn7p, a response-regulator transcription factor.

Skn7p shows homology to bacterial response-regulator proteins (Brown *et al*., 1994; Morgan *et al*., 1995), which function in two-component systems with histidine kinases (reviewed in Parkinson, 1993; Loomis *et al*., 1998). In a typical prokaryotic two-component signaling pathway, a sensor histidine kinase, located at the cell membrane, autophosphorylates on a histidine residue upon stimulation. This phosphate is then transferred to an aspartyl residue on the receiver protein, which often, but not always, contains a DNA-binding domain and initiates transcription. Skn7p has been shown to participate in yeast two-component signaling through its canonical response-regulator aspartate residue (Asp427) with the histidine kinase Sln1p and the small phospho-transfer protein Ypd1p (Ketela *et al*., 1998; Li *et al*., 1998). Skn7p also contains a coiled-coil domain, which is necessary for several protein–protein interactions (Alberts *et al*., 1998; Bouquin *et al*., 1999).

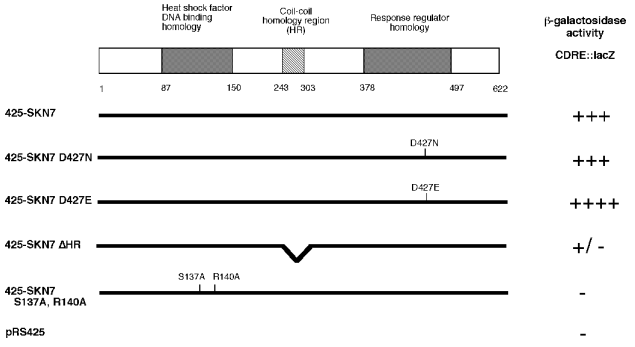

Fig. 1. Schematic diagram of Skn7p with alleles used to identify regions of Skn7p important for CDRE::*lacZ* activation. The HR region (see text) and the DNA-binding domain are essential for activation of the CDRE::*lacZ* by the overexpression of Skn7p. Multicopy plasmids were transformed into KWY242, containing the 2×CDRE::*lacZ* reporter gene. β-galactosidase activity was assessed by plate assay. All plasmids are derivatives of pRS425. 425-*SKN7ΔHR* is pKW47; 425-*SKN7 S137A, R140A* is pKW37. Western blot analysis revealed approximately equal levels of expression from these plasmids (data not shown).

Skn7p functions in a multiplicity of pathways, including cell wall stress, oxidative stress, heat stress and the cell cycle, although its role in this variety of processes is not completely understood. First, Skn7p helps the cell to respond to cell wall stress. Skn7p has genetic and biochemical interactions with cell wall synthesis genes (Brown *et al*., 1993) and members of a PKC-mediated cell-integrity pathway (Levin and Bartlett-Heubusch, 1992; Alberts *et al*., 1998), and transcribes at least one cell wall synthesis gene, *OCH1* (S.Li and J.S.Fassler, in preparation). Secondly, Skn7p also plays a role in adaptation to oxidative stress; the mutants are sensitive to hydrogen peroxide (Krems *et al*., 1996), and Skn7p induces the transcription of many genes upon exposure to oxidative stress (Kuge and Jones, 1994; Morgan *et al*., 1997; Lee *et al*., 1999). Thirdly, Skn7p responds to heat stress. *skn7* mutants are sensitive to acute heat stress and Skn7p contributes to the transcription of several heat shock proteins (Raitt *et al*., 2000). Finally, in the cell cycle, Skn7p has also been shown to activate G_1 transcriptional events in the absence of normal G_1–S regulatory transcription factors (Morgan *et al*., 1995; Bouquin *et al*., 1999).

Thus, Skn7p plays a part in numerous biological stress responses. In this paper we further elucidate a role for Skn7p in stress-activated signal transduction. We present biochemical and genetic evidence that Skn7p affects calcineurin-mediated signaling and physiology. Specifically, Skn7p modulates calcineurin-dependent transcriptional output *in vivo*, by affecting the rate of Crz1p turnover. Furthermore, we show that Skn7p binds to both Crz1p and calcineurin *in vitro*, suggesting that Skn7p modulates Crz1p stability through direct protein–protein interactions. Thus, these studies identify a novel activity of Skn7p as a regulator of protein stability and establish a new mechanism for the regulation of calcineurin/Crz1p-dependent transcription. We also demonstrate for the first time a phosphatase-independent role for the calcineurin protein.

Table I. Multicopy SKN7 activates the CDRE::*lacZ* reporter gene

	CDRE::*lacZ* β-galactosidase activity[a]			
	Wild type[b]	*crz1Δ*[c]	*cnb1Δ*[d]	Wild type[e] + FK520
Vector[f]	–	–	–	–
2μ *CRZ1*	++++	++++	++++	++++
2μ *SKN7*	+++	–	–	+++
2μ *SWI5*	+++	+++	+++	+++

[a]β-galactosidase activity was determined by plate assay.
[b]KWY242.
[c]ASY834.
[d]ASY461 and KWY246.
[e]ASY459 and KWY242; plates contain 2 μg/ml FK520.
[f]Vector, YEP351; 2μ *CRZ1*, pAMS435; 2μ *SKN7*, pRS425-*SKN7* and library plasmids; 2μ *SWI5*, M1485 (D.Stillman) and library plasmids. None of the plasmids identified in the screen activated the mutant CDRE::*lacZ* reporter.

Results

Identification of SKN7 as a multicopy enhancer of calcineurin- and Crz1p-dependent transcription

A wild-type strain containing a previously characterized CDRE fused to the *lacZ* gene (Stathopoulos and Cyert, 1997) (KWY242) was transformed with two multicopy genomic libraries to identify genes whose overexpression caused increased calcineurin-dependent transcription (see Materials and methods). To determine whether the reporter gene activity resulted specifically from calcineurin- and Crz1p-dependent transcription, the plasmids were re-transformed into strains with a mutant version of the CDRE as well as strains that lacked either the calcineurin regulatory subunit, Cnb1p, or the transcription factor Crz1p. For each plasmid isolated, the open reading frame (ORF) responsible for reporter gene activation was identified by subcloning.

Five plasmids contained *SKN7*. Skn7p is a previously characterized response-regulator protein with a DNA-binding domain homologous to heat shock factor (HSF) (Brown *et al*., 1993) (Figure 1). Multicopy *SKN7* did not activate the CDRE::*lacZ* reporter gene in calcineurin (*cna1Δcna2Δ* or *cnb1Δ*) or *crz1Δ* mutant strains, indicating that the Skn7p-mediated increase in CDRE::*lacZ* activity reflected signaling through the calcineurin pathway (Table I). Only one other gene, *SWI5*, was identified multiple times. *SWI5*, however, activated the CDRE::*lacZ* reporter gene independently of both calcineurin and Crz1p (Table I). Swi5p, like Crz1p, is a C_2H_2 zinc finger protein whose DNA-binding domain is almost identical to that of Crz1p. Thus, when present at high levels, Swi5p likely binds to the CDRE and activates transcription. Similarly, when overexpressed, Swi5p activates transcription of genes regulated by Ace2p, another transcription factor with a DNA-binding domain highly related to that of Swi5p (Dohrmann *et al*., 1992). Swi5p was not characterized further. Additional plasmids that were only isolated once will not be discussed further in this paper. These plasmids all had similar phenotypes in that they caused an increase in CDRE::*lacZ* activity in the wild-type strain, but not in the *cnb1Δ* or *crz1Δ* strains, or in the wild type + FK520. None of the plasmids identified in the screen activated the mutant CDRE::*lacZ* reporter gene. Because

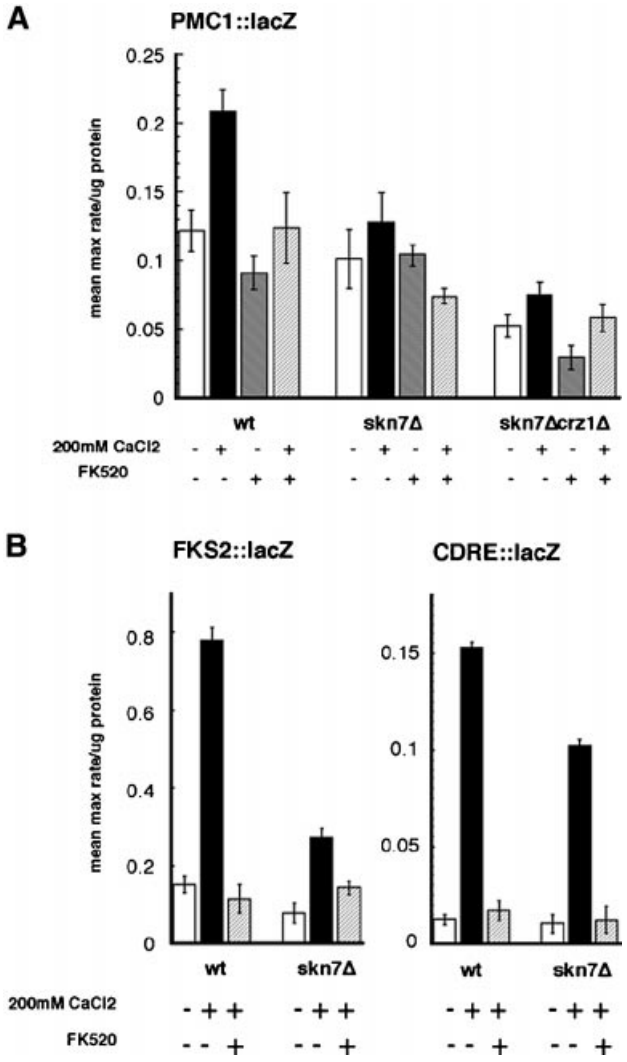

Fig. 2. *skn7Δ* strains can not fully activate Crz1p- and calcineurin-dependent transcription in response to Ca^{2+}. Strains contain plasmid-based reporter genes (**A**) *PMC1::lacZ* (pAMS381), (**B**) *FKS2::lacZ* (pAMS317) and *CDRE::lacZ* (pAMS342) (Stathopoulos and Cyert, 1997). β-galactosidase activities are shown for wild-type (YPH499), *skn7Δ* (KWY266) and *skn7Δcrz1Δ* (KWY289) cell extracts, in either the presence or absence of 200 mM $CaCl_2$ and 2 μg/ml FK520. For each condition, three cell extracts were assayed three times each; the SD is the error between the samples.

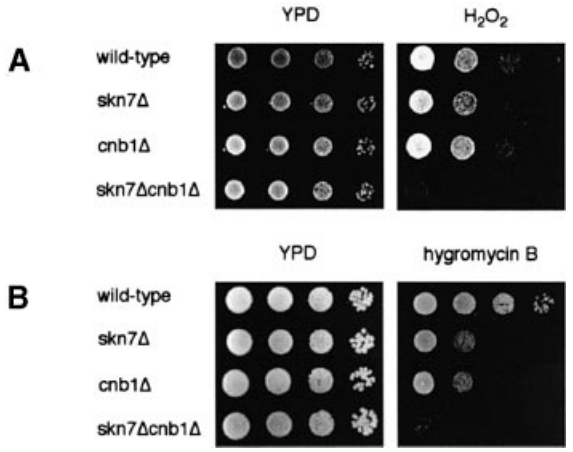

Fig. 3. Skn7p and calcineurin interact genetically. (**A**) Deletion of *CNB1* exacerbates the hydrogen peroxide sensitivity of the *skn7Δ* strain. (**B**) *skn7Δcnb1Δ* mutants are more hygromycin B sensitive than *skn7Δ* and *cnb1Δ* strains. Wild-type (YPH499), *skn7Δ* (KWY266), *cnb1Δ* (DD12) and *skn7Δcnb1Δ* (KWY267) strains were grown to saturation, then diluted to an OD_{600} of 1. Ten-fold serial dilutions were spotted onto YPD plates with either no added chemicals, 1.8 mM hydrogen peroxide (A), or 70 μg/ml hygromycin B (B), and grown at 30°C for 1–3 days.

of its frequency of identification, Skn7p was further characterized by examining the effects of the *skn7* mutation on calcineurin-dependent transcription.

skn7Δ reduces Ca^{2+}-, calcineurin- and Crz1p-dependent transcription

Ca^{2+} stimulates the expression of many genes in a calcineurin- and Crz1p-dependent fashion, including the P-type ATPases *PMC1* and *PMR2* (Rudolph *et al*., 1989; Haro *et al*., 1991; Cunningham and Fink, 1994, 1996), as well as the β1-3 glucan synthase *FKS2* (Mazur *et al*., 1995; Matheos *et al*., 1997; Stathopoulos and Cyert, 1997). The effect of Skn7p on calcineurin signaling was measured by examining *pmc1::lacZ* and *fks2::lacZ* reporter gene activity in different strain backgrounds. FK520, a calcineurin phosphatase inhibitor (Liu *et al*., 1992), was used to examine the calcineurin dependence of this transcription. Skn7p was required for the full Ca^{2+} activation of these genes in a calcineurin- and Crz1p-dependent fashion (Figure 2). The reduced expression of *pmc1::lacZ* observed in the *skn7Δcrz1Δ* strain was equivalent to its expression in the *crz1Δ* strain (data not shown). Similar effects were observed with a *pmr2::lacZ* reporter gene (data not shown). In addition, the CDRE/Crz1p binding region of the *FKS2* promoter was sufficient for this effect, as a decrease in Ca^{2+}-activated transcription of a CDRE(24 bp)::*lacZ* reporter gene was seen in the *skn7Δ* strain (Figure 2B).

Skn7p and calcineurin interact genetically

We further explored the relationship between Skn7p and calcineurin by examining the phenotypes of *skn7Δcnb1Δ* double mutants. Skn7p is required for the response to oxidative stress, and *skn7* mutants are sensitive to several oxidizing agents including hydrogen peroxide (Krems *et al*., 1996) (Figure 3A). In contrast, *cnb1Δ* mutants exhibit no sensitivity to hydrogen peroxide (Figure 3A). However, the *skn7Δcnb1Δ* strain was dramatically more sensitive to hydrogen peroxide than the *skn7Δ* strain, suggesting that calcineurin does participate in the oxidative stress response *in vivo* in combination with Skn7p.

Interestingly, previous studies have established that both calcineurin and Skn7p exhibit genetic interactions with the PKC pathway, which mediates the response to cell wall stress (Heinisch *et al*., 1999). Calcineurin mutants are synthetically lethal with *pkc1* mutants and other downstream members of the Pkc1p-regulated MAP kinase cascade (Garrett-Engele *et al*., 1995). In addition, overexpressing a constitutively active calcineurin allele ameliorates the *pkc1Δ* growth defect on low osmolarity media (Garrett-Engele *et al*., 1995). *skn7* mutations are

similarly synthetically lethal with *pkc1Δ*, and overexpressing Skn7p rescues a *pkc1Δ* cell lysis defect (Brown *et al*., 1994). To identify possible interactions between calcineurin and Skn7p with respect to cell wall stress, we tested their effects on hygromycin B sensitivity. Several mutants that have defects in cell wall synthesis or structure exhibit sensitivity to this drug (Dean, 1995; Lussier *et al*., 1997), and calcineurin and *skn7* mutants are both sensitive to hygromycin B (Withee *et al*., 1998; S.Li and J.S.Fassler, in preparation) (Figure 3B). Notably, the *skn7Δcnb1Δ* strain is significantly more sensitive than either single mutant (Figure 3B). Thus, Skn7p and calcineurin act synergistically to provide hygromycin B resistance. Together, these phenotypic observations indicate that calcineurin and Skn7p participate in similar physiological functions and work together to regulate the yeast response to several different types of stress.

Skn7p stabilizes Crz1p

These genetic observations, as well as the effect of Skn7p on calcineurin-dependent transcription, suggested that Skn7p and calcineurin could be acting together in a signal transduction pathway. Several approaches were used to determine the mechanism of the Skn7p effect on calcineurin signaling. First, electrophoretic mobility shift assays (EMSA) were performed to determine whether Skn7p could bind to the CDRE DNA element. As previously reported (Stathopoulos and Cyert, 1997), a DNA–protein complex containing Crz1p was observed when extracts were incubated with oligonucleotides containing the CDRE sequence. However, the mobility of this complex was identical in extracts of wild-type and *skn7Δ* cells and was unaffected by addition of Skn7p-specific antiserum (data not shown). Thus, Skn7p does not appear to bind to the CDRE. Secondly, green fluorescent protein (GFP) microscopy was also used to test the effect of Skn7p on GFP–Crz1p localization. As reported previously, adding Ca^{2+} causes GFP–Crz1p to translocate to the nucleus in a calcineurin-dependent manner (Stathopoulos-Gerontides, 1999). The deletion or overexpression of Skn7p had no discernible effect on Crz1 protein localization, either in the absence of presence of Ca^{2+} (data not shown).

Finally, we did observe an effect of Skn7p on Crz1 protein levels. In whole-cell extracts, multicopy *SKN7* increased and *skn7Δ* decreased Crz1 protein levels, respectively (Figure 4A). In contrast, calcineurin protein levels were unaffected by Skn7p (data not shown). Surprisingly, Skn7p had no effect on *CRZ1* mRNA levels (data not shown), thus we investigated the effect of Skn7p on Crz1 protein stability. Crz1 protein levels were examined at a series of time intervals after incubation of wild-type and *skn7Δ* cells with cycloheximide. Crz1p in the wild-type strain was stable even 90 min after cycloheximide treatment, but Crz1 protein declined more rapidly in the *skn7Δ* strain, and exhibited significantly reduced levels after 45 min (Figure 4B).

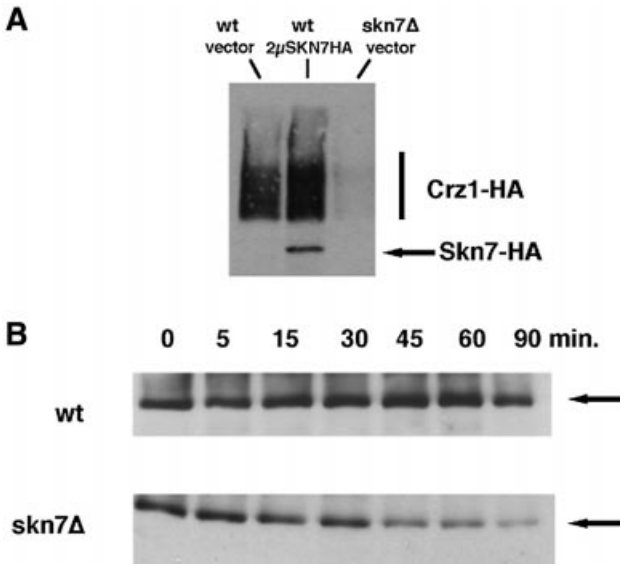

Fig. 4. Skn7p stabilizes the Crz1 protein. (**A**) The overexpression of Skn7p results in more Crz1 protein; the deletion of Skn7p results in less Crz1 protein. Whole-cell extracts from strains either overexpressing or deleting Skn7 [YPH499 with YEP352, YPH499 with YEP352-*SKN7-HA*, *skn7Δ::TRP1* (KWY266)] were subjected to SDS–PAGE and anti-HA western analysis. All strains contain pRS315-*CRZ1-HA* (pAMS450). These results were reproduced using anti-Crz1p antibodies to examine endogenous Crz1p levels (data not shown). (**B**) Crz1p is less stable in *skn7Δ*. Samples were taken at the indicated times after the addition of 100 μg/ml cycloheximide from both wild type (YPH499) and *skn7Δ* (KWY266), and subjected to anti-Crz1p western analysis.

Calcineurin, but not its phosphatase activity, is required for Skn7p-mediated Crz1p stabilization

The finding that Skn7p regulates Crz1p at the level of protein turnover led us to analyze further potential regulators of Skn7p-mediated Crz1p stability. As phosphorylation often affects transcription factor protein stability (reviewed in Hochstrasser and Kornitzer, 1998), we analyzed the effect of calcineurin on Skn7p-mediated Crz1p stabilization. The overexpression of Skn7p caused an increase in Crz1 protein level in a wild-type background. However, when this experiment was performed in a *cnb1Δ* strain, the Crz1p levels not only failed to increase, but actually decreased (Figure 5). This observation is consistent with the fact that multicopy *SKN7* failed to activate the CDRE::*lacZ* in a calcineurin mutant strain (Table I). To understand better the role of calcineurin in Skn7p-mediated Crz1p stability, the effects of a calcineurin inhibitor were analyzed. FK520 is a pharmaceutical closely related to FK506 that inhibits the phosphatase activity of calcineurin (Liu *et al*., 1992). In all previously reported cases, the addition of FK506 or FK520 causes wild-type cells to acquire calcineurin mutant phenotypes (Foor *et al*., 1992; Breuder *et al*., 1994). However, in this case, multicopy *SKN7* did activate the expression of CDRE::*lacZ* in strains grown with FK520, in contrast to its failure to activate the same reporter construct in the *cnb1Δ* mutant strain (Table I). This difference was not caused by a dosage- or drug-specific effect as either increasing the concentration of FK520 or using cyclosporin A, another calcineurin inhibitor, produced similar results (data not shown). In addition, these treatments were effective in reducing the activation of CDRE::*lacZ* by other plasmids isolated from this genetic screen (data not shown). Furthermore, extracts of FK520-treated cells expressing multicopy *SKN7* displayed increased Crz1 protein levels (Figure 5), whereas adding FK520 in the absence of

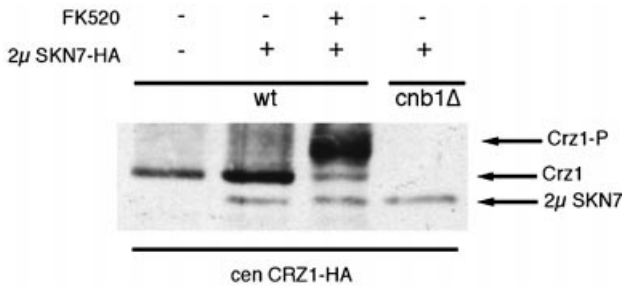

Fig. 5. Calcineurin, but not its phosphatase activity, is essential for Skn7p to stabilize Crz1p. Anti-HA western blot analysis of either wild-type (YPH499) or *cnb1Δ* (DD12) strains containing the plasmids pAMS450, YEP352 or YEP352-*SKN7-HA*, with or without FK520 (2 μg/ml).

multicopy *SKN7* did not cause increased Crz1p levels (data not shown). These findings are consistent with the observation that multicopy *SKN7* increased CDRE::*lacZ* activity in the presence of FK520 (Table I). Notably, Crz1p displayed the reduced mobility that represents its phosphorylated form in extracts of FK520-treated cells (Figure 5) (Stathopoulos-Gerontides *et al*., 1999). This confirms that FK520 treatment effectively inhibits calcineurin phosphatase activity. Thus, Skn7p stabilized Crz1p in the absence of calcineurin phosphatase activity caused by the inhibitor FK520, but not in the absence of the calcineurin B subunit gene product (*cnbl1Δ*). This suggests that the physical presence of the calcineurin polypeptides, as opposed to their phosphatase activity, is essential for Skn7p-mediated Crz1p stabilization.

Skn7p, Crz1p and calcineurin interact physically

The fact that the presence of calcineurin was required for maintenance of Crz1 protein levels suggested that protein–protein interactions might be required for Skn7p-dependent Crz1p stabilization. Several experiments were performed to test for interactions between calcineurin, Crz1p and Skn7p. We were unable to detect such interactions by co-immunoprecipitation, but Skn7p from yeast extracts bound weakly to recombinant glutathione *S*-transferase (GST)–calcineurin immobilized on glutathione-Sepharose beads (data not shown). To develop a more sensitive assay for Skn7p, Crz1p and calcineurin protein–protein interactions, two additional experiments were performed. First, to determine whether Skn7p binds to Crz1p and/or calcineurin, recombinant GST–Crz1p, GST–calcineurin or GST purified from *Escherichia coli* was immobilized on glutathione-Sepharose beads and incubated with recombinant His_6-tagged Skn7p (see Materials and methods). Western analysis indicated that Crz1p and calcineurin, but not GST, bound to Skn7p (Figure 6A). Secondly, in the reverse experiment, His_6-tagged Skn7p was immobilized on Ni^+-NTA-agarose beads, and incubated with either purified GST, Crz1p or calcineurin. Immunoblotting revealed that again calcineurin and Crz1p, but not the non-specific protein GST, interact with Skn7p (Figure 6B). These results indicate that *in vitro* Skn7p forms a specific interaction with both calcineurin and Crz1p.

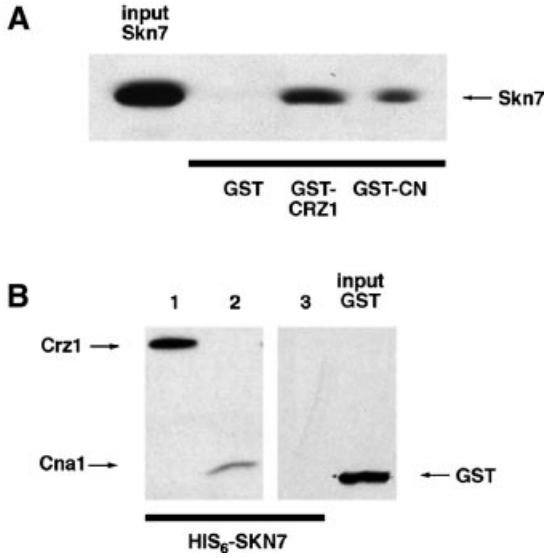

Fig. 6. Skn7p binds to calcineurin and Crz1p *in vitro*. (**A**) Immobilized GST–Crz1p and GST–calcineurin, but not GST, can bind Skn7p. GST, GST–Crz1p and GST–calcineurin were purified from *E.coli* on glutathione-Sepharose, and then incubated with recombinant His_6-Skn7p. Following washing, bound proteins were eluted and fractionated by SDS–PAGE. Skn7p was detected using anti-His_4 antibody. (**B**) Immobilized His_6-Skn7p binds calcineurin and Crz1p, but not GST. His_6-Skn7p was purified on Ni^+-NTA resin and then incubated with recombinant GST–Crz1p (lane 1), GST–calcineurin (lane 2), or GST (lane 3). Samples were processed as in (A), except that proteins were detected using anti-GST antibody.

A functional Skn7p DNA-binding domain is required for transcriptional, stability and protein–protein interaction effects

A functional Skn7p DNA-binding domain is essential for its ability to stimulate transcription. Two residues that are essential for DNA binding have been structurally determined in the DNA-binding domain of HSF (Hubl *et al*., 1994), which is highly homologous to the DNA-binding domain of Skn7p. Mutations in the analogous Skn7p residues (S137A, R140A) eliminate activation of the Skn7p-dependent promoter elements in *OCH1* (S.Li and J.S.Fassler, in preparation). Interestingly, these two residues in the Skn7p DNA-binding domain proved essential for all aspects of Skn7p-mediated effects on calcineurin/Crz1p signaling. First, the multicopy *SKN7 S137A,R140A* plasmid completely failed to activate the CDRE *in vivo* (Figure 1). Secondly, *SKN7 S137A,R140A* completely failed to complement the low levels of Crz1p in the *skn7Δ* strain (Figure 7A). Finally, recombinant Skn7p with DNA-binding domain mutations (S137A, R140A) consistently failed to bind to either GST–Crz1p or GST–calcineurin (Figure 7B). Therefore, these residues are essential for Skn7p function in Crz1p- and calcineurin-dependent transcription, Skn7p stabilization of Crz1 protein, and Skn7p binding to calcineurin and Crz1p.

The Skn7p homology region (HR) is required for CDRE activation and Crz1p stabilization

The coiled-coil (HR) region in the center of the Skn7 protein is required for its binding to both the Mbp1p transcription factor (Bouquin *et al*., 1999) and to Rho1p

(Alberts *et al*., 1998), a GTPase that activates Pkc1p as well as other targets (Nonoka *et al*., 1995). This part of the protein is essential for most Skn7p functions as a protein lacking this region fails to complement most *skn7Δ* phenotypes (Alberts *et al*., 1998). We consequently tested the *SKN7ΔHR* allele for its ability to affect CDRE::*lacZ* transcription, Crz1p stabilization, and binding to Crz1p and calcineurin. When multicopy *SKN7ΔHR* was transformed into the CDRE::*lacZ*-expressing strain, it failed to activate transcription (Figure 1). In addition, this allele was tested for its effects on Crz1 protein stability. *SKN7ΔHR* also failed to complement the low Crz1 protein levels observed in extracts of a *skn7Δ* strain (Figure 7A). These two observations indicated that the coiled-coil HR domain of Skn7p is essential for its ability both to activate calcineurin-dependent transcription and to stabilize Crz1p *in vivo*. Surprisingly, when this allele was tested for *in vitro* biochemical interaction with both calcineurin and Crz1p, purified recombinant Skn7ΔHRp bound as well as the wild-type Skn7p allele (Figure 7B) (see Discussion).

Skn7p response-regulator phosphorylation is not required for CDRE activation, Crz1p stabilization, or binding to Crz1p

In response-regulator proteins, a canonical aspartate residue is phosphorylated by a histidine kinase. The phosphorylation of this aspartate residue in Skn7p (Asp427) is not essential for all Skn7p functions in yeast. Several experiments were performed to determine whether Skn7p response-regulator aspartate phosphorylation is required to mediate Crz1p stabilization. First, to test the effects of this residue on CDRE::*lacZ* reporter gene activation, multicopy *SKN7 D427N*, an unphosphorylatable allele, and *SKN7 D427E*, an allele that mimics aspartate phosphorylation, were transformed into the KWY242 wild-type CDRE::*lacZ* reporter strain to compare their activities with wild-type *SKN7*. The activated *D427E* allele showed greater reporter activity than the wild-type allele. However, the *D427N* construct resulted in reporter gene activity indistinguishable from wild type, indicating that Asp427 is not required for increased CDRE::*lacZ* expression (Figure 1).

Secondly, these two alleles were tested for their effects on Crz1p stabilization. Plasmids containing either the *SKN7 D427N* or the *SKN7 D427E* allele were transformed into the *skn7Δ* strain. Western blot analysis was performed to determine whether either allele would complement the reduced levels of Crz1 protein displayed by the *skn7* mutant. The levels of Crz1p in the strains expressing either *SKN7 D427N* or wild-type *SKN7* were equivalent, indicating that for Crz1 protein stability, as for CDRE::*lacZ* reporter activation, response-regulator aspartate phosphorylation is not required (Figure 7A). Interestingly, slightly higher Crz1 protein levels were consistently observed in the D427E-expressing strain, indicating that this residue may play some functional role in Crz1p stability, although it is not required to complement the *skn7Δ* strain (Figure 7A).

Finally, the Skn7p D427N and D427E alleles were tested for their ability to bind *in vitro* to recombinant calcineurin and Crz1p. Binding of Skn7p D427E to both GST–Crz1p and GST–calcineurin was equivalent to that of wild-type Skn7p (Figure 7B). Skn7p D427N also bound to GST–Crz1p comparably to wild-type Skn7p. In contrast, Skn7p D427N showed reduced binding to GST–calcineurin (Figure 7B).

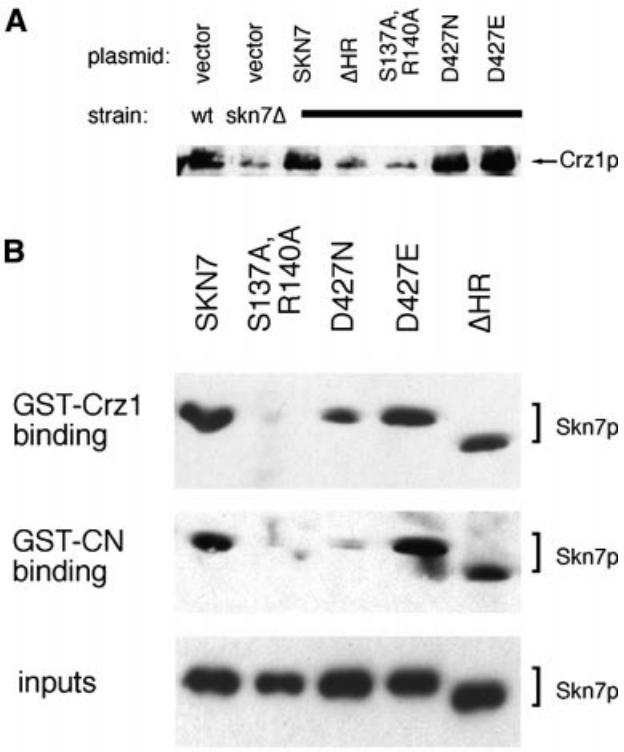

Fig. 7. The Skn7p HR and DNA-binding domain are required for Crz1p stabilization; the DNA-binding domain is essential for binding to Crz1p and calcineurin. (**A**) Anti-Crz1p western blot analysis of YPH499 or KWY266 with Skn7p plasmids as in Figure 1. All plasmids are derivatives of pRS425. *SKN7ΔHR* is pKW47; *S137A,R140A* is pKW37. Each Skn7p allele was expressed at comparable levels (data not shown). (**B**) GST–Crz1p and GST–calcineurin were purified on glutathione-Sepharose, and then incubated with 0.2 μg of His-tagged Skn7p, Skn7p S137A,R140A, Skn7p D427N, Skn7p D427E, or Skn7pΔHR (from pKW25, 39, 40, 42 and 44, respectively). The Sepharose was washed, and the bound proteins eluted and analyzed by SDS–PAGE and anti-His_4 western blotting (top two panels). Purified Skn7 protein (0.1 μg) (expressed from pKW25, 39, 40, 42 and 44) was similarly run on a gel (bottom panel).

Discussion

We have demonstrated that Skn7p, a previously identified response-regulator protein, modulates Crz1p/calcineurin-dependent signaling. First, overexpression of Skn7p increases Crz1p- and calcineurin-dependent reporter gene activity, and *skn7Δ* strains show reduced Ca^{2+}-, calcineurin- and Crz1p-dependent transcription of multiple genes. Secondly, overexpression of Skn7p increases Crz1 protein levels, and *skn7Δ* cells show greatly reduced Crz1 protein levels due to increased Crz1 protein turnover. Thirdly, the elimination of calcineurin subunits, but not the addition of a calcineurin phosphatase inhibitor, blocks both the Skn7p-mediated activation of Crz1p-dependent transcription and its effect on Crz1p stability. Finally, Skn7p binds specifically to both calcineurin and Crz1p *in vitro*. A functional Skn7p DNA-binding domain, but not Asp427 phosphorylation, was required for all of these functions. The Skn7p coiled-coil region was required for

both Skn7p-mediated *in vivo* CDRE activation and Crz1p stabilization, but not for *in vitro* protein–protein interactions.

These observations show that Skn7p affects Crz1p-dependent transcription by modifying Crz1 protein stability. The *in vitro* binding of Skn7p, Crz1p and calcineurin suggests that Skn7p may modulate Crz1p stability through direct interactions with these proteins. This role for Skn7p is different from previous work on the protein in one key regard: we implicate Skn7p in the regulation of protein stability. Skn7p has three well characterized domains: a response-regulator region, which resembles two-component signaling proteins; a coiled-coil domain, which mediates protein–protein interactions; and an HSF-like DNA-binding domain, which interacts with specific promoter regions. It is particularly interesting to consider the different motifs present in Skn7p in light of this novel role for the protein.

Skn7p is a response regulator implicated in protein stability

Skn7p is the first eukaryotic response regulator to be implicated in protein stability. However, a prokaryotic equivalent exists that shares several key similarities with our model. Like Skn7p, the prokaryotic response regulator RssB/SprE binds to and affects the stability of a transcriptional regulator. RssB/SprE destabilizes the RNA polymerase subunit σ^s under specific environmental conditions, and RssB/SprE can bind directly to σ^s to modulate its degradation by the ClpXP protease (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Zhou and Gottesman, 1998). In contrast though, many details of the two systems are different. First, while Skn7p has an HSF-like DNA-binding domain and can bind to DNA, RssB/SprE does not have any type of previously characterized DNA-binding domain (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Zhou and Gottesman, 1998).

Secondly, phosphorylation of the conserved response-regulator aspartate plays different roles in RssB/SprE and Skn7p. For RssB/SprE, aspartyl phosphorylation greatly enhances binding to σ^s, the protein whose stability it modulates (Becker *et al*., 1999). On the other hand, Skn7p is an unusual response-regulator protein in that many of its functions operate independently of receiver phosphorylation. Although aspartyl phosphorylation of Skn7p is required for its suppression of *kre9Δ* (Brown *et al*., 1994) and its activation of *OCH1* transcription (S.Li and J.S.Fassler, in preparation), Skn7p D427N, an allele that can not be phosphorylated by two-component proteins, is fully functional in many situations. For example, Skn7p D427N complements the sensitivity of *skn7Δ* strains to oxidative stress (Morgan *et al*., 1997) and, when overexpressed, rescues a *pkc1Δ* growth defect (Brown *et al*., 1994).

In our *in vivo* studies, Skn7p D427N resembles the wild-type allele in its ability to stabilize Crz1p and activate CDRE::*lacZ* transcription. However, expression of the D427E allele did lead to slightly increased Crz1p levels and CDRE::*lacZ* activity, indicating that regulation of Skn7p through this residue, while not required, may still play some role in this response. In contrast, Skn7p D427N bound less well than wild-type Skn7p to calcineurin *in vitro*. This binding, though weaker, may still be sufficient for Skn7p-mediated Crz1p stabilization *in vivo*. Alternatively, the presence of other proteins may improve the association of Skn7p D427N with Crz1p/calcinerin *in vivo*.

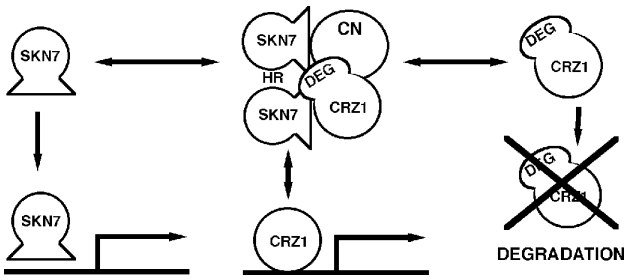

Fig. 8. Model describing the Skn7p interaction with calcineurin-mediated signal transduction (see Discussion).

Finally, the mechanism of Skn7p-regulated Crz1p stabilization is distinct from RssB/SprE-mediated turnover in requiring the physical presence of the eukaryotic phosphatase calcineurin, as well as the DNA-binding domain of Skn7p. These features outline a unique mechanism of transcription factor stabilization. Future studies may reveal whether other Skn7p binding proteins are regulated by Skn7p-mediated stabilization, and whether other response-regulator proteins are implicated in protein stability.

The Skn7p coiled-coil domain is essential for in vivo function

Interestingly, Skn7p has been shown to bind to several other signaling proteins, including several transcription factors, Mbp1p, Hsf1p and itself (Bouquin *et al*., 1999; Raitt *et al*., 2000). Coiled-coil domains typically mediate protein–protein interaction, and the coiled-coil homology region (HR) is essential for Skn7p binding to both Rho1p and Mbp1p (Alberts *et al*., 1998; Bouquin *et al*., 1999). Hence, we were surprised to observe that while the coiled-coil region is essential for the effect of Skn7p on both Crz1p- and calcineurin-dependent transcription and Crz1p stabilization *in vivo*, it was not necessary for Skn7p binding to either Crz1p or calcineurin *in vitro*.

Several models may explain this result. First, Raitt *et al*. (2000) observed that while the Skn7p coiled-coil domain is not homologous to the HSF multimerization domain at the amino acid level, both regions are helical in nature and are at the same proximity from their HSF DNA-binding domains. Thus, the HR domain may mediate Skn7p–Skn7p dimerization. Skn7p alleles interact similarly with both Crz1p and calcineurin, so it is possible that Skn7p dimerization allows one molecule of Skn7p to interact with calcineurin and another to interact with Crz1p, forming a tetramolecular complex that could be required for Crz1p stabilization *in vivo* (Figure 8). Secondly, the coiled-coil domain may mediate protein–protein interaction with other proteins *in vivo* that are required for Crz1p stabilization but are not present in our *in vitro* binding reaction. Future experiments will attempt to identify *in vivo* protein complexes containing Skn7p and Crz1p to address this possibility. In addition to mediating *in vivo* effects on Crz1p stability, the HR domain is essential for binding to several additional signaling

proteins including Rho1p, a GTPase that activates Pkc1p as well as other targets, and Mbp1p, a G_1–S transcription factor (Alberts *et al*., 1998; Bouquin *et al*., 1999). Hence, Skn7p may integrate responses between different signaling pathways through the HR domain.

A multipurpose Skn7p DNA-binding domain

Skn7p is a transcription factor and a DNA-binding protein, and although the CDRE was sufficient for the effect of Skn7p on Crz1p-dependent transcription, we were unable to detect Skn7p or Skn7p complex binding to the CDRE. In addition, Li *et al*. have observed Skn7p binding to an *OCH1* promoter element which was unaffected in a *crz1Δ* mutant (S.Li and J.S.Fassler, personal communication). Interestingly, a functional Skn7p DNA-binding domain was completely essential for every Skn7p-mediated effect on calcineurin signaling that we examined. The Skn7p DNA-binding domain was required for activation of Crz1p-dependent transcription through the CDRE, Crz1p stabilization, and binding to both Crz1p and calcineurin *in vitro*.

Thus, the Skn7p DNA-binding domain is required for binding to both Crz1p and calcineurin as well as binding to promoters involved in stress responses. This suggests that Skn7p may exist in an equilibrium between protein–protein and protein–DNA complexes *in vivo*. Regulation of the relative abundance of these complexes may modulate the amount of Skn7p-dependent transcription available to the many different signal-transduction pathways in which it participates (Figure 8).

A phosphatase-independent role for calcineurin

We found that Skn7p was able both to activate Crz1p- and calcineurin-dependent transcription and to stabilize Crz1p in the presence of the calcineurin phosphatase inhibitor FK520, but not in the *cnb1Δ* calcineurin mutant. Consequently, we conclude that Skn7p requires calcineurin, but not its phosphatase activity, for these functions. This is the first example of a phosphatase-independent role for calcineurin. Catalysis-independent roles for signaling molecules have been uncovered in other pathways, such as the scaffolding function of the yeast MAPKK Pbs2p (Posas and Saito, 1997) and the repressive function of the inactive MAPK Kss1p (Cook *et al*., 1997). The fact that the presence of the complete calcineurin holoenzyme is required for Skn7p-dependent stabilization of Crz1p suggests that protein–protein interactions may be present. We found that Skn7p binds to both calcineurin and Crz1p; however, Skn7p can bind to Crz1p in the absence of calcineurin in our *in vitro* binding assay. Our data are consistent with the possibility that calcineurin, Crz1p and Skn7p form a complex that stabilizes Crz1p. Without the presence of all of the calcineurin subunits, Skn7p may not completely mask a degradation signal on Crz1p. However, we can not exclude the possibility that calcineurin may have some other, uncharacterized phosphatase-independent function that may affect Crz1p *in vivo* stability.

A novel mechanism of Crz1p regulation

The Skn7p-mediated control of Crz1p stability represents a new mechanism of regulating calcineurin signaling. Calcineurin also stabilizes Crz1p in a phosphorylation-dependent manner, independently of Skn7p (our unpublished observations). In addition, Crz1p has been previously shown to be regulated at the levels of nuclear localization (Stathopoulos-Gerontides *et al*., 1999) and transcription, since Crz1p initiates its own transcription upon calcineurin-responsive stress (Matheos *et al*., 1997). We show that the control of turnover represents a new mechanism of Crz1p regulation that affects Crz1p- and calcineurin-dependent transcription. We postulate that the complex regulation of Crz1p may enable the cell to 'fine-tune' the stress response.

The physiology of Skn7p

We have described a novel function for Skn7p as a regulator of protein stability, and have shown that Skn7p modulates stress-activated calcineurin signaling. Previously, the role of Skn7p in several stress responses, including cell integrity, oxidative stress and heat shock, has been documented (Brown *et al*., 1993; Kuge and Jones, 1994; Krems *et al*., 1996; Morgan *et al*., 1997; Lee *et al*., 1999; Raitt *et al*., 2000) as well as its role in the cell cycle (Morgan *et al*., 1995; Bouquin *et al*., 1999). While the precise connection between these responses remains unclear, the fact that Skn7p participates in this variety of functions suggests that they are physiologically related. In particular, Skn7p and calcineurin both affect aspects of cell wall function. Skn7p and calcineurin each activate transcription of cell wall synthesis genes and display extensive genetic interactions with *PKC1*, a major regulator of cell wall structure and function (Levin and Bartlett-Heubusch, 1992; Brown *et al*., 1993; Garrett-Engele *et al*., 1995; S.Li and J.S.Fassler, in preparation). In this report we show that cells lacking both Skn7p and calcineurin are synergistically sensitive to hygromycin B and hydrogen peroxide (Figure 3). These observations indicate that Skn7p and calcineurin function together in response to oxidative stress and cell wall damage, and thus regulate similar physiological processes *in vivo*.

The major role of Skn7p *in vivo* may be to integrate different stress signaling events. Some domains of Skn7p are clearly required for more than one of its functions. This may indicate that regulation of these functions is coordinated, or instead, that Skn7p signaling is modulated by direct competition for Skn7p by its different effectors. The discovery that Skn7p affects both protein stability and calcineurin signaling will contribute to future studies that elucidate mechanisms of global stress-response coordination.

Materials and methods

Yeast strains and culture conditions

Yeast strains KWY242 and KWY246 were created from YPH499, DD12 (*cnb1Δ*) (Cyert and Thorner, 1992), respectively, by using the pKW11 integration vector to introduce two tandem copies of the CDRE upstream of *lacZ* at the *URA3* locus. *skn7Δ::TRP1* strains, as described (Brown *et al*., 1993), were generated in the YPH499 background using standard techniques (Sherman *et al*., 1986). Yeast cells were grown on standard YPD or SCD media (Sherman *et al*., 1986), except that amino acids were added at approximately twice the recommended level to SCD. FK520 (Merck), an FK506 analog and calcineurin inhibitor (Liu *et al*., 1992), in 90% ethanol, 10% Tween-20, was added to a final concentration of 2 μg/ml where noted. In protein stability studies, cycloheximide was added to log phase cells to a final concentration of 100 μg/ml. Recombinant DNA procedures were performed according to standard techniques (Ausubel *et al*., 1987). Sequencing was performed either using

Table II. Yeast strains used in this work

Strain[a]	Genotype	Reference
DD12	*cnb1::hisG*	Cyert and Thorner (1992)
ASY472	*crz1Δ::loxP-kanMX-loxP*	Stathopoulos and Cyert (1997)
KWY242	*ura3-52*::2×CDRE::*lacZ*	this study
KWY246	DD12, except *ura3-52*::2×CDRE::*lacZ*	this study
ASY459	*ura3-52*::4×CDRE::*lacZ*	Stathopoulos and Cyert (1997)
ASY460	*ura3-52*::mutant 4×CDRE::*lacZ*	Stathopoulos and Cyert (1997)
ASY461	DD12, except 4×CDRE::*lacZ*	Stathopoulos and Cyert (1997)
ASY834	ASY472, except 4×CDRE::*lacZ*	Stathopoulos and Cyert (1997)
KWY266	*skn7Δ::TRP1*	this study
KWY267	DD12, except *skn7Δ::TRP1*	this study
KWY289	ASY472, except *skn7Δ::TRP1*	this study

[a]All strains are in the YPH499 (*MAT**a***, *ura3-52*, *lys2-801*, *ade2-101*, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) background (Sikorski and Hieter, 1989).

the Sequenase system (US Biochemical Corporation) with [α-^{35}S]dATP (Amersham) or at the Stanford University PAN facility.

Growth assays

Saturated cultures were diluted to an OD_{600} of 1. Ten-fold serial dilutions were spotted onto YPD plates that contained either no added chemicals, 1.8 mM hydrogen peroxide (Fischer) or 70 μg/ml hygromycin B (Roche Diagnostics). Cells were grown at 30°C for 1–3 days.

Plasmids

pKW11 was constructed from pAMS363 (Stathopoulos and Cyert, 1997), a 2 micron *URA3* vector containing two copies of the CDRE upstream of the minimal promoter *CYC1* fused to *lacZ*. The 2 micron sequence was removed by *Hin*dIII digestion and subsequent re-ligation to generate an integration vector. pKW25, pKW39, 40, 42 and 44 are His_6-tagged Skn7p bacterial expression vectors. *SKN7* coding sequence was amplified from pRS425-*SKN7* (Ketela *et al*., 1998), pSL1108 (S.Li and J.S.Fassler, in preparation), pRS425-*SKN7D427N*, pRS425-*SKN7D427E* (Ketela *et al*., 1998), pAB93 (Bouquin *et al*., 1999), respectively, introducing an *Nde*I site before the ATG and a *Bam*HI site after the TAA, with the exception of pKW44, which introduces a *Xho*I site at the TAA. These fragments were introduced into the *Nde*I, *Bam*HI or *Xho*I sites of pET15b (Novagen). pKW37 was created by inserting a 3.5 kb *Sal*I–*Hin*dIII fragment from pSL1108 (S.Li and J.S.Fassler, in preparation) into pRS425. pKW47 was created by amplifying the *SKN7ΔHR* allele and promoter region from pAB93 (Bouquin *et al*., 1999), introducing *Xba*I and *Hin*dIII sites outside the YEPlac195 polylinker. This piece was introduced into the same sites of pRS425. BJP3003 was created by introducing *Bam*HI sites at the ATG and after an introduced stop codon after Ser417 in *CNA1*; this fragment was inserted into the *Bam*HI site of pGEX-5X-1 (Amersham Pharmacia), a GST bacterial expression vector (B.Jiang and M.Cyert, unpublished data). BJP3003 expresses a truncated version of *CNA1* that removes the autoinhibitory domain, producing a constitutively active calcineurin allele. This is referred to in the text as GST–*CNA1*. pLMB117 was created by using PCR mutagenesis to introduce an in-frame *Hin*dIII site at the ATG and a *Sal*I site after the stop codon of *CRZ1*. This fragment was inserted on the C-terminal side of GST into the respective sites in pRD56, a pRS316 *GAL1*–GST vector (L.Boustany and M.Cyert, unpublished data). A *Bam*HI–*Sal*I GST–*CRZ1* fragment from pLMB117 was cloned into the *Bam*HI–*Xho*I sites of pGEX4T-3 (Amersham Pharmacia) to form pGEX-*CRZ1*, a bacterial expression vector (K.Saltsman and M.Cyert, unpublished data). pAMS435 is YEP351-*CRZ1* (Stathopoulos and Cyert, 1997); M1485 is YEPlac181-SWI5 (D.Stillman, personal communication); YEP352-SKN7HA contains the *SKN7* ORF in-frame with an HA epitope tag (H.Bussey, personal communication).

β-galactosidase assays

Quantitative assays. To examine the effect of various stimuli on reporter gene activity, cells were grown to log phase in synthetic media, then spun down and diluted to an OD of 0.2–0.3 in YPD pH 5.5, 50 mM succinic acid. $CaCl_2$ (200 mM) and FK520 (2 μg/ml) or control solvent (90% ethanol, 10% Tween-20) were added, and the cells were grown for 4 h. Protein extracts were made from the pelleted cells as described previously (Withee *et al*., 1997), breaking the cells with glass beads in 0.1 M Tris pH 8.0, 10% glycerol. Protein concentration was determined by BCA assay (Pierce). Activity was determined at room temperature in a microplate reader (Bio-Rad) using 10 μl of 4 mg/ml ONPG (*O*-nitrophenyl-β-D-galactopyranoside; Sigma) and 90 μl of Z buffer (100 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.027% β-mercaptoethanol). Maximum rates are given in rate of OD_{415} change per minute per μg protein.

Qualitative assays. Colonies were scored for β-galactosidase activity as described (Stathopoulos and Cyert, 1997), except that blue colonies were selected at a time point before the negative control (KWY242 with the empty vector YEP351) turned blue—usually at ~7 h. Xgal (Sigma) was used at a concentration of 50 μg/ml.

Genetic screening

KWY242 was transformed with two multicopy genomic libraries. Cells were grown at room temperature. Approximately 160 000 colonies transformed with the 2J351 library (Hill *et al*., 1986; Engebrecht *et al*., 1990) and 79 000 colonies transformed with the YEP13 library (Nasmyth and Reed, 1980) were screened. Plasmids from blue colonies were retransformed into ASY461 (4×CDRE::*lacZ*) and ASY462 (4× mutant CDRE::*lacZ*) to eliminate enhancers of general transcription. Plasmids were plated on media containing 2 μg/ml FK520 (Merck) to assess calcineurin dependence. The plasmids were also re-transformed into the original strain (KWY242), a *cnb1Δ* strain (KWY246), and a *crz1Δ* strain (ASY834), to ensure that the β-galactosidase activity was plasmid-, calcineurin- and Crz1p-dependent. Inserts were identified by sequencing the ends of the genomic DNA and matching this sequence to regions of the genome with BLAST (Altschul *et al*., 1990) and the *Saccharomyces* Genome Database (Cherry *et al*., http://genome-www.stanford.edu/Saccharomyces).

Protein purification

Recombinant proteins were prepared from French press *E.coli* extracts (BL21pLysS with His-*SKN7* constructs, BLR with GST–*CRZ1* and GST–*CNA1*) according to the manufacturers' directions (GST tags, Amersham Pharmacia; His tags, Qiagen). After elution, proteins were dialyzed into Buffer 88 (20 mM HEPES pH 6.8, 150 mM potassium acetate, 250 mM sorbitol, 2 mM magnesium acetate) with a 10 000 MWCO DispoDialyzer (Amersham Pharmacia). Recombinant yeast calcineurin was purified by first mixing two bacterial strains containing either BJP3003 (GST–*CNA1*) or pET-*CNB1* (Okano *et al*., 1998), followed by lysis and glutathione chromatography (as above).

Binding assays

Recombinant protein binding assays were performed as in Lai *et al*. (1998). Approximately 0.2 μg of recombinant protein were added to protein immobilized on beads in 500 μl binding buffer [50 mM Tris–HCl pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2% Triton-X, 0.5 mg/ml bovine serum albumin, aprotinin (5 μg/ml), leupeptin (5 μg/ml), pepstatin A (5 μg/ml), benzamidine (1.25 mM) and PMSF (0.5 mM) (Sigma) as well as 1 mM dithiothreitol where glutathione-Sepharose was included] and rotated at 4°C for 2–3 h. Twenty microliters of 50% (w/v) Sepharose were used in all binding assays, except that 5 μl of beads with GST–CRZ1 were used to pull down His_6-tagged Skn7 alleles. Beads were washed three times with 800 μl binding buffer, before adding loading buffer, boiling, and performing SDS–PAGE.

Western blotting

SDS–PAGE and western blotting were performed by standard procedures. BCA assays (Pierce) were used to normalize protein loading (15–40 μg) for westerns of whole-cell extracts. Immunoblots were performed with either anti-HA 12CA5 antibodies (Roche Molecular Diagnostics), anti-Crz1 (H-X.Li and M.Cyert, unpublished data), anti-GST (Berkeley Antibody Co.), or anti-tetra-His antibodies (Qiagen), and anti-mouse-HRP or anti-rabbit-HRP (Amersham Pharmacia) and ECL detection reagents (Amersham Pharmacia).

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