A conserved family of calcineurin regulators

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The protein phosphatase calcineurin mediates many cellular responses to calcium signals. Using a genetic screen in yeast, we identified a new family of proteins conserved in fungi and animals that inhibit calcineurin function when overexpressed. Overexpression of the yeast protein Rcn1p or the human homologs DSCR1 or ZAKI-4 inhibited two independent functions of calcineurin in yeast: The activation of the transcription factor Tcn1p and the inhibition of the H⁺/Ca²⁺ exchanger Vcx1p. Purified recombinant Rcn1p and DSCR1 bound calcineurin in vitro and inhibited its protein phosphatase activity. Signaling via calmodulin, calcineurin, and Tcn1p induced Rcn1p expression, suggesting that Rcn1p operates as an endogenous feedback inhibitor of calcineurin. Surprisingly, rcn1 null mutants exhibited phenotypes similar to those of Rcn1p-overexpressing cells. This effect may be due to lower expression of calcineurin in rcn1 mutants during signaling conditions. Thus, Rcn1p levels may fine-tune calcineurin signaling in yeast. The structural and functional conservation between Rcn1p and DSCR1 suggests that the mammalian Rcn1p-related proteins, termed calcipressins, will modulate calcineurin signaling in humans and potentially contribute to disorders such as Down Syndrome.

[Key Words: Calcineurin; calcium signaling; Rcn1p; DSCR1; ZAKI-4]

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The calcium and calmodulin-activated protein phosphatase calcineurin regulates a variety of developmental and cellular processes. Calcineurin helps control T-cell activation (Liu et al. 1992; Crabtree 1999), skeletal and cardiac muscle growth and differentiation (Chin et al. 1998; Hughes 1998; Molkentin et al. 1998; Sussman et al. 1998), memory (Mansuy et al. 1998; Winder et al. 1998), and apoptosis (Shibasaki and McKeon 1995; Krebs 1998). Calcineurin is highly conserved in fungi and animals, becoming activated on binding calcium and calmodulin when cytosolic calcium rises, and inhibited on binding the immunosuppressants Cyclosporin A and FK506 in complexes with their respective cellular receptors (Liu et al. 1991a; Klee et al. 1998; Hemenway and Heitman 1999). Feedback regulators of calcineurin have not yet been identified in any cell type.

In the budding yeast Saccharomyces cerevisiae, calcineurin regulates gene expression and ion transport in response to calcium signals (Fig. 1A) but the genes encoding calcineurin (CNA1, CNA2, and CNB1) are not essential for viability (Cyert et al. 1991; Kuno et al. 1991; Liu et al. 1991b; Cyert and Thorner 1992; Ye and Bretsch 1992). Calcineurin promotes growth in high calcium environments by dephosphorylating the transcription factor cytoplasmic Tcn1p (also called Crz1p and Hal8p), which then accumulates in the nucleus and induces expression of the calcium ATPases Pmc1p and Pmr1p (Cunningham and Fink 1994b; Cunningham and Fink 1996; Matheos et al. 1997; Stathopoulos and Cyert 1997; Mendizabal et al. 1998; Stathopoulos-Gerontides et al. 1999). Calcineurin also appears to inhibit a vacuolar H⁺/Ca²⁺ exchanger Vcx1p by a posttranslational mechanism thereby preventing its function in calcium tolerance (Cunningham and Fink 1996, Pozos et al. 1996). The exogenous inhibitors of calcineurin, FK506 and Cyclosporin A, restore calcium tolerance to pmc1 mutants by permitting Vcx1p function (Cunningham and Fink 1996). We took advantage of this phenomenon to screen for factors that can inhibit calcineurin function when they are overexpressed. We report the identification of a previously uncharacterized family of proteins, termed calcipressins, will modulate calcineurin signaling in humans and potentially contribute to disorders such as Down Syndrome.

Results

A genetic screen for endogenous inhibitors of calcineurin

If endogenous inhibitors of calcineurin are produced in yeast, we reasoned that such molecules would confer calcium tolerance phenotypes similar to those observed with FK506 and Cyclosporin A (Cunningham and Fink 1994b). To identify endogenous calcineurin inhibitors or factors that produce them, we screened a high-dosage library of yeast genomic DNA for genes that conferred calcium tolerance to pmc1 mutants in a Vcx1p-dependent manner. From this screen (Cunningham and Fink 1996) we recovered 20 plasmids with overlapping inserts spanning the previously uncharacterized open reading...
frame (ORF) RCN1 (formerly YKL159c, GenBank accession no. Z28159). Subclones containing only the RCN1 gene conferred strong calcium tolerance to a pmc1 mutant but did not confer calcium tolerance to a pmc1 vcx1 double mutant (Fig. 2A) at any concentration (data not shown). The requirement for VCX1 suggested that overexpression of RCN1 promoted calcium tolerance not through buffering or efflux but through the direct or indirect activation of the H+/Ca2+ exchanger Vcx1p.

If the activation of Vcx1p by RCN1 overexpression was a consequence of calcineurin inhibition, RCN1 overexpression would also be expected to inhibit the activation of Tcn1p, a calcineurin-dependent transcription factor. Indeed, RCN1 overexpression partially blocked calcineurin- and Tcn1p-dependent induction of a PMC1–lacZ reporter gene (Fig. 2B). A pmc1 vcx1 double mutant was used in this experiment to eliminate any differences in calcium tolerance or sequestration secondary to calcineurin inhibition, though similar effects were observed in wild-type strains (data not shown). No effect of RCN1 overexpression was observed when a constitutively active variant of Tcn1p was coexpressed (Fig. 2B), ruling out the possibility that RCN1 might affect the PMC1 promoter independent of calcineurin. Overexpression of RCN1 also inhibited the expression of a CDRE–lacZ reporter gene (Fig. 2C) containing a single Tcn1p binding site upstream of an inert minimal promoter (Stathopoulos and Cyert 1997). These findings suggest RCN1 overexpression disrupts Tcn1p activation. The ability of RCN1 overexpression to inhibit two independent activi-
ties of calcineurin suggests that RCN1 exerts its negative effect at the level of calcineurin or further upstream in the calcium-signaling cascade. Experiments described below confirm that the protein product of RCN1 directly binds and inhibits calcineurin in vitro, and therefore is a direct regulator of calcineurin (RCN).

Rcn1p-related proteins

The RCN1 gene encodes a hydrophilic protein (Rcn1p) of 212 amino acids exhibiting significant homology to predicted proteins in other fungi, invertebrate animals, and mammals [aligned in Fig. 1B] all of which express calcineurin homologs. All proteins in the Rcn1p family share a highly conserved central segment containing a novel consensus sequence motif LxxPxRxKFLISPPxSPPxxW. The human DSCR1 cDNA was identified during analysis of chromosome 21 as a gene mapping near the Down Syndrome critical region [Fuentes et al. 1995]. The human ZAKI-4 cDNA was identified in a screen for genes responsive to thyroid hormone in fibroblasts [Miyazaki et al. 1996] and a third closely related human gene termed DSCR1L was recently deposited in GenBank. None of the Rcn1p family members have been characterized functionally.

To determine whether Rcn1p-related proteins retain a similar function, human DSCR1 and ZAKI-4 cDNAs were cloned into yeast expression plasmids and introduced into various yeast strains. As observed with RCN1, overexpression of DSCR1 or ZAKI-4 increased calcium tolerance in pmc1 mutants but not pmc1 vcx1 double mutants (Fig. 2A). Additionally, expression of DSCR1 and ZAKI-4 diminished calcineurin-dependent induction of PMC1–lacZ by 84% and 95%, respectively, and also strongly inhibited induction of the CDRE–lacZ reporter gene (Fig. 2C). Thus, despite their limited sequence similarity to Rcn1p, DSCR1 and ZAKI-4 retained the ability to inhibit calcineurin function when expressed in yeast.

Rcn1p and DSCR1 bind and inhibit calcineurin in vitro

Rcn1p and DSCR1 were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli, purified by affinity chromatography, and tested for interactions with calcineurin purified from bovine brain. Bovine calcineurin specifically bound to both GST–DSCR1 and GST–Rcn1p on glutathione–agarose beads (Fig. 3A), though the crosskingdom interaction between Rcn1p and calcineurin appeared weaker. Calcineurin bound to GST–DSCR1 in buffers containing either 2 mM EGTA or 2 mM CaCl₂ + calmodulin (Fig. 3B) and therefore was independent of the calcium concentration. Furthermore, FK506/FKBP12 complexes exhibited a calmodulin-dependent interaction with the DSCR1/calcineurin complex (Fig. 3C). These results suggest DSCR1 binds calcineurin at a site [or sites] distinct from those that bind calmodulin and FK506/FKBP12.

The functional significance of DSCR1 and Rcn1p interaction with calcineurin was investigated using standard protein phosphatase assays with phospho-RII peptide as substrate. Addition of purified GST–DSCR1 strongly inhibited the calcium/calmodulin-dependent dephosphorylation of RII peptide by bovine calcineurin, whereas the same quantities of GST had no effect on calcineurin activity [Fig. 3D]. Increasing calmodulin concentration 10-fold did not overcome inhibition by GST–DSCR1, confirming that DSCR1 does not compete with calmodulin. Much higher levels of GST–Rcn1p were required to inhibit bovine calcineurin [Fig. 3E] consistent with its lower binding (Fig. 3A). Finally, a synthetic 24-
residue peptide surrounding the most conserved motif of DSCR1 (peptide DS-24) also inhibited calcineurin in a dose-dependent manner [Fig. 3F]. The DS-24 peptide was much less potent than full-length DSCR1, suggesting that other regions of DSCR1 also contribute to interactions with calcineurin. These results demonstrate that two divergent members of the Rcn1p family of proteins can directly bind and inhibit calcineurin.

Calcineurin-dependent expression of Rcn1p

Calcium signals increased the expression of DSCR1 mRNA in mammalian cell lines (Crawford et al. 1997; Leahy et al. 1999; Fuentes et al. 2000). Therefore, we tested whether RCN1 transcription and Rcn1p accumulation were regulated in yeast. A RCN1-lacZ reporter gene containing 2-kb of the RCN1 promoter region was expressed at low levels in wild-type cells during growth in standard medium and was induced 20-fold after shift to high calcium conditions [Fig. 4A]. Induction of RCN1-lacZ in response to calcium was completely blocked by addition of FK506 or by deletion of the genes encoding Tcn1p [Fig. 4A] or calcineurin [data not shown], suggesting that Rcn1p may be another downstream target of the calcineurin-dependent transcription factor. Western blot analysis of cells expressing an epitope-tagged Rcn1p–HA protein from a low-dosage plasmid confirmed this pattern of expression. Growth in high calcium stimulated Rcn1p–HA accumulation in wild-type cells [Figs. 4B, lanes 1,2] but not in tcn1 mutants [lanes 5,6]. Induction of Rcn1p–HA was also blocked in cna1 cna2 double mutants that lack the two catalytic A subunits of calcineurin (not shown), in cna1 mutants that lack the single regulatory B subunit of calcineurin [Fig. 4C, lanes 9,10], and in cmd1-6 mutants that express a defective calmodulin [Fig. 4C, lanes 5,6] that is unable to bind calcium or activate calcineurin [Geiser et al. 1991]. Thus, calcium signaling through calmodulin, calcineurin, and Tcn1p may be another downstream target of the calcineurin-dependent transcription factor.

The phenotype of rcn1 null mutants

The above results all suggest that Rcn1p may operate as a feedback inhibitor of calcineurin signaling in vivo. If this were the only role of Rcn1p in yeast, mutants lacking Rcn1p would likely exhibit characteristics of enhanced calcineurin activity. To test this hypothesis, a rcn1 null mutant was constructed by homologous recombination and assayed for Tcn1p activation and Vcx1p inhibition. Surprisingly, both assays revealed reduced calcineurin activity in rcn1 null mutants. First, the low calcium tolerance of pmc1 mutants but not pmc1 vcx1 double mutants was suppressed partially by
the deletion of RCN1 (Fig. 5A). The addition of FK506 increased the calcium tolerance of pmc1 mutants to the same level with or without Rcn1p, indicating that Rcn1p and calcineurin function within a common pathway. Secondly, calcineurin-dependent induction of PMC1–lacZ, RCN1–lacZ, and FKS2–lacZ in rcn1 mutants decreased by 93%, 87%, and 62%, respectively, relative to wild type. Similar effects were observed in pmc1 vcx1 double mutants (Fig. 5B). Induction of CDRE–lacZ was also largely dependent on Rcn1p [Fig. 5C] but expression of a calcineurin-independent CYC1–lacZ reporter was unaffected by Rcn1p function [Fig. 5B]. Calcineurin-dependent dephosphorylation of Tcn1p causes a shift in its mobility on SDS gels even in wild-type cells [Stathopoulos and Gerontides 1999]. Using this method, we found that epitope-tagged Tcn1p–HA from rcn1 mutants migrated similar to that of cnb1 mutants (Fig. 5D), indicating that Rcn1p was required for calcineurin-dependent dephosphorylation of Tcn1p. Thus, at least two independent outputs of calcineurin were specifically impaired in rcn1 mutants. Interestingly, the positive role of Rcn1p on calcineurin may also be conserved in mammalian cells because expression of DSCR1 in rcn1 mutants partially complemented the defect in PMC1–lacZ expression (Fig. 5B).

The nature of Rcn1p’s positive contribution to calcineurin signaling was investigated further by monitoring calcineurin expression and stability. Neither deletion nor overexpression of Rcn1p affected expression of an epitope-tagged Cna1p–MYC protein in nonsignaling conditions [Fig. 6A]. In high calcium conditions however, Cna1p–MYC consistently declined to lower levels in rcn1 mutants compared to wild type and remained higher in Rcn1p-overexpressing strains. The calcium-dependent decline of Cna1p–MYC was more pronounced in pmc1 vcx1 mutants [Fig. 6B] where cytosolic calcium increases to higher levels than wild type [Miseta et al. 1999]. Inhibition of calcineurin through FK506 addition did not prevent the loss of Cna1p–MYC in the presence of high calcium. To determine if Rcn1p affected Cna1p–MYC stability, Cna1p expression was assayed in the presence of cycloheximide. Surprisingly, cycloheximide blocked the down-regulation of Cna1p–MYC with or without Rcn1p and/or FK506 (Fig. 6C; data not shown). Thus, Cna1p stability appeared to be insensitive to calcium, FK506, and Rcn1p. Instead, the elevated accumulation of Cna1p–MYC caused by Rcn1p during signaling conditions may reflect a positive effect of Rcn1p on calcineurin expression. This hypothesis was confirmed through analysis of CNA1–lacZ, CNA2–lacZ, and CNB1–lacZ expression [Fig. 7]. In standard medium with or without calcium and FK506, these reporter genes were expressed respectively at ~50%, ~35%, and ~150% higher levels in wild-type cells relative to rcn1 mutants. Interestingly, high calcium conditions diminished expression of all three reporter genes in a FK506-sensitive fashion. These findings reveal a significant role for Rcn1p in stimulating calcineurin expression. This effect provides at least a partial explanation for the positive role of Rcn1p on calcineurin signaling in yeast.

Discussion

This study reports the identification of a conserved family of proteins that appear to function as feedback inhibi-
tors of calcineurin during calcium signaling. Recombinant Rcn1p and DSCR1 proteins bound and inhibited bovine calcineurin activity in vitro while overexpression of Rcn1p and DSCR1 inhibited at least two independent functions of yeast calcineurin in vivo, including the activation of Tcn1p and the inhibition of Vcx1p [see Fig. 1A]. RCN1 transcription and Rcn1p accumulation in yeast were strongly induced by calcineurin-dependent activation of Tcn1p, supporting the hypothesis that Rcn1p operates as an endogenous feedback inhibitor of calcineurin signaling. Recent studies suggest that DSCR1 functions as a feedback inhibitor of calcineurin signaling in human cells. DSCR1 transcription in human astrocytoma cells was strongly stimulated by calcineurin signaling and DSCR1 overexpression inhibited calcineurin-dependent activation of NFAT (Rothermel et al. 2000, Fuentes et al. 2000). Together these findings suggest broad conservation of the Rcn1p-related proteins as feedback inhibitors of calcineurin.

The analysis of rcn1 null mutants also revealed a stimulatory role of Rcn1p on calcineurin signaling. Calcineurin-dependent regulation of both Tcn1p and Vcx1p was clearly reduced in rcn1 mutants but not completely abolished as judged by the more severe consequences of adding FK506. The apparent deficiency of calcineurin signaling in rcn1 mutants may be the result of decreased calcineurin expression. For example, Cna1p levels were lower in rcn1 mutants and higher in Rcn1p-overexpressing strains as compared to wild-type strains grown in high calcium conditions. We detected no obvious effects of Rcn1p on either Cna1p accumulation in nonsignaling conditions or on Cna1p stability in any conditions tested. Additionally, we could not detect any effect of Rcn1p on Vcx1p or Tcn1p function when calcineurin had been inactivated by FK506, although Rcn1p is unstable under these conditions. The simplest model consistent with these results is one where Rcn1p stimulates calcineurin expression during calcium signaling.

Calcineurin expression in yeast has not yet been studied in detail. Rcn1p increased expression of CNA1, CNA2, and CNB1 reporter genes whereas calcium decreased expression through an FK506-sensitive mechanism [Fig. 7]. Cna1p levels also declined during growth in high calcium conditions, an effect that was enhanced in rcn1 mutants [Fig. 6] and diminished in cmd1-6 mutants [data not shown]. These results suggest calcineurin activation may down-regulate expression of its structural genes, an effect that would be stimulated by calmodulin and inhibited by Rcn1p. However, the decline of Cna1p was not blocked by FK506 addition as if another calcium-dependent mechanism contributed to calcineurin down-regulation in yeast. Therefore, calcineurin expression, accumulation, and function appear to be regulated by calcium at multiple levels. Further analysis of calcineurin dynamics is warranted in order to understand the significance of this unexpected complexity and to fully explain the calcineurin-deficient phenotype of rcn1 mutants.

Additional roles for Rcn1p in promoting calcineurin function in yeast can not be ruled out. For example,
Rcn1p may actually stimulate calcineurin signaling to some degree in vivo through a mechanism that was not reconstituted or detectable in our in vitro assays, even at levels 100-fold lower than those required to inhibit calcineurin activity. Low doses of these proteins might increase calcineurin activity in vivo but such effects might escape detection in vitro if oxidative inactivation of calcineurin was also stimulated (Wang et al. 1996). Alternatively, these proteins might promote interactions between calcineurin and its natural substrates, much like the targeting or scaffolding subunits of type 1 protein phosphatase [Hubbard and Cohen 1993; Sim and Scott 1999]. For example, the inhibitor-2 proteins prevent some activities of PP1 while stimulating others (Alessi et al. 1993), possibly by altering the partitioning of active PP1 molecules. Remarkably, genetic analysis of inhibitor-2 function in yeast revealed positive and negative effects on PP1 function [Tung et al. 1995] much like the effects of Rcn1p on calcineurin function reported here. Two proteins in mammals, AKAP79 [Coghlan et al. 1995; Kashishian et al. 1998] and Cabin1/cain [Lai et al. 1998; Sun et al. 1998; Youn et al. 1999], are known to bind calcineurin at sites distinct from the FK506/FKBP12 binding sites, to inhibit calcineurin phosphatase activity, and to also bind other cellular factors which may include substrates. The calcineurin-binding domains of both proteins are rather basic in character and not obviously related to the conserved domains of the Rcn1p family members. These structural differences and the dramatic up-regulation of Rcn1p and DSCR1 in response to calcineurin signaling distinguish this new family of calcineurin regulators from proteins described previously.

Finally, the possibility that Rcn1p and DSCR1 act as downstream effectors of calcineurin signaling remains to be fully explored. We have been unable to detect any effect of Rcn1p on Vcx1p or Tcn1p function in various contexts, but negative results of this nature do not rule out the possibility that Rcn1p mediates the regulation of other factors that respond to calcineurin signaling. The most conserved segment KxFLISPPxSPPx bears some resemblance to the conserved SPxxSPxxSPxx motifs repeated several times in NFAT proteins [Rao et al. 1997].

Figure 6. Down-regulation of calcineurin during calcium signaling conditions. (A) Cna1p expression correlates with Rcn1p in high calcium conditions. Western blots of Cna1p–MYC in rcn1 mutants, wild type, and Rcn1p-overexpressing strains were performed on total cell protein after 4 hr growth in YPD medium at pH 5.5 supplemented with 100 mM CaCl2 as indicated. (B) Endogenous Rcn1p increases Cna1p expression in pmc1 vcx1 mutants in high calcium conditions. Experimental conditions were as described in A except 0.3 µM FK506 was added as indicated. (C) Rcn1p is not required to stabilize Cna1p. Cna1p–MYC levels were monitored in pmc1 vcx1 double and rcn1 pmc1 vcx1 triple mutants after a pretreatment with 100 µM cycloheximide for 20 min followed by addition of 100 mM CaCl2 and 0.3 µM FK506. Total cell protein was extracted at 30 min intervals and analyzed by Western blotting as in Figure 3.

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Figure 7. Involvement of Rcn1p in expression of calcineurin structural genes. (A) CNA1–lacZ, (B) CNA2–lacZ, and (C) CNB1–lacZ reporter genes were introduced into wild type and rcn1 deletion mutant. β-Galactosidase activity was assayed in three independent transformants following 4 hr growth at 30°C in YPD medium at pH 5.5 supplemented with 100 mM CaCl2 and 0.3 µM FK506 as indicated.
These SP repeats are thought to be functionally important for NFAT regulation by calcineurin, serving as substrates of calcineurin after phosphorylation by protein kinases in the nucleus (Beals et al. 1997; Chow et al. 1997; Zhu et al. 1998; Crabtree 1999). Therefore, it is essential for NFAT regulation by calcineurin, serving as substrates. Any effector functions of Rcn1p would also be influenced by interactions with calcineurin after phosphorylation by protein kinases. It is conceivable that calcineurin activation dephosphorylates Rcn1p family members as a mechanism for regulating additional downstream factors. Because Rcn1p stability and expression depend on interactions with calcineurin, any effector functions of Rcn1p would also be affected by FK506.

The findings reported here and elsewhere (Fuentes et al. 2000) suggest that feedback inhibition of calcineurin is conserved from yeast to humans. The function of this feedback mechanism may be to fine-tune calcineurin signaling over a spectrum of intervals and conditions. Improper regulation of Rcn1p family members might lead to the disruption of calcineurin function in humans and contribute disease. For example, the increased dosage of DSCR1 in trisomy-21 individuals may contribute to the neurological, cardiac, or immunological defects observed in Down syndrome patients (Epstein 1995) through inhibition of calcineurin signaling. It will be interesting to determine if the interactions between DSCR1 and calcineurin in human cells are as complex as those we have observed for Rcn1p in yeast. It is not yet known if endogenous levels of DSCR1 are required to promote calcineurin function in vivo, or if calcineurin expression is down-regulated during prolonged calcium signaling. If FK506 and Cyclosporin A destabilize DSCR1 or its homologs in human cells, the efficacy or side effects of these drugs in transplantation therapies might be attributed to loss of these proteins. Understanding the relationship between the human Rcn1p family members and calcineurin in vivo will not only enhance our understanding of calcineurin function but also potentially provide novel therapeutic targets to control calcineurin function in humans.

**Materials and methods**

A library of yeast genomic DNA carried on high-dosage plasmids was screened for potential inhibitors of calcineurin by selecting for plasmids that could restore growth of a pmc1 null mutant (strain K473) on solid YPD medium at pH 5.5 supplemented with 200 mM CaCl2 (Cunningham and Fink 1996). Of 24 plasmids that were recovered, two carried the Ca2+ pumps Pmc1p and Pmr1p, two carried Vcx1p, and 20 carried the uncharacterized yeast gene RCNI/YKL159c plus flanking sequences. Subcloning demonstrated the active gene was RCNI. The entire RCNI coding sequence was deleted from the genome by homologous recombination using plasmid pTJK93 linearized by EcoRI digestion. The resulting rcn1::HIS3 null mutant was crossed to other mutants in an isogenic background [Matheos et al. 1997] to generate strains bearing multiple mutations (see Table 1).

**Recombinant DNA**

All recombinant DNA work was conducted with standard techniques with enzymes purchased from New England Biolabs or GIBCO BRL. The rcn1::HIS3 disruption plasmid pTJK93 was constructed by sequentially ligating two PCR products corresponding to the 5′ and 3′ flanking regions of RCNI that had been amplified from genomic DNA using the primers CCGAATTCGCCATCTATCAAAATG and GGGATCCCTGCCAGTTCGTTGTTT [for 5′ sequences] and CCCTCGAGGATGCAGGGAGCCATTGTG and CGGAATTCGAAATAGAAATAAAGAT [for 3′ sequences] into vector pRS303 digested with EcoRI + BamHI and EcoRI + XhoI, respectively. The RCNI–HA expression plasmid pTJK29 was generated by subcloning the 3xHA tag from pBSHA3 (Cunningham and Fink 1994a) into pRS316 (Sikorski and Hieter 1989) containing 5′ regulatory and coding sequences from the RCNI gene lacking a stop codon that had been amplified by PCR using primers GATCTTCACAAATCTTTGAGG and GCCATCTCTCTCTAGAATCTCATCGTCATCAG. A downstream stop codon was reconstructed by generating a frameshift at the SpeI site in the polylinker. Plasmids bearing CNA1–lacZ, CNA2–lacZ, CNB1–lacZ, and RCNI–lacZ reporter genes were constructed by subcloning PCR-generated DNA segments corresponding to nucleotides –2000 and +3 relative to the initiator codon of each gene into plasmid pLG178 using the following primers: for CNA1, CTCGAGAAGCGGAGTGGCACTTG and GGATCCCATTTGCGTTGAAGAG; for CNA2, CTCGAGAAGCGGAGTGGCACTTG and GGATCCCATTTGCGTTGAAGAG; and for CNB1, CTCGAGAAGCGGAGTGGCACTTG and GGATCCCATTTGCGTTGAAGAG. The GST–DSCR1 expression plasmid pTJK1 was constructed by subcloning the Smal–NsiI fragment of phZAKI-4-3.2 (courtesy of H. Seo, Nagoya University, Japan) into pRS425MET digested with Smal + PstI. The DSCR1 expression plasmid pTJK37 was constructed by subcloning a PCR product from DSCR1-1pBS [courtesy of X. Estivill, I.R.O., Barcelona, Spain] using primers GCGAGGATCCATTTGCGTTGAAGAG and CCCCACAGAGGAGTGGCACTTG and CCCCACAGAGGAGTGGCACTTG into pRS425MET digested with BanHI + XhoI. The GST–DSCR1 expression plasmid pTJK92 was constructed from the same PCR product after subcloning a BanHI–BglII fragment into the BanHI site of pGEX3X. The GST–Rcn1p expression plasmid

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### Table 1. *S. cerevisiae* strains used in this study

| Strains | Genotype | Reference |
|---------|----------|-----------|
| DMY14   | tcn1::G418 | Matheos et al. [1997] |
| JGY148  | cmd1-6    | Moser et al. [1996] |
| K482    | pmc1::TRP1 | Cunningham and Fink [1994] |
| K537    | cna1::URA3 cna2::HIS3 | this study |
| K601    | +         | Cunningham and Fink [1994] |
| K603    | cna1::LEU2 | Cunningham and Fink [1994] |
| K651    | pmc1::TRP1 vcx1Δ | Cunningham and Fink [1996] |
| K665    | pmc1::TRP1 vcx1Δ | Cunningham and Fink [1996] |
| TKY268  | rcn1::HIS3 pmc1::TRP1 | this study |
| TKY275  | rcn1::HIS3 | this study |
| TKY278  | rcn1::HIS3 pmc1::TRP1 vcx1Δ | this study |

All strains are isogenic to W303-1A [ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1].
pTJK93 was constructed by subcloning into the BamHI site of pGEX-3X a BglII + BamHI-digested PCR product using primers CTGGAGGGATCCGGATCCGGAGG. The Cna1p–MYC expression plasmid pTJK93 was constructed by subcloning the 3xMYC sequences from pKB241 into pRS315 containing the promoter and coding sequences of CNA1 that had been amplified using the primers CTGGAGGGATCCGGATCCGGAGG. The Cna1p–MYC expression plasmid pTJK93 was constructed by subcloning the 3xMYC sequences from pKB241 into pRS315 containing the promoter and coding sequences of CNA1 that had been amplified using the primers CTGGAGGGATCCGGATCCGGAGG.

Protein analysis and purification

The consensus Aspergillus nidulans and Dictyostelium discoideum sequences were compiled from overlapping cDNA and gDNA sequences at GenBank and the D. discoideum Genome Project. Protein sequences were aligned using the Clustal program from DNAstar based on the PAM250 weight table. GST–DSCR1, GST–Rcn1p, and GST were expressed in TOPP2 cells as suggested by the manufacturer [Stratagene] and purified on GgTGAAGGAGGT. The Cna1p–MYC expression plasmid pTJK93 was constructed by subcloning the 3xMYC sequences from pKB241 into pRS315 containing the promoter and coding sequences of CNA1 that had been amplified using the primers CTGGAGGGATCCGGATCCGGAGG. The Cna1p–MYC expression plasmid pTJK93 was constructed by subcloning the 3xMYC sequences from pKB241 into pRS315 containing the promoter and coding sequences of CNA1 that had been amplified using the primers CTGGAGGGATCCGGATCCGGAGG.

Log-phase cell cultures were harvested, extracted with trichloroacetic acid, and processed for SDS-PAGE, Western blotting, and ECL detection as described previously [Cunningham and Fink 1996]. The average β-galactosidase activity measured from three independent transfectants is plotted [±S.D.].

A conserved family of calcineurin regulators

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