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^+/Ca^{2+} 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 Bretscher 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^+/Ca^{2+} 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, conserved in fungi and animals, which can diminish calcineurin function in vivo and can bind and inhibit calcineurin in vitro directly. The Rcn1p family may serve as feedback regulators of calcineurin signaling and thereby modulate a variety of calcineurin-dependent functions.

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

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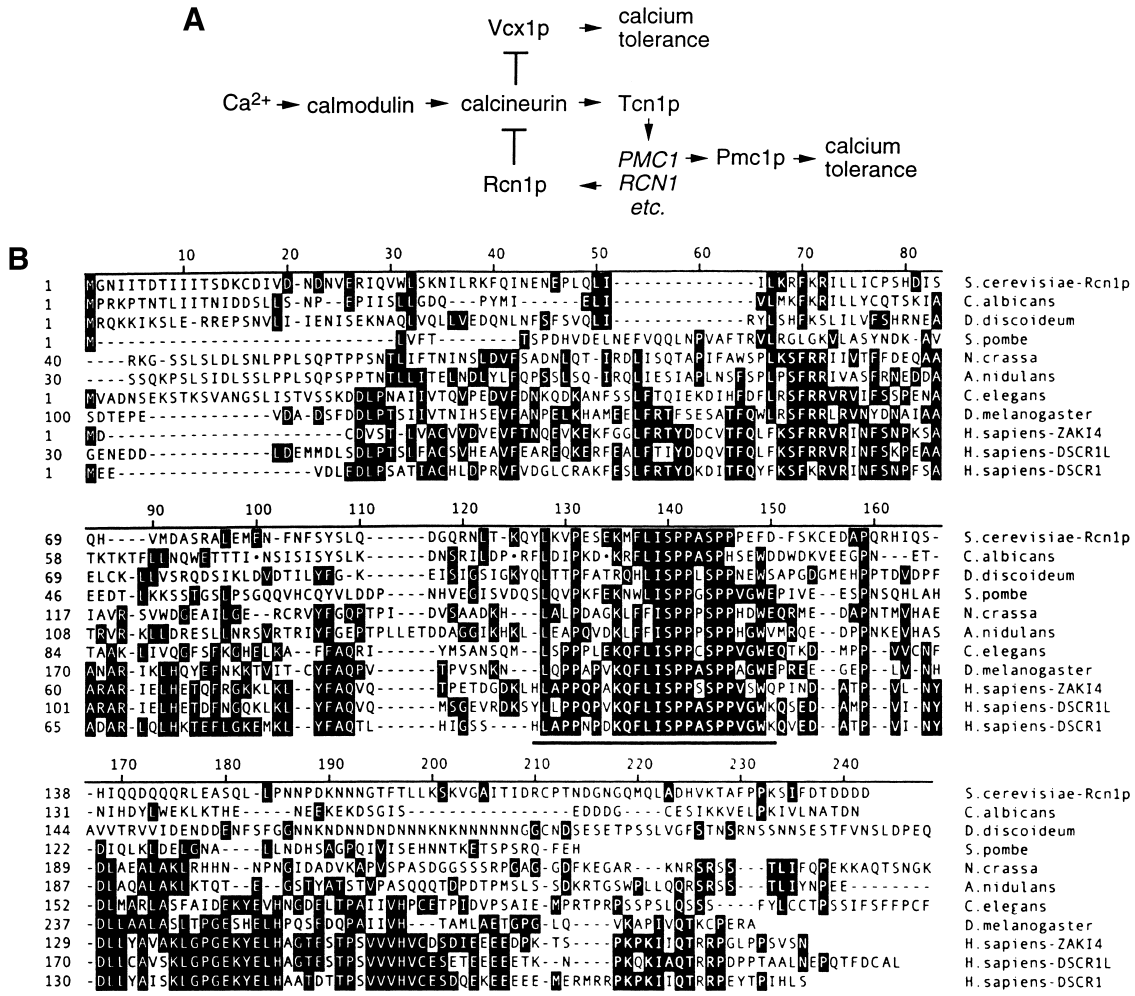

Figure 1. (*A*) Model of the calcium signaling pathway in yeast. Calcineurin activation by binding calcium and calmodulin inhibits the H^+/Ca^{2+} exchanger Vcx1p and induces expression of the Ca^{2+} pump Pmc1p through activation of the transcription factor Tcn1p. (*B*) Multiple sequence alignment of Rcn1p from *S. cerevisiae* and predicted proteins from other fungi (*Candida albicans*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Aspergillus nidulans*), protozoans (*Dictyostelium discoideum*), and animals (*Caenorhabditus elegans*, *Drosophila melanogaster*, and *Homo sapiens*) was generated using the Clustal algorithm. Residues identical in at least three of the seven sequences are highlighted and the most conserved central motif corresponding to the DS-24 peptide is underlined. GenBank accession numbers are NP_012763 (*S.c.*), Q09791 (*S.p.*), P53806 (*C.e.*), AAD33987 (*D.m.*), D83407 (*H.s.* ZAKI-4), AAF01684 (*H.s.* DSCR1L), and U85266 (*H.s.* DSCR1). Other sequences were obtained from ongoing genome sequencing projects (*C.a.*, *D.d.*, and *N.c.*) or from a compilation of EST sequences at GenBank (*A.n.* and *D.d.*). Note that some sequences were truncated at amino- and/or carboxyl termini and three small deletions (●) were introduced into the *C. albicans* sequence.

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^+/Ca^{2+} 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-

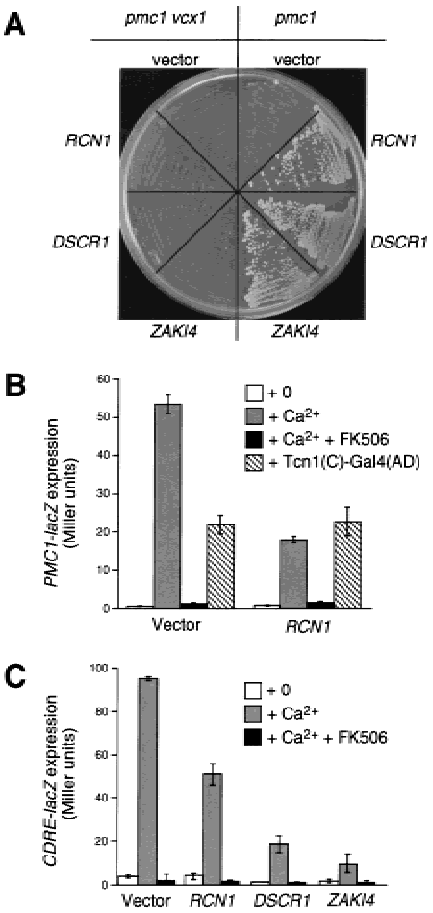

Figure 2. Rcn1p family members inhibit calcineurin function in yeast. (*A*) Overexpression of yeast *RCN1*, human DSCR1, or human *ZAKI4* genes in yeast blocked calcineurin-dependent inhibition of Vcx1p, thereby restoring growth to *pmc1* mutants but not *pmc1 vcx1* mutants in high calcium medium (YPD at pH 5.5 + 200 mM $CaCl_2$). Photographs of colonies were taken after two days incubation at 30°C. (*B*) Overexpression of *RCN1* inhibited calcineurin-dependent induction of *PMC1–lacZ*. Reporter gene expression was monitored in *pmc1 vcx1* double mutants carrying either empty vector or *RCN1* overexpression plasmid after 4 hr growth in medium supplemented with 100 mM $CaCl_2$ and 0.3 μM FK506 as indicated. Expression was also monitored in wild-type strains containing a constitutively active Tcn1p(C)–Gal4(DB) transcription factor. (*C*) Overexpression of *RCN1*, *DSCR1*, or *ZAKI-4* inhibited expression of *CDRE–lacZ*. Betagalactosidase assays were performed as indicated in *B*.

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 LxxPxxxKxFLISPPxSPPxxW. 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 glutathionine *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 $CaCl_2$ + 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-

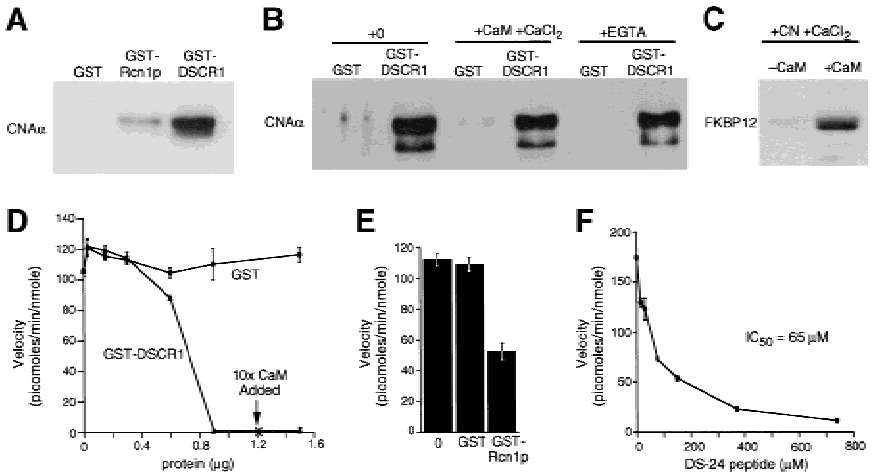

Figure 3. Binding and inhibition of calcineurin in vitro by Rcn1p, DSCR1, and DS-24 peptide. (*A*) GST, GST–Rcn1p, and GST–DSCR1 were immobilized on glutathione-sepharose beads and assayed for the ability to bind purified bovine brain calcineurin. (*B*) Immobilized GST–DSCR1, but not GST alone bound calcineurin in buffer A, or buffer A supplemented with either 2 mM $CaCl_2$ or 2 mM EGTA. (*C*) GST–DSCR1/ calcineurin bound to FK506/ FKBP12 in the presence of calmodulin and 2 mM $CaCl_2$. (*D–F*) Dephosphorylation of RII peptide by calcineurin was inhibited by purified GST–DSCR1, GST–Rcn1p (100 μg/ml), and DS-24 synthetic peptide. Tenfold excess calmodulin failed to reverse calcineurin inhibition by DSCR1.

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 was 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 *RCN1* 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 *cnb1* 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 was required for stimulation of Rcn1p expression above a basal level. Strong up-regulation of Rcn1p in yeast and DSCR1 in mammals in response to calcineurin signaling may therefore constitute a negative-feedback mechanism modulating calcineurin activity in vivo.

An additional effect of calcineurin on Rcn1p stability was revealed through analysis of Rcn1p–HA levels in calcineurin-deficient mutants. In nonsignaling conditions, accumulation of Rcn1p–HA was greatly reduced in *cnb1* mutants and *cna1 cna2* double mutants, relative to wild-type and *tcn1* mutants and *cmd1-6* mutants (Fig. 4B,C; data not shown). These results reveal a role of calcineurin A/B heterodimers on Rcn1p stability or basal expression that is independent of calcium, calmodulin, and Tcn1p. Basal expression of *RCN1–lacZ* was identical in wild-type and *cnb1* mutants, suggesting that Rcn1p stability might be increased by the presence of calcineurin in the cell (data not shown). Stability of Rcn1p–HA was difficult to quantitate in *cnb1* mutants because of the low initial levels of the protein. However, we noticed that addition of FK506 plus calcium caused Rcn1p–HA levels to decline in wild type, *tcn1* mutants, and *cmd1-6* mutants to levels approaching that of *cnb1* mutants whereas FK506 alone had no effect (Fig. 4B,C). Treatment with FK506 plus calcium accelerated the disappearance of Rcn1p–HA in wild-type cells that had been pretreated with cycloheximide to inhibit protein synthesis (Fig. 4D). These findings demonstrate that calcineurin stabilizes Rcn1p in vivo, even in the absence of activation by Ca^{2+}/calmodulin.

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

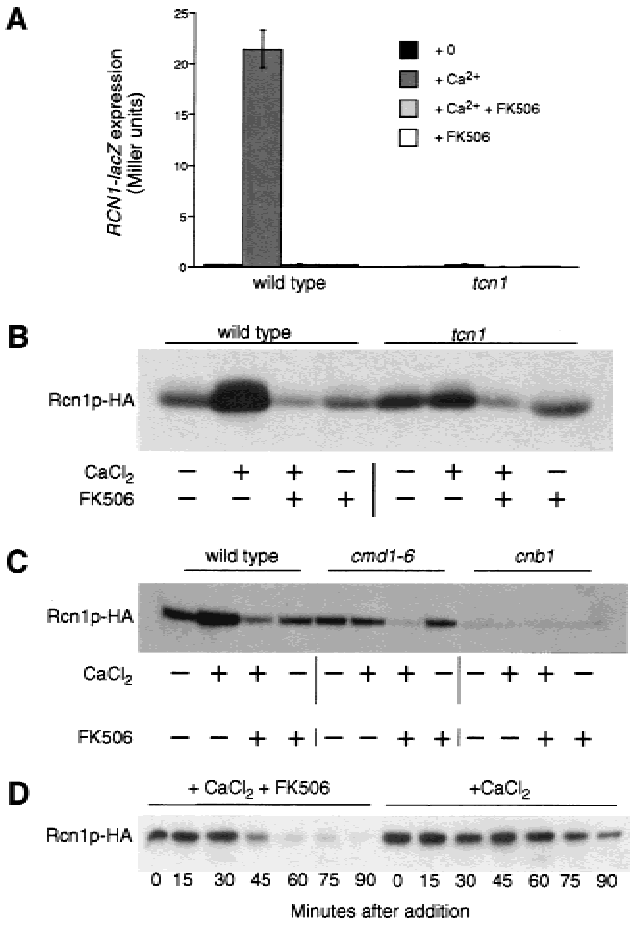

Figure 4. Calcineurin regulates *RCN1* transcription and Rcn1p stability in yeast. (*A*) Induction of a *RCN1–lacZ* reporter gene required calcium, calcineurin, and Tcn1p. A *RCN1–lacZ* reporter gene was introduced into wild-type and *tcn1* mutants. β-Galactosidase activity was assayed in three independent transformants following 4 hr growth at 30°C in YPD at pH 5.5 medium supplemented with 100 mM $CaCl_2$ and 0.3 μM FK506 as indicated. (*B*) Rcn1p–HA protein levels increased in response to calcium via the activation of calcineurin and Tcn1p. A low-dosage plasmid containing the epitope-tagged *Rcn1p–HA* gene was introduced into wild-type yeast and *tcn1* mutants. Cells were grown as in *A* and then total cell protein was fractionated by SDS-PAGE and analyzed by Western blotting. (*C*) Basal accumulation of Rcn1p–HA required calcineurin but not calcium, calmodulin, or Tcn1p. Total cell extracts were prepared and analyzed as in *B* using wild-type cells, *cnb1* mutants lacking the regulatory B subunit of calcineurin, or *cmd1–6* mutants lacking Ca^{2+}-binding sites in calmodulin. (*D*) FK506 destabilized Rcn1p–HA in high calcium conditions. Wild-type yeast expressing Rcn1p–HA were treated with 100 μM cycloheximide for 20 min and then treated with 100 mM $CaCl_2$ in either the presence or absence of FK506. Total cell protein was extracted at 15 min intervals and analyzed by Western blotting as in *B*.

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-

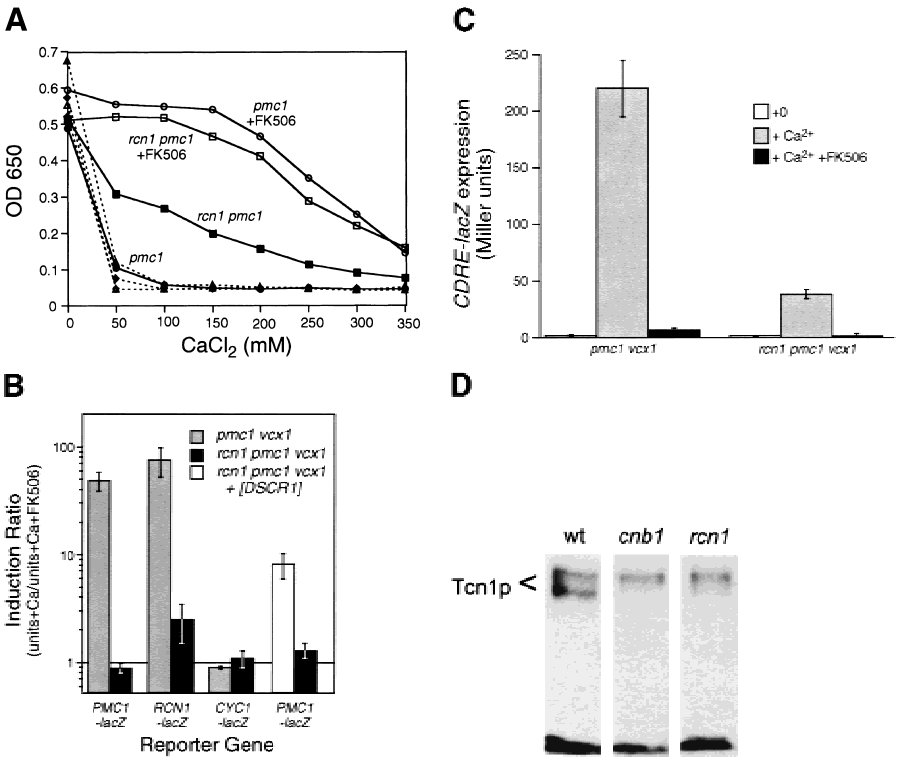

Figure 5. Rcn1p promotes calcineurin function and expression. (*A*) Rcn1p promotes calcineurin-dependent inhibition of Vcx1p. The optical density of *pmc1* mutant cultures grown for 20 hr at 30°C was measured at 650 nm and plotted as a function of added $CaCl_2$. Solid lines represent growth of *pmc1* or *rcn1 pmc1* mutants and dashed lines represent growth of *pmc1 vcx1* or *rcn1 pmc1 vcx1* mutants in the presence and absence of 0.3 μM FK506. (*B*) Calcineurin-dependent induction ratios were calculated for various reporter genes expressed in *pmc1 vcx1* double mutants or *rcn1 pmc1 vcx1* triple mutants as indicated. Expression of DSCR1 partially complemented the *rcn1* defect for *PMC1–lacZ* expression. (*C*) Induction of the calcineurin-dependent minimal *CDRE–lacZ* reporter gene was reduced in the absence of Rcn1p. The *CDRE–lacZ* reporter gene was introduced into *pmc1 vcx1* and *rcn1 pmc1 vcx1*. 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 $CaCl_2$ and 0.3 μM FK506 as indicated. (*D*) Western blot analysis of Tcn1p–HA extracted from wild type and *rcn1* mutants. Total protein was extracted from log phase cells grown in YPD medium at pH 5.5 and analyzed by Western blotting using 12CA5 monoclonal antibody.

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,

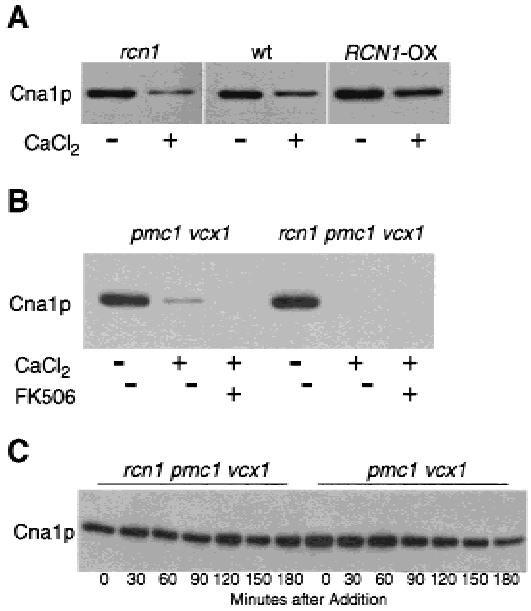

Figure 6. Down-regulation of calcineurin during calcium signaling conditions. (*A*) Cna1p expression correlates with Rcn1p in high calcium conditions. Western blots of Cna1p–MYC in *rcn1* mutants, wild type, and Rcn1p-overexpressing strains were performed on total cell protein after 4 hr growth in YPD medium at pH 5.5 supplemented with 100 mM $CaCl_2$ 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 $CaCl_2$ and 0.3 μM FK506. Total cell protein was extracted at 30 min intervals and analyzed by Western blotting as in Figure 3.

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).

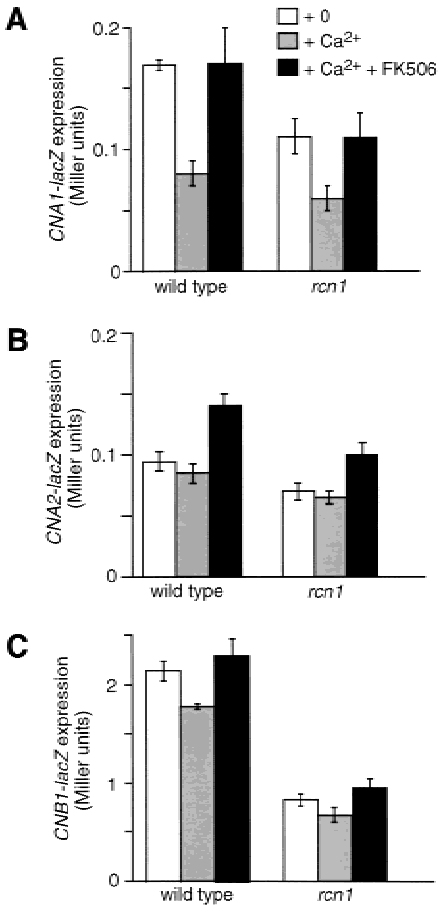

Figure 7. Involvement of Rcn1p in expression of calcineurin structural genes. (*A*) *CNA1–lacZ*, (*B*) *CNA2–lacZ*, and (*C*) *CNB1–lacZ* reporter genes were introduced into wild type and rcn1 deletion mutant. β-Galactosidase activity was assayed in three independent transformants following 4 hr growth at 30°C in YPD medium at pH 5.5 supplemented with 100 mM $CaCl_2$ and 0.3 μM FK506 as indicated.

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	*cnb1::LEU2*	Cunningham and Fink (1994)
K651	*pmc1::TRP1 vcx1*Δ	Cunningham and Fink (1996)
K665	*pmc1::TRP1 vcx1*Δ	Cunningham and Fink (1996)
TKY268	*rcn1::HIS3 pmc1::TRP1*	this study
TKY275	*rcn1::HIS3*	this study
TKY278	*rcn1::HIS3 pmc1::TRP1 vcx1*Δ	this study

All strains are isogenic to W303-1A (ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1).

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 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

Genetic procedures

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 $CaCl_2$ (Cunningham and Fink 1996). Of 24 plasmids that were recovered, two carried the Ca^{2+} pumps Pmc1p and Pmr1p, two carried Vcx1p, and 20 carried the uncharacterized yeast gene *RCN1/YKL159c* plus flanking sequences. Subcloning demonstrated the active gene was *RCN1*. The entire *RCN1* coding sequence was deleted from the genome by homologous recombination using plasmid pTJK39 linearized by *Eco*RI 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 pTJK39 was constructed by sequentially ligating two PCR products corresponding to the 5′ and 3′ flanking regions of *RCN1* that had been amplified from genomic DNA using the primers CCGAATTCGCCATACTATCAAATG and GGGGATCCCTGCAGTTCTGTGTTT (for 5′ sequences) and CCCTCGAGGATGGCGAGGCGATTTG and CCGAATTCGAATAGTAATAAAGAT (for 3′ sequences) into vector pRS303 digested with *Eco*RI + *Bam*HI and *Eco*RI + *Xho*I, respectively. The *RCN1–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 *RCN1* gene lacking a stop codon that had been amplified by PCR using primers GATCTTCACAAATCTTGGGG and GCCATCTTATCTAGAATCATCGTCATCAG. A downstream stop codon was reconstructed by generating a frameshift at the *Spe*I site in the polylinker. Plasmids bearing *CNA1–lacZ*, *CNA2–lacZ*, *CNB1–lacZ*, and *RCN1–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 pLGΔ178 using the following primers: for *CNA1*, CTCGAGAACGGAAGTGGCAACTTG and GGATCCCATTGGCGTTGAGAGTGT; for *CNA2*, CTCGAGTAAAGCTGGAGCCAAGAC and GGATCCCATTGCGGGTTCAAGAAG; for *CNB1*, CTCGAGGACTAGTTCAAAGGTAAA and AGATCTCATTTTAAGAAATAAAAATGC; and for *RCN1*, CTGCTCGAGCAGAAAATTCGTGAAC and GGGATCCCATCTGCAGTTCTGTGT. The *ZAKI4* expression plasmid pTJK1 was constructed by subcloning the *Xmn*I–*Nsi*I fragment of phZAKI-4-3.2 (courtesy of H. Seo, Nagoya University, Japan) into pRS425MET digested with *Sma*I + *Pst*I. 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 GCGAGGATCCGTATGGAGGAGGTGGACCTG and CCCTCACTCGAGGCTGAGGTGGATCGGCGTGTA into pRS426MET digested with *Bam*HI + *Xho*I. The GST–DSCR1 expression plasmid pTJK92 was constructed from the same PCR product after subcloning a *Bam*HI–*Bgl*II fragment into the *Bam*HI site of pGEX3X. The GST–Rcn1p expression plasmid

pTJK93 was constructed by subcloning into the *Bam*HI site of pGEX-3X a *Bgl*II + *Bam*HI-digested PCR product using primers CTGCAGGGGATCCGTATGGGTAATATTATAAC and CGAAATAGATCTGATGAAGAGGAGGT. The Cna1p–MYC expression plasmid pTJK91 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 CTCGAGAACGGAAGTGGCAACTTG plus GGATCCCATTGGCGTTGAGAGTGT and AGATCTAGAATGTCGAAAGACTTGAATTCT plus GGCTGATGGTGGTGTTCA. Plasmid pAMS342 (Stathopoulos and Cyert 1997) harboring the *CDRE–lacZ* reporter gene and pDM16 harboring the activated *TCN1* allele (Matheos et al. 1997) were described previously.

Protein analysis and purification

The consensus *Aspergillis 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 glutathione-agarose beads according to instructions (Pharmacia Biotech). β-Galactosidase assays were conducted as described previously (Cunningham and Fink 1996). The average β-galactosidase activity measured from three independent transformants is plotted (±S.D.).

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). Monoclonal antibodies 12CA5 (Boehringer) or 9E10 (Santa Cruz Biotechnology) were used to detect HA and MYC-tagged proteins respectively. Calcineurin was detected using polyclonal antibodies (gift of Claude Klee, NIH). For studies of Rcn1p–HA stability, yeast cells were grown to log phase overnight in SC minus uracil medium and then shifted to YPD medium at pH 5.5. After 1 hr incubation, 100 μg/ml cycloheximide was added and cultures were split and diluted into the same medium containing 100 mM $CaCl_2$ with or without 0.3 μM FK506. Samples were processed for Western blotting as described above.

Purified bovine brain calcineurin, calmodulin, and FKBP12 were obtained from Sigma Chemical Corp. Polyclonal anti-FKBP12 was from BIOMOL. Calcineurin binding experiments were performed as described (Lai et al. 1998) followed by SDS-PAGE and Western blot detection. Calcineurin activity assays using ^{32}P-labeled RII peptide as substrate were performed as described (Fruman et al. 1996; Sagoo et al. 1996) except 25 nM calcineurin was used in 15 min reactions at 30°C. The DS-24 peptide HLAPPNPDKQFLISPPASPPVGWKC was synthesized at Pocono Rabbit Farm and Laboratory.

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