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Domain Architecture of the Regulators of Calcineurin (RCANs) and Identification of a Divergent RCAN in Yeast▽†

Sohum Mehta,[1] Huiming Li,[2,3] Patrick G. Hogan,[3] and Kyle W. Cunningham[1]*

Department of Biology, Johns Hopkins University, 3400 N. Charles Street, Baltimore, Maryland 21218,[1] and Department of Pathology[2] and Immune Disease Institute,[3] Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

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Regulators of calcineurin (RCANs) in fungi and mammals have been shown to stimulate and inhibit calcineurin signaling in vivo through direct interactions with the catalytic subunit of the phosphatase. The dual effects of RCANs on calcineurin were examined by performing structure-function analyses on yeast Rcn1 and human RCAN1 (a.k.a. DSCR1, MCIP1, and calcipressin 1) proteins expressed at a variety of different levels in yeast. At high levels of expression, the inhibitory effects required a degenerate PxIxIT-like motif and a novel LxxP motif, which may be related to calcineurin-binding motifs in human NFAT proteins. The conserved glycogen synthase kinase 3 (GSK-3) phosphorylation site was not required for inhibition, suggesting that RCANs can simply compete with other substrates for docking onto calcineurin. In addition to these docking motifs, two other highly conserved motifs plus the GSK-3 phosphorylation site in RCANs, along with the E3 ubiquitin ligase SCF^{Cdc4}, were required for stimulation of calcineurin signaling in yeast. These findings suggest that RCANs may function primarily as chaperones for calcineurin biosynthesis or recycling, requiring binding, phosphorylation, ubiquitylation, and proteasomal degradation for their stimulatory effect. Finally, another highly divergent yeast RCAN, termed Rcn2 (*YOR220w*), was identified through a functional genetic screen. Rcn2 lacks all stimulatory motifs, though its expression was still strongly induced by calcineurin signaling through Crz1 and it competed with other endogenous substrates when overexpressed, similar to canonical RCANs. These findings suggest a primary role for canonical RCANs in facilitating calcineurin signaling, but canonical RCANs may secondarily inhibit calcineurin signaling by interfering with substrate interactions and enzymatic activity.

The Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin is conserved in most eukaryotic species. In humans and other mammals, calcineurin regulates several developmental and physiological processes (64), often through dephosphorylation and nuclear translocation of NFAT family transcription factors (17, 33). Inappropriate levels of calcineurin signaling are associated with various pathologies, from heart disease to neurological and psychological disorders (41, 48, 53). Calcineurin could therefore be a useful target in treating these diseases. In fungal pathogens of humans, calcineurin also promotes virulence and resistance to antifungal drugs (23, 56, 67). Specific inhibitors of calcineurin, such as FK506 and cyclosporine A, have been shown to augment the potency of azole class antifungal drugs (49, 50), but their utility in antifungal therapy is hampered by well-known activities against human calcineurin, causing immunosuppression and unwanted effects on other tissues (24, 46, 60). A better understanding of how calcineurin activity is regulated within different fungal and animal cell types could engender more-selective ways of controlling calcineurin function.

Calcineurin operates as a stable heterodimer composed of a catalytic A subunit and a regulatory B subunit. The A subunit of calcineurin contains a catalytic domain homologous to PPP family serine/threonine phosphoprotein phosphatases as well as domains important for binding the regulatory B subunit, binding Ca^{2+}/calmodulin, and autoinhibition (64). As cytosolic free Ca^{2+} concentrations rise, the binding of Ca^{2+} to the B subunit and Ca^{2+}/calmodulin to the A subunit causes conformational changes that displace the autoinhibitory domain from the active site and allow access to substrates (40, 78). Calcineurin activity can be further modified by inhibitors and scaffolding proteins, such as AKAP79/150, calsarcin, Bcl-2, and Cabin1/cain (reviewed in reference 47). Additionally, Cu-Zn superoxide dismutase (SOD1) is able to directly bind calcineurin, stimulating its phosphatase activity in vitro and in vivo (2).

Regulators of calcineurin (RCANs) are a novel family of calcineurin regulators that have been suggested as key factors contributing to Down syndrome in humans (7, 25). RCANs were first identified in fungi on the basis of their ability to interact with and inhibit calcineurin when overexpressed (27, 38). Overexpressed human RCAN1 also inhibited calcineurin function in cultured mammalian cells (25, 61). Additionally, RCAN1 overexpression in mouse heart was shown to prevent calcineurin-dependent cardiac hypertrophy in response to multiple stimuli (31, 62). Overexpression of endogenous RCANs in the nematode *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster* caused developmental and physiological defects that mimicked calcineurin deficiencies (34, 42, 73). In the budding yeast *Saccharomyces cerevisiae*, overexpression of either yeast Rcn1 or human RCAN1 inhibited the effects of

* Corresponding author. Mailing address: Department of Biology, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218. Phone: (410) 516-7844. Fax: (410) 516-5213. E-mail: kwc@jhu.edu.

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calcineurin on two independent targets (38). These findings suggest that calcineurin inhibition may be a conserved function of RCANs. Remarkably, expression of RCANs can be strongly induced in yeast, nematodes, mammals, and humans by calcineurin-dependent transcription factors (38, 42, 80). Mouse mutants lacking the *RCAN1* gene but retaining *RCAN2* and *RCAN3* genes also exhibited elevated calcineurin signaling in some tissues (66). These findings suggest that RCANs may serve as feedback inhibitors of calcineurin signaling.

However, RCANs also appear to stimulate calcineurin signaling in many circumstances. *RCAN1*$^{-/-}$ mice exhibited diminished calcineurin signaling in hypertrophic heart (75), and mice lacking both *RCAN1* and *RCAN2* genes exhibited calcineurin signaling deficiencies in many cell types (68). Complete loss of *RCN1* in yeast also resulted in diminished calcineurin signaling toward several targets (38). When expressed at low levels in yeast, human RCAN1 complemented the *rcn1* mutation and restored calcineurin signaling to the wild-type level. The idea that RCANs function as stimulators of calcineurin signaling was further advanced by the discovery that glycogen synthase kinase 3 (GSK-3)-related protein kinases directly phosphorylate a conserved site in RCANs after phosphorylation of an adjacent site by mitogen-activated protein kinases (1, 30, 76). GSK-3 kinases were required for the stimulatory effects of Rcn1 and RCAN1 on yeast calcineurin (30). Mutation of the GSK-3 phosphorylation site in yeast Rcn1 and human RCAN1 to nonphosphorylatable residues specifically abolished their ability to stimulate calcineurin signaling and increased their ability to inhibit calcineurin signaling when expressed at low levels (26, 30, 39). GSK-3-phosphorylated RCANs exhibit decreased affinity for calcineurin and increased affinity for 14-3-3 proteins (1) and the E3 ubiquitin ligase SCF^{Cdc4} (39), which destabilizes the proteins (26, 30). Thus, calcineurin signaling appears to be stimulated by low levels of phosphorylated RCANs.

The molecular mechanisms by which RCANs stimulate and inhibit calcineurin signaling have not been elucidated. The model most commonly reported in the field establishes RCANs simply as phosphorylation-sensitive calcineurin buffers. This model explains how calcineurin signaling can be increased by phosphorylation of RCANs, but it cannot explain the observations where RCAN deficiencies result in decreased calcineurin signaling. Mathematical modeling of this scheme fit poorly to experimental observations, except when the loss of RCANs was accompanied by a loss of calcineurin (71), an effect that has not been observed experimentally. An alternative model proposed that RCANs may function as phosphorylation-dependent chaperones that promote calcineurin maturation, recycling, or activation without altering calcineurin abundance (30). This model fits all the data when it is assumed that free phospho- and dephospho-RCANs can rebind calcineurin and either inhibit its catalytic activity or interfere with binding of other substrates. A similar model has been proposed previously for the stimulation and inhibition of protein phosphatase 1 by inhibitor-2 (3). The two models make different predictions about the behavior of mutant RCAN proteins. The inhibition-only model predicts that mutations in RCANs that increase or decrease calcineurin inhibition will have the opposite effect on calcineurin stimulation. The chaperone model predicts that inhibitory and stimulatory effects of RCANs may involve different noninteracting motifs that can be independently altered.

Here, we identify conserved domains and motifs in a large collection of eukaryotic RCANs, and we perform detailed structure-function studies of yeast and human RCANs expressed in yeast cells, where stimulatory and inhibitory effects can be easily quantified. Docking motifs conserved in RCANs and other substrates were necessary for inhibition of calcineurin signaling, suggesting that overexpressed RCANs effectively compete with other substrates. Several additional motifs in RCANs were specifically required for stimulatory effects and not required for inhibitory effects. Additionally, GSK-3 and the E3 ubiquitin ligase SCF^{Cdc4} were specifically required for Rcn1 to stimulate calcineurin signaling. These findings argue against the inhibition-only hypothesis that was used to explain the diminished calcineurin signaling in *cdc4-1* mutants (39) and support a model where phosphorylation and degradation of RCANs are required to promote calcineurin function. Finally, we identify a novel yeast protein, Rcn2, which is related to Rcn1 but has lost stimulation-specific motifs and stimulatory activity but not the docking motifs or inhibitory activity. These findings clarify the roles of RCANs in calcineurin signaling and open new opportunities for controlling calcineurin in the treatment of disease.

TABLE 1. Yeast strains used in this study

Name[a]	Description or genotype	Source or reference
K601	Wild-type W303-1A	77
K1373	*rcn1*::*ADE2 vcx1*Δ	This study
K1377	*rcn1*::*ADE2 mck1*::*HIS3 vcx1*Δ	This study
DDY156	*pmc1*::*HIS3 crz1*::*G418*	This study
MTY668	*cdc4-1*	58
RBY19	*rcn2*::*HIS3*	This study
RBY255	*rcn1*::*LEU2*	This study
RBY257	*rcn1*::*LEU2 rcn2*::*HIS3*	This study
SM005	*rcn1*::*ADE2 rcn2*::*NAT vcx1*Δ	This study
SM006	*cna1*Δ *cna2*::*NAT*	This study
SM012	*RCN2*-HA_3::*HIS3*	This study
SM013	*cnb1*::*LEU2 RCN2*-HA_3::*HIS3*	This study
SM014	*cna1*Δ *cna2*::*NAT RCN2*-HA_3::*HIS3*	This study
SM015	*crz1*::*G418 RCN2*-HA_3::*HIS3*	This study
SM016	*cmd1-6 RCN2*-HA_3::*HIS3*	This study
SM019	*cdc4-1 rcn1*::*HIS3*	This study
SM030	*RCN1*-HA_3::*LEU2*	This study
SM032	*RCN2*-HA_3::*LEU2*	This study
TKY275	*rcn1*::*HIS3*	38

[a] All strains are isogenic derivatives of strain K601/W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,14 leu2-3,112 trp1-1 ura3-1*).

MATERIALS AND METHODS

Yeast strains, plasmids, and growth media. All yeast strains used in this study (Table 1) were derived from the parental strain W303-1A (77), using standard methods of molecular and classical genetics. Strains were grown in rich yeast extract-peptone-dextrose (YPD) culture medium or synthetic complete (SC) culture medium lacking methionine or other nutrients, as described previously (70). In experiments involving the addition of $CaCl_2$, either YPD medium was buffered to pH 5.5 by addition of 5 mM succinic acid or SC medium containing half the standard amount of yeast nitrogen base was prepared.

The plasmids and oligonucleotides used in this study are listed in Tables 2 and 3. Plasmid pSM010, used for construction of all RCAN expression plasmids, was constructed by PCR amplification of a fragment encoding the triple-hemagglutinin (HA_3) epitope and stop codon from pZH300 (30), using the forward primer

TABLE 2. Plasmids used in this study

Name	Description	Source or reference
pAMS342	*CDRE-lacZ*	72
pSM010	p415MET25-HA_3	This study
pSM012	p415MET25-*RCN1*(1-196)-HA_3	This study
pSM013	p415MET25-*RCN1*(1-172)-HA_3	This study
pSM015	p415MET25-*RCN1*-S113A(1-196)-HA_3	This study
pSM016	p415MET25-*RCN1*-S113A(1-172)-HA_3	This study
pSM019	p415MET25-RCAN1.4-HA_3	This study
pSM022	p415MET25-RCAN1.4(1-153)-HA_3	This study
pSM023	p415MET25-*RCN1*-PxAxAT-HA_3	This study
pSM029	p415MET25-*RCN1*(99-211)-HA_3	This study
pSM031	p415MET25-*RCN1*(120-211)-HA_3	This study
pSM036	p415MET25-*RCN1*-S113A(99-211)-HA_3	This study
pSM042	p415MET25-RCAN1.4(94-199)-HA_3	This study
pSM044	p415MET25-RCAN1.4(115-199)-HA_3	This study
pSM065	p415MET25-*RCN2*-HA_3	This study
pSM066	p415MET25-*RCN2*(1-255)-HA_3	This study
pSM109	p415MET25-RCAN1.4-PxAxAT-HA_3	This study
pSM110	p415MET25-*RCN2*-PxAxAT-HA_3	This study
pSM112	p415MET25-*RCN2*(86-265)-HA_3	This study
pSM115	p415MET25-RCAN1.4-S108A-HA_3	This study
pSM116	p415MET25-*RCN1*-PVIVIT-HA_3	This study
pSM117	p415MET25-*RCN2*-PVIVIT-HA_3	This study
pSM118	p415MET25-RCAN1.4-PVIVIT-HA_3	This study
pSM119	p415MET25-*RCN1*-S113A,PVIVIT-HA_3	This study
pSM121	p415MET25-RCAN1.4-S108A,PVIVIT-HA_3	This study
pSM132	p415MET25-*RCN1*-S113A,PxAxAT-HA_3	This study
pSM133	p415MET25-*RCN1*-ExxP-HA_3	This study
pSM134	p415MET2-*RCN1*-S113A,ExxP-HA_3	This study
pSM135	p415MET25-*RCN1*-LxxP-HA_3	This study
pSM136	p415MET25-*RCN1*-S113A,LxxP-HA_3	This study
pSM142	p415MET25-RCAN1.4(1-180)-HA_3	This study
pSM143	p415MET25-RCAN1.4-S108A(1-180)-HA_3	This study
pSM146	p415MET25-RCAN1.4-S108A(1-153)-HA_3	This study
pSM147	p415MET25-RCAN1.4-S108A(94-199)-HA_3	This study
pSM151	p415MET25-RCAN1.4-ExxP-HA_3	This study
pSM153	p415MET25-RCAN1.4-LxxP-HA_3	This study
pSM154	p415MET25-RCAN1.4-S108A,LxxP-HA_3	This study
pSM176	p415MET25-*RCN1*-LxxP,PVIVIT-HA_3	This study
pSM177	p415MET25-*RCN1*-LxxP,S113A, PVIVIT-HA_3	This study
pSM178	p415MET25-RCAN1.4-LxxP,PVIVIT-HA_3	This study
pSM179	p415MET25-RCAN1.4-LxxP,S108A, PVIVIT-HA_3	This study
pSM200	p405MET25-*RCN1*-HA_3	This study
pSM201	p405MET25-*RCN2*-HA_3	This study
pZH300	p415MET25-*RCN1*-HA_3	30
pZH301	p415MET25-*RCN1*-S113A-HA_3	30

3HASalI-5′ and the reverse primer 3HAXhoI-3′, followed by digestion with SalI and XhoI and ligation into p415MET25 (54). The resulting p415MET25-HA_3 plasmid was digested with PstI and SalI and ligated with similarly digested PCR products of yeast *RCN1* and *RCN1*-S113A that had been amplified from pZH300 and pZH301 (30), respectively, using the forward primer Rcn1PstI-5′and the reverse primers Rcn1(1-196)SalI-3′ (for inserts truncated before the TxxP motif [pSM012 and pSM015]) and Rcn1(1-172)SalI-3′ (for inserts truncated before the PxIxIT-like motif [pSM013 and pSM016]). N-terminal truncations lacking the RRM and SP domains were generated using forward primers Rcn1(+99)PstI-5′ (pSM029 and -036) and Rcn1(+120)PstI-5′ (pSM031), respectively, and the reverse primer Rcn1SalI-3′. Wild-type yeast *RCN2* (pSM065) was amplified from yeast genomic DNA by using forward primer Rcn2PstI-5′ and reverse primer Rcn2SalI-3′. A fragment lacking the C-terminal PxIxIT-like motif (pSM066) was also amplified, using the reverse primer Rcn2(1-255)SalI-3′, and an N-terminally truncated fragment lacking the RRM domain (pSM112) was amplified using the forward primer Rcn2(+86)PstI-5′. The full-length exon 4 variant of human RCAN1 (pSM019) was amplified from DSCR1-4pBS (courtesy of X. Estivill, I.R.O., Barcelona, Spain), using forward primer RCAN1PstI-5′ and reverse primer RCAN1SalI-3′. The RCAN1(1-153)SalI-3′ (pSM022) or RCAN1(1-180)SalI-3′ (pSM142) reverse primer was also used to amplify inserts lacking the PxIxIT-like or TxxP motif, respectively. N-terminal truncations were constructed using the RCAN1(+94)PstI-5′ (pSM042) or RCAN1(+115)PstI-5′ (pSM044) forward primer with the reverse primer RCAN1SalI-3′, generating fragments lacking the RRM or SP domain, respectively. Site-directed mutagenesis was performed to introduce the S108A mutation at the GSK-3 phosphorylation site of RCAN1 (pSM115, -143, -146, and -147). Core hydrophobic residues in the PxIxIT-like motifs of Rcn1 (GAITID), Rcn2 (PSITVN), and RCAN1 (PSVVVH) were replaced with alanines (GAATAD, PSATAN, and PSAVAH), yielding plasmids pSM023, -109, -110, -132, and -180. Each PxIxIT-like motif was also mutated in its entirety to the sequence PVIVIT (pSM116, -117, -118, -119, and -121). Alanine substitutions were introduced into the ExxP (pSM133 and -151) and LxxP (pSM135, -136, -153, -154, -176, -177, -178, and -179) motifs of Rcn1 and RCAN1. All site-directed mutagenesis was performed using a QuikChange site-directed-mutagenesis kit (Stratagene) according to the manufacturer's instructions. Site-directed mutants were verified by DNA sequencing. To generate pSM200 and pSM201, ~2.8-kb SacI-BstEII fragments cut from pZH300 and pSM065 (respectively) were subcloned into the yeast integrating plasmid pRS405.

Fluorescence polarization. Binding of synthetic peptides to the PxIxIT-docking site of calcineurin was monitored in competitive binding assays in which unlabeled peptides were titrated at increasing concentrations into a reaction mixture containing 100 nM Oregon Green-labeled 14-mer PVIVIT peptide and 1 to 4 μM of a recombinant catalytic domain of human calcineurin or a truncated, constitutively active form of a recombinant human calcineurin heterodimer (6). The mixtures were incubated until equilibrium was achieved and analyzed for fluorescence polarization (43), using either a Synergy 2 multimode microplate reader (BioTek Instruments, Highland Park, VT) or an Analyst plate reader (LJL Biosystems). Samples with no competing peptide or with no calcineurin were used to define the dynamic range of the assay. For each peptide tested, the 50% inhibitory concentration (determined from nonlinear regression of the experimental data by use of four-parameter sigmoid functions) was converted to K_i (apparent K_d) by using an online calculator specifically designed for competitive fluorescence polarization assays (55).

β-Galactosidase assays. To assess calcineurin signaling, yeast strains were transformed with the plasmid pAMS342, bearing the *CDRE-lacZ* reporter gene (72); grown overnight to mid-log phase in SC medium lacking uracil; harvested by centrifugation; and resuspended in modified YPD or SC medium containing 100 mM $CaCl_2$ with or without 0.3 μM FK506. After shaking for 4 hours at 30°C, cells were harvested, permeabilized, and assayed for β-galactosidase activity as described previously (19). In some cases, expression of yeast Rcn1 or human RCAN1 (or their derivatives) was repressed to various degrees by growth overnight in SC medium containing 0 to 8 mg/ml (4×) L-methionine. The saturated cultures were diluted approximately 40-fold into identical medium and regrown to early log phase (~6 h) before addition of 100 mM $CaCl_2$ and measurement of β-galactosidase activity. Unless otherwise indicated, all assays were performed on at least three independent transformants, normalized to cell density, and plotted as means ± standard deviations (SD).

VCX1-dependent growth assays. Calcineurin-dependent inhibition of Vcx1 function was assessed by monitoring the growth of *pmc1 crz1* double mutants transformed with Rcn1 or RCAN1 expression plasmids. Transformed strains were grown overnight at 30°C in SC medium lacking leucine. Saturated cultures were then diluted to an optical density of 0.5 (at 600 nm) using fresh culture medium, subjected to fivefold serial dilutions in the same medium, pinned onto 2% agar plates containing modified SC medium lacking leucine and containing 300 mM $CaCl_2$ plus the indicated concentrations of methionine, and incubated at room temperature for 4 days before being photographed.

Protein extraction and Western blotting. Equal numbers of log-phase cells (normalized for optical density), grown and treated as described elsewhere in the text, were lysed for 10 min at 0°C with 2 M NaOH and 7.4% beta-mercaptoethanol and extracted with 50% trichloroacetic acid for 1 hour at 0°C. Trichloroacetic acid-insoluble material was pelleted in a benchtop centrifuge (for 10 min at 13,000 rpm), washed once with 1 ml of ice cold water, resuspended in 50 μl of the same, and dissolved in 60 μl of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 0.02% beta-mercaptoethanol). Extracts were then boiled for 5 min and centrifuged at room temperature for 10 min, and the detergent-soluble proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride, and probed with anti-HA monoclonal antibodies (16B12; Covance). Cross-reacting proteins were visualized using a HyGLO chemiluminescence detection kit (Denville) and HyBlot CL autoradiography

TABLE 3. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)[a]
3HASalI-5′	GATGATGTCGACTCTAGATACCCATAC
3HAXhoI-3′	GACCTCGAGCTAGCTAGTTCTAGAAGC
Rcn1PstI-5′	GCCCTGCAGATGGGTAATATTATAACGGAT
Rcn1(1-196)SalI-3′	GATGATGTCGACTTTAACGTGGTCCGCTAA
Rcn1(1-172)SalI-3′	GATGATGTCGACCACCTTTGATTTCAATAA
Rcn1(+99)PstI-5′	GCCCTGCAGATGCAATACTTGAAGGTA
Rcn1(+120)PstI-5′	GCCCTGCAGATGCCCGAATTCGATTTC
Rcn1SalI-3′	GATGATGTCGACATCATCGTCATC
Rcn2PstI-5′	GCCCTGCAGATGGCAAACCAAAAGCAAATG
Rcn2SalI-3′	GATGATGTCGACATGGAAAAACTCGTTAAC
Rcn2(1-255)SalI-3′	GATGATGTCGACACTTTTTGGAGGATTTGA
Rcn2(+86)PstI-5′	GCCCTGCAGATGAAGTTATTTGTAACTGAG
RCAN1PstI-5′	GCCCTGCAGATGCATTTTAGAAACTTTAAC
RCAN1SalI-3′	GATGATGTCGACGCTGAGGTGGATCGGCGT
RCAN1(1-153)SalI-3′	GATGATGTCGACAGTGGTGTCAGTCGCTGC
RCAN1(1-180)SalI-3′	GATGATGTCGACCTTAGGTCTCCTCATTCTTTC
RCAN1(+94)PstI-5′	GCCCTGCAGATGTCACACCTGGCTCCG
RCAN1(+115)PstI-5′	GCCCTGCAGATGGTGGGATGGAAACAAGTG
RCAN1(S108A)F	GCAGTTCCTGATC**GC**CCCTCCCGCCTCTC
RCAN1(S108A)R	GAGAGCCGGGAGG**GGC**GATCAGAAACTGC
Rcn1(AxA)F	CAAAGGTGGGCGCA**GCA**ACC**GCA**GATAGATGTCCCAC
Rcn1(AxA)R	GTGGGACATCTATC**TGC**GGT**TGC**TGCGCCCACCTTTG
Rcn2(AxA)F	CCAAAAAGTCCAAGC**GCA**ACG**GCA**AACGAGTTTTTCCAT
Rcn2(AxA)R	ATGGAAAAACTCGTT**TGC**CGT**TGC**GCTTGGACTTTTTGG
RCAN1(AxA)F	GACACCACTCCCAGC**GCA**GTG**GCA**CATGTATGTGAGAGT
RCAN1(AxA)R	ACTCTCACATACATG**TGC**CAC**TGC**GCTGGGAGTGGTGTC
Rcn1(VIVIT)F	TTGAAATCAAAGGTG**CCTGTTATTGTTATTACT**AGATGTACCACGAAC
Rcn1(VIVIT)R	GTTCGTGGTACATCT**AGTAATAACAATAACAGG**CACCTTTGATTTCAA
Rcn2(VIVIT)F	CCTCCAAAAAGT**CCAGTTATAGTTATTACT**GAGTTTTTCCAT
Rcn2(VIVIT)R	ATGGAAAAACTC**AGTAATAACTATAACTGG**ACTTTTTGGAGG
RCAN1(VIVIT)F	GCGACTGACACCACT**CCCGTTATTGTGATTACT**GTATGTGAGAGTGAT
RCAN1(VIVIT)R	ATCACTCTCACATAC**AGTAATCACAATAACGGG**AGTGGTGTCAGTCGC
Rcn1(ExxP-ala)F	TTCGATTTCAGCAAATGT**GCAGCAGCGGCA**CAAAGGCATATACAAAGT
Rcn1(ExxP-ala)R	ACTTTGTATATGCCTTTG**TGCCGCTGCTGC**CACATTTGCTGAAATCGAA
RCAN1(ExxP-ala)F	GTGGGATGGAAACAAGTG**GCAGCTGCGGCCGCA**GTCATAAACTATGATCTC
RCAN1(ExxP-ala)R	GAGATCATAGTTTATGAC**TGCGGCCGCAGCTGC**CCACTTGTTTCCATCCCAC
Rcn1(LxxP-ala)F	GAATTTAACTAAACAATAC**GCG**AAGGTA**GCA**GAGAGTGAAAAAATGTTCC
Rcn1(LxxP-ala)R	GGAACATTTTTTCACTCTC**TGC**TACCTT**CGC**GTATTGTTTAGTTAAATTC
RCAN1(LxxP-ala)F	CATAGGAAGCTCACAC**GCG**GCTCCG**GCA**AATCCAGACAAGCAGT
RCAN1(LxxP-ala)R	ACTGCTTGTCTGGATT**TGC**CGGAGC**CGC**GTGTGAGCTTCCTATG

[a] Cleavage sites for resrtiction endonucleases are underlined. Altered codon sequences used in site-directed mutagenesis are in boldface.

film (Denville) after incubation with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (Jackson ImmunoResearch).

Phylogenetic analyses. RCANs from many species of fungi and animals were identified using BLAST (4) searches of publicly available databanks, using the standard genetic code. Multiple sequence alignments were performed using CLUSTAL W (74) after truncation of the C termini of most proteins just before the PxIxIT-like motif. A simple neighbor-joining tree was generated from the multiple-sequence alignment by using MEGAlign 7.0 (DNASTAR, Inc.) and colored according to species groups.

RESULTS

Domain structure of RCANs. To identify features, motifs, and domains that are conserved among RCAN proteins, we assembled a collection of predicted RCAN sequences from dozens of animal, fungal, and protozoal species and performed a multiple-sequence alignment by using CLUSTAL W (see Fig. S1 in the supplemental material). Four highly conserved regions separated by poorly conserved indels were evident, as represented schematically in Fig. 1A: (i) an N-terminal domain that was recently shown to adopt a three-dimensional fold resembling an RRM domain (http://www.pdb.org/pdb/explore.do?structureId=1WEY), (ii) a highly conserved SP domain containing the site for phosphorylation by GSK-3 (76), (iii) a [PG]x[IV]x[IVL][EDNHT] motif resembling the well-known PxIxIT motif responsible for docking many substrates to calcineurin (5), and (iv) a previously unrecognized C-terminal TxxP motif of unknown function. With the exception of three closely related budding yeasts (*Candida albicans*, *Candida dubliniensis*, and *Candida tropicalis*), two fission yeasts (*Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus*), and a protist (*Dictyostelium discoideum*), the TxxP motif was universally conserved in fungal and animal RCANs.

A segment of human RCAN1 was previously assumed to function as a PxIxIT-like motif (PKIIQT, residues 181 to 186) (8, 12, 61). However, this sequence does not conform to the consensus sequences of known PxIxIT motifs, is not conserved outside of mammals, and overlaps the conserved TxxP motif at residues 186 to 189. To determine if the conserved PxIxIT-like motif and the nonconserved PKIIQT motif can function like PxIxIT motifs, synthetic peptides corresponding to residues 150 to 163 and 178 to 191 of RCAN1 were tested in vitro for their ability to compete with the binding of the fluorescent PVIVIT peptide to human calcineurin, using a fluorescence

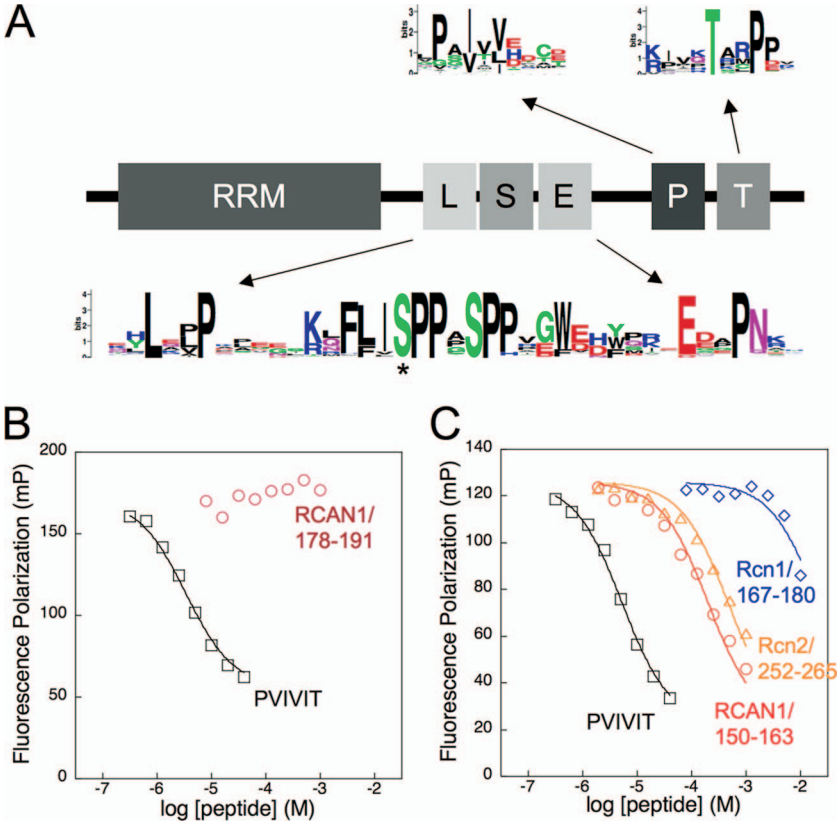

FIG. 1. Schematic representation of domains and motifs conserved in RCANs. (A) The LxxP motif (L), GSK-3 phosphorylation site (S; *), ExxP motif (E), PxIxIT-like motif (P), and TxxP motif (T) are indicated. Sequence logos were generated from a multiple-sequence alignment (see Fig. S1 in the supplemental material) by using WebLogo (18). (B, C) Identification of a conserved PxIxIT-like motif in RCANs. The effects of synthetic peptides on the polarization of a fluorescently labeled PVIVIT peptide bound to purified human calcineurin are shown. Unlabeled synthetic peptides corresponding to conserved PxIxIT-like motifs from human RCAN1 and yeast Rcn1 and Rcn2 (RCAN1/150-163, Rcn1/167-180, and Rcn2/252-265) and the PKIIQTxxP motif of human RCAN1 (RCAN1/178-191) were added at various concentrations, as described in Materials and Methods.

polarization assay (43). The peptide containing PKIIQT (RPKPKIIQTRRPEY) did not compete with fluorescent PVIVIT at concentrations up to 1 mM (Fig. 1B), whereas the peptide containing the PxIxIT-like motif (TDTTPSVVVHVCES) competed, with a K_i of approximately 45 μM (Fig. 1C). A synthetic peptide spanning the PxIxIT-like motif of yeast Rcn1 (sequence KSKVGAITIDRCPT) was also able to compete with binding of the PVIVIT peptide to human calcineurin, albeit with ~75-fold-lower affinity than the homologous segment of RCAN1 (Fig. 1C). These results, together with those described below, suggest that the PxIxIT-like motif present in all RCANs (consensus sequence [PG]x[IV]x[IVL][EDNHT]) can bind to the substrate-docking groove of calcineurin much more effectively than the previously proposed nonconserved, nonconforming PKIIQT sequence of RCAN1.

System for measuring RCAN functions in yeast. Two different readouts of calcineurin signaling in yeast have been used to study the stimulatory and inhibitory effects of RCANs (Fig. 2A). One measures the expression of a *CDRE-lacZ* reporter gene that can be induced by the calcineurin-dependent transcription factor Crz1 (52, 72). The other measures the growth of *pmc1* mutants in high-calcium-concentration environments, which depends on the calcineurin-sensitive Ca^{2+}/H^{+} exchanger Vcx1 (19). Both readouts were used as described below to estimate the stimulatory and inhibitory effects of RCAN-related proteins.

The inhibitory effects of RCANs are observed in yeast only when the proteins are overexpressed in strains containing wild-type Rcn1. The stimulatory effects of RCANs are observed only when the proteins are expressed at physiological levels in strains lacking Rcn1. To determine how the PxIxIT-like motif and other motifs in RCANs may influence calcineurin signaling in vivo, wild-type and mutant RCANs were expressed from low-copy-number plasmids under the control of a methionine-repressible promoter derived from the *MET25* gene (54). By use of this expression system, a 10-fold range of Rcn1 expression was achieved by varying the concentration of methionine in the medium (Fig. 2B). This overlapped the range of expression observed for Rcn1 under the control of its own promoter (lanes 1 and 2). Overlapping expression ranges were also achieved for Rcn2 (Fig. 2C), a protein that will be discussed later.

Domains of Rcn1 necessary for inhibition of calcineurin signaling in yeast. GSK-3 and the GSK-3 phosphorylation site are required for the stimulatory effects but not the inhibitory effects of RCANs on calcineurin signaling in yeast (30). In

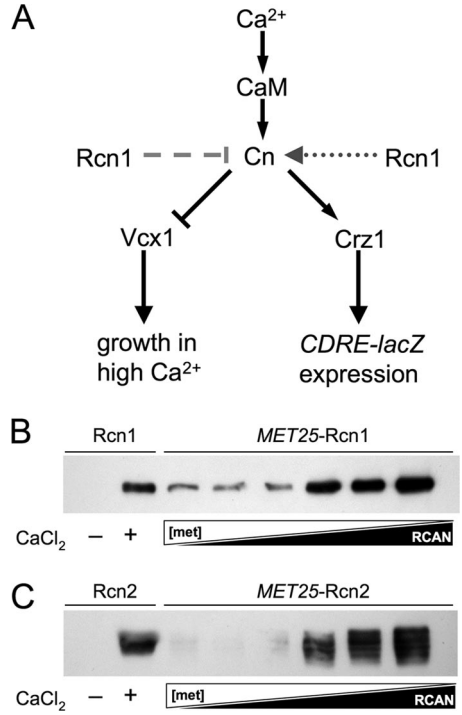

FIG. 2. A system for evaluating RCAN function in yeast. (A) Diagram showing two independent readouts of calcineurin signaling in yeast. Calcineurin inhibits the ability of Vcx1 to promote growth in high-Ca^{2+}-concentration environments (19), and calcineurin activates the Crz1 transcription factor, which induces many genes such as the *CDRE-lacZ* reporter (52, 72). In high-Ca^{2+}-concentration environments, factors that inhibit calcineurin signaling will increase growth and decrease gene expression and factors that stimulate calcineurin signaling will slow growth and induce gene expression. (B) Variable expression of Rcn1 (top) and Rcn2 (bottom) expressed in strain DDY156 (*pmc1 crz1* double knockout mutant) from the methionine-repressible *MET25* promoter. Cells were grown in medium supplemented with various amounts of L-methionine as described in Materials and Methods and analyzed by Western blotting. For comparison, wild-type cells (strain K601) bearing the same epitope tag-encoding sequence in the chromosomal genes for Rcn1 and Rcn2 were analyzed before and after exposure to a high-calcium-concentration environment (100 mM $CaCl_2$ for 4 h).

order to focus specifically on inhibitory mechanisms without interference from stimulatory mechanisms, structure-function analyses were first performed on the Rcn1-S113A mutant that lacks the GSK-3 phosphorylation site and calcineurin stimulation but retains the ability to inhibit calcineurin signaling. As described above, each derivative of Rcn1-S113A was expressed at high or low levels in yeast by growth in medium lacking or containing methionine (Fig. 3B). For convenience, the Vcx1-dependent growth assay was used to measure calcineurin inhibition (Fig. 3A), though similar results were obtained using the *CDRE-lacZ* reporter (data not shown).

At both low and high levels of expression, full-length Rcn1-S113A increased the growth of *pmc1* mutants on medium containing high concentrations of calcium chloride relative to cells bearing the empty expression plasmid (Fig. 3A, rows 1 and 10), indicating that Rcn1-S113A could inhibit calcineurin signaling at either level of expression. In contrast, Rcn1-S113A proteins containing two alanine substitutions within the PxIxIT-like motif were unable to grow in high-calcium-concentration medium at either level of expression (row 6). Similar substitutions in the PxIxIT motifs of other calcineurin substrates have been shown to block docking onto and dephosphorylation by calcineurin (20). Therefore, docking of Rcn1-S113A onto calcineurin via the conserved PxIxIT-like motif was required for calcineurin inhibition.

To elucidate the role of the other conserved domains, a series of N-terminal and C-terminal truncations of Rcn1-S113A was constructed and tested for calcineurin inhibition using the same readout. A C-terminal truncation that removed the conserved TxxP motif did not alter the inhibitory activity of Rcn1-S113A (Fig. 3A, row 4), but a shorter protein that additionally lacked the PxIxIT-like motif lost nearly all of its ability to inhibit calcineurin (row 5), consistent with the effect of the I-to-A substitution mutations (row 6). An N-terminal truncation that removed the conserved RRM domain significantly diminished the inhibitory activity of Rcn1-S113A (row 2), but this effect can be attributed to the lower expression level of this variant relative to those of the others (Fig. 3B). Interestingly, a larger N-terminal truncation that also removed the conserved SP domain exhibited no inhibitory activity (row 3), even though the protein was expressed at nearly the same level as the ΔRRM variant. These findings suggest that the SP domain and the PxIxIT motif are jointly required for inhibition of calcineurin signaling. The SP domain contains two conserved motifs in addition to the GSK-3 phosphorylation site. Replacement of the conserved ExxP motif with AxxA had no effect on the inhibitory activity of Rcn1-S113A (data not shown). On the other hand, a similar substitution of the conserved LxxP motif weakened inhibitory activity at both levels of expression (row 8). The effects of this mutation were partially reversed by replacement of the low-affinity PxIxIT-like motif with the high-affinity PVIVIT sequence (row 9). These findings show that both the PxIxIT-like motif and the LxxP motif in the SP domain contribute to the inhibitory activity of Rcn1-S113A on calcineurin signaling.

These same mutations were also introduced into wild-type Rcn1 to determine their roles in the inhibition of calcineurin signaling by the phosphorylatable protein. Unlike Rcn1-S113A, wild-type Rcn1 was unable to inhibit calcineurin signaling at the lower level of expression (Fig. 3C), even though the expression of Rcn1 was similar to that of Rcn1-S113A (Fig. 3B and D). As above, inhibition required the PxIxIT-like motif but not the TxxP motif or the RRM domain (Fig. 3C, rows 1, 2, and 4 to 6). The inhibition of calcineurin signaling also required the SP domain and the LxxP motif within it (rows 3 and 8). Replacement of the PxIxIT-like motif with a PVIVIT sequence increased the inhibitory activity of Rcn1 (row 7) as well as the derivative lacking the LxxP motif (row 9) without altering expression in either case. The inverse correlation between docking affinity and calcineurin signaling observed across all these experiments suggests that overexpressed Rcn1 inhibits calcineurin signaling in vivo by binding to substrate-docking sites and interfering with the productive binding of other substrates.

Domains of Rcn1 necessary for stimulation of calcineurin signaling in yeast. When expressed at low levels in *rcn1* knockout mutants of yeast, Rcn1 stimulated calcineurin-dependent expression of *CDRE-lacZ* approximately threefold (38). This

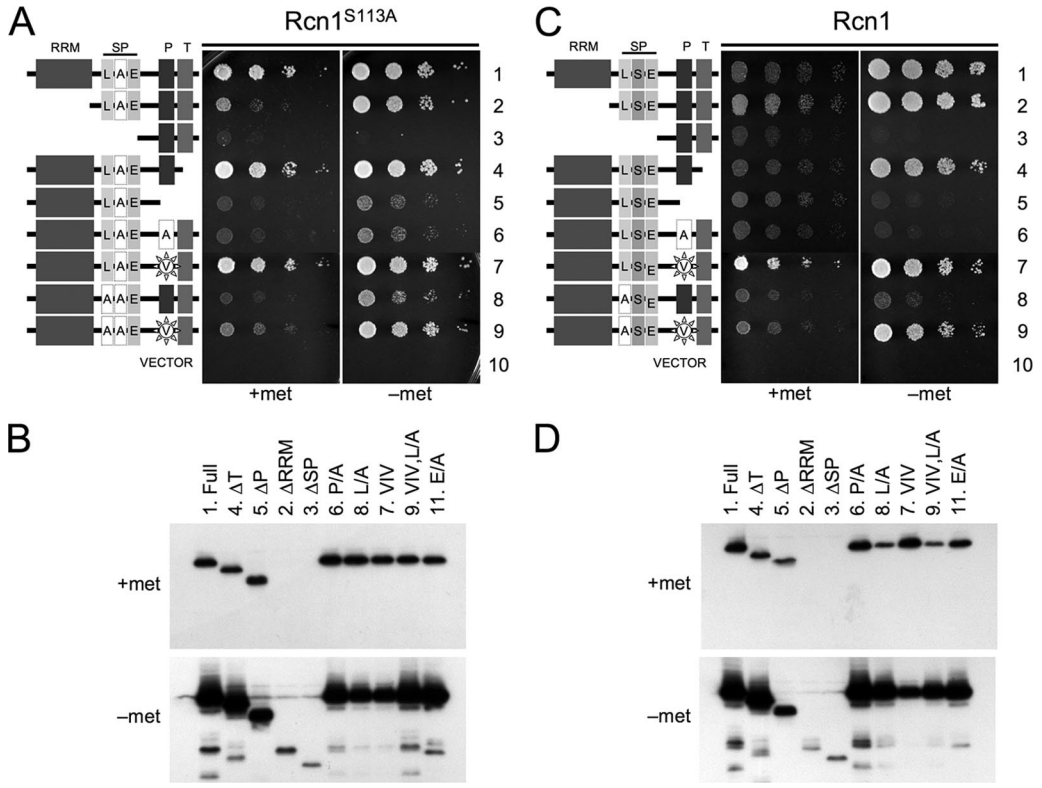

FIG. 3. Domains of Rcn1 required for calcineurin inhibition. Yeast strain DDY156 (*pmc1 crz1* double knockout mutant) transformed with derivatives of nonphosphorylatable Rcn1-S113A (A, B) or phosphorylatable Rcn1 (C, D), expressed from the methionine-repressible *MET25* promoter, were assayed for growth in the presence of 300 mM extracellular $CaCl_2$ in the presence or absence of 2 mg/ml L-methionine to maintain low or high levels of expression, respectively. Serial fivefold dilutions of each culture are shown. (B and D) Accumulation of nonphosphorylatable Rcn1-S113A (B) and phosphorylatable Rcn1 (D) and their derivates expressed at low and high levels.

stimulatory effect was shown to require GSK-3 kinases as well as the GSK-3 phosphorylation site located within the SP domain (30). To determine if this stimulatory activity requires different domains and motifs than the inhibitory activity, many of the Rcn1 derivatives described above were assayed for their ability to stimulate calcineurin after expression at a variety of levels from the *MET25* promoter. To remove Vcx1 activity as a variable, these experiments were performed with double knockout mutants that lacked both Vcx1 and Rcn1. As observed previously, low expression levels of Rcn1 induced expression of the reporter gene approximately fourfold relative to the level for the empty plasmid control (Fig. 4A). When Rcn1 was expressed at the highest level, calcineurin signaling dropped to or below the level observed in the control cells, consistent with the growth assays of calcineurin signaling described earlier. The N-terminally truncated variant that lacked the RRM domain retained full stimulatory activity (Fig. 4A) in spite of its much lower expression level (Fig. 3D). The stimulatory activity of the ΔRRM variant of Rcn1 was abolished by an additional S113A substitution or by deletion of the entire SP domain (data not shown). The simplest interpretation of these findings is that the RRM domain stabilizes Rcn1 and facilitates expression but does not contribute to the stimulatory effects of Rcn1 on calcineurin signaling.

Remarkably, the LxxP and ExxP motifs within the SP domain were both required for Rcn1 to stimulate calcineurin signaling, as alanine substitutions yielded proteins that could not stimulate *CDRE-lacZ* expression at any level of expression (Fig. 4A). These mutations did not interfere with phosphorylation at the nearby site targeted by GSK-3 (see below), suggesting that they play critical roles in docking and/or stimulation of calcineurin. Alanine substitutions within the PxIxIT-like motif of Rcn1 also diminished stimulatory activity (Fig. 4B), but the effect was not as severe as those for the alanine substitutions in the LxxP motif, ExxP motif, or GSK-3 phosphorylation sites. The PxIxIT-like motif may be much more important for calcineurin stimulation at very low (physiological) levels of Rcn1 expression, which were not achievable using the methionine-repressible expression system. Therefore, the stimulatory effects of Rcn1 on calcineurin signaling required the ExxP motif in addition to the LxxP and PxIxIT-like motifs involved in docking and interference.

Surprisingly, a C-terminal truncation that removed the TxxP motif strongly diminished the ability of Rcn1 to stimulate calcineurin at all levels of expression (Fig. 4B), while having no effect on the inhibition at high levels of expression (Fig. 3). The behavior of the TxxP-deficient variant was remarkably similar to the behavior of wild-type Rcn1 expressed in yeast cells lacking GSK-3 (29, 39). If GSK-3 and the TxxP motif independently stimulated calcineurin signaling, the TxxP motif would stimulate *CDRE-lacZ* expression even in the absence of GSK-3. To test this hypothesis, Rcn1 and its ΔTxxP variant

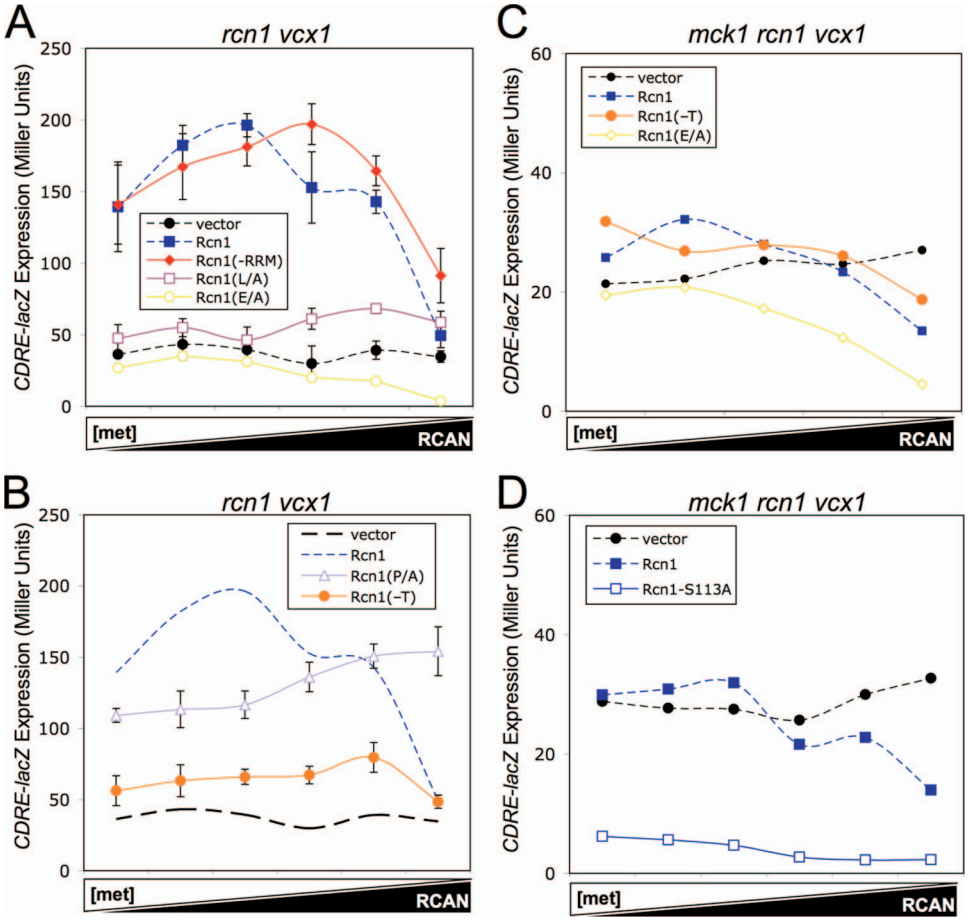

FIG. 4. Domains of Rcn1 necessary for stimulation of calcineurin signaling. In these experiments, yeast strains K1373 (*rcn1 vcx1* double knockout mutant) (A and B) and K1377 (*mck1 rcn1 vcx1* triple knockout mutant) (C and D) bearing *CDRE-lacZ* and the indicated expression plasmids were grown to log phase in medium containing various concentrations of L-methionine (see Materials and Methods) and then shifted to the same medium containing 100 mM $CaCl_2$. After 4 hours of incubation, β-galactosidase activity was measured. Truncations that remove the N-terminal RRM domain (−RRM) or the C-terminal TxxP motif (−T) and alanine substitutions that destroy the PxIxIT-like motif (P/A), the LxxP motif (L/A), and the ExxP motif (E/A) are indicated. Panels A and B depict averages (± SD) for three independent transformants. Dashed black and blue lines in panel B were copied from panel A. Data in panels C and D are representative of three independent experiments.

were expressed in *mck1* mutant cells lacking the GSK-3 kinase primarily responsible for phosphorylating S113 of Rcn1 (30). In the absence of GSK-3, both wild-type Rcn1 and the ΔTxxP mutant were unable to stimulate calcineurin and equivalently inhibited calcineurin signaling at high expression levels (Fig. 4C), suggesting that GSK-3 and the TxxP motif function in a common pathway to stimulate calcineurin signaling. The ExxP motif may also function in this common pathway because, in GSK-3-deficient *mck1* mutants, the E/A variant behaved like wild-type Rcn1 (Fig. 4C), albeit with increased inhibitory activity. In contrast, the S113A substitution at the GSK-3 phosphorylation site resulted in strong inhibition of calcineurin signaling in the *mck1* mutant (Fig. 4D). In summary, the TxxP motif, the ExxP motif, and GSK-3 seem to function in a pathway that specifically stimulates calcineurin signaling, whereas the LxxP motif and the PxIxIT motif participate in both stimulatory and inhibitory pathways.

Motifs involved in Rcn1 phosphorylation, ubiquitylation, and degradation. The TxxP, ExxP, and LxxP motifs of Rcn1 might be required for phosphorylation by GSK-3, for recognition by SCF^{Cdc4} and subsequent ubiquitylation, or for degradation by the proteasome (39). To test this possibility, we overexpressed Rcn1 and its variants in *cna1 cna2* double mutants that lack the catalytic subunits of calcineurin and examined the abundance of the proteins by Western blotting at different times following the addition of cycloheximide. Calcineurin-deficient cells were used in these experiments because calcineurin can stabilize a pool of Rcn1 in its inactive state (38) and can dephosphorylate the GSK-3 site in its active state (30), thus complicating the analyses. Under these conditions, Rcn1 was unstable during the cycloheximide chase whereas Rcn1-S113A was much more stable (Fig. 5), similar to what was found in experiments where the proteins were overexpressed in wild-type cells (39). Rcn1 variants lacking the TxxP motif, the ExxP motif, or the LxxP motif were similarly unstable when the GSK-3 phosphorylation site was present and stable when the GSK-3 phosphorylation site was substituted (Fig. 5). Therefore, we obtained no support for the idea that disrupting the TxxP, ExxP, or LxxP motif of Rcn1 diminishes phosphorylation by GSK-3, polyubiquitylation by SCF^{Cdc4}, or degradation by the proteasome.

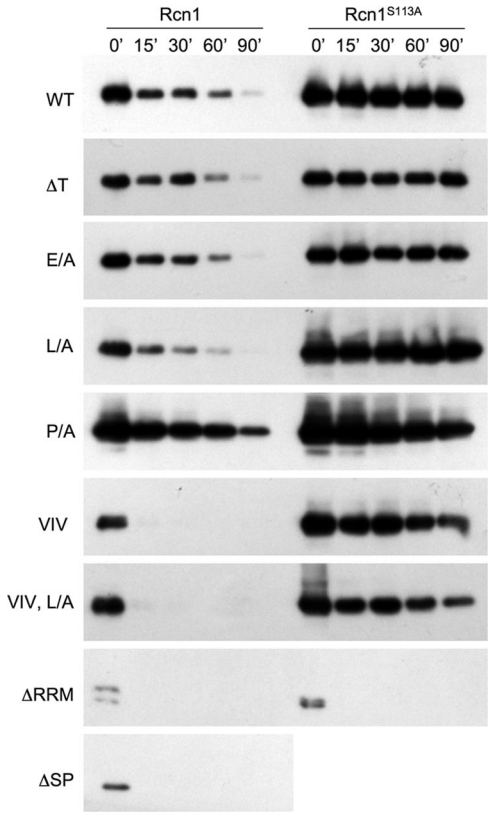

FIG. 5. Phosphorylation-dependent degradation of Rcn1 mutants. Epitope-tagged Rcn1 proteins and derivatives lacking the GSK-3 phosphorylation site (S113A), TxxP motif (ΔT), ExxP motif (E/A), PxIxIT-like motif (P/A), LxxP motif (L/A), RRM domain (ΔRRM), or SP domain (ΔSP), as well as PVIVIT mutants (VIV), were expressed in strain SM006 (*cna1 cna2* double knockout mutant) and visualized by Western blotting after treatment with 100 mM $CaCl_2$ for 15 min, followed by treatment with 100 μM cycloheximide for the indicated times. WT, wild type.

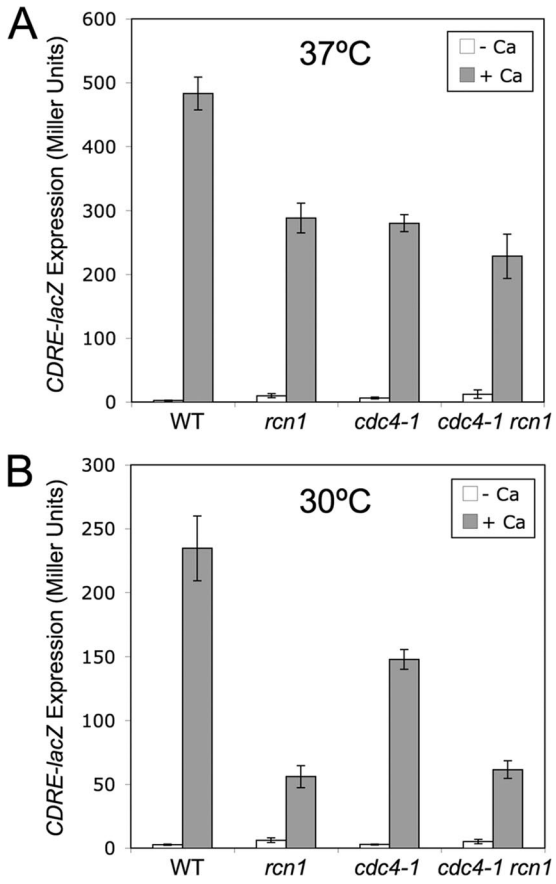

FIG. 6. Involvement of Cdc4 in stimulation of calcineurin by Rcn1. Log-phase cultures of wild-type (WT; K601), *rcn1* mutant (TKY275), *cdc4-1* mutant (MTY668), and *cdc4-1 rcn1* double mutant (SM019) yeast strains bearing the *CDRE-lacZ* reporter gene were shifted to a nonpermissive temperature (37°C) (A) or a permissive temperature (30°C) (B) for 1 hour, incubated at the same temperatures for 4 hours in medium with or without 100 mM $CaCl_2$, and then assayed for β-galactosidase activity. Averages for six replicates (± SD) are shown.

Substitutions at the PxIxIT-like motif of Rcn1 did not alter the stabilities of the nonphosphorylatable S113A proteins (Fig. 5, right). Unexpectedly, alanine substitutions that destroyed the PxIxIT-like motif increased the stability of the phosphorylatable protein, whereas the PVIVIT substitution decreased the stability of the phosphorylatable protein. These effects cannot be attributed to differential interactions with calcineurin and therefore suggest interactions between the PxIxIT motifs and GSK-3, SCF^{Cdc4}, or some other factor involved in Rcn1 phosphorylation, ubiquitylation, and degradation. The decreased stability of Rcn1 with the PVIVIT substitution apparently did not mitigate its increased potency as a calcineurin inhibitor. An additional S113A substitution in this variant dramatically stabilized the protein and rendered it an even more potent inhibitor of calcineurin signaling in vivo. These findings indicate that all of the Rcn1 variants can be phosphorylated by GSK-3, with the possible exception of the ΔRRM variant, which was highly unstable with or without the GSK-3 phosphorylation site.

Role for Cdc4 in calcineurin stimulation by Rcn1. The E3 ubiquitin ligase SCF^{Cdc4} was shown to bind phospho-Rcn1 and promote its polyubiquitylation and degradation by the proteasome (39). Because calcineurin signaling was lower and Rcn1 abundance was higher in *cdc4-1* mutants at the nonpermissive temperature than in wild-type cells, it was proposed that SCF^{Cdc4} promotes calcineurin signaling by inhibiting the accumulation of inhibitory Rcn1. However, the authors did not consider the simpler hypothesis that SCF^{Cdc4} activates the stimulatory activity of Rcn1. The first hypothesis predicts that calcineurin signaling will increase in *cdc4-1 rcn1* double mutants relative to that in *cdc4-1* single mutants at the nonpermissive temperature, whereas the alternative hypothesis predicts that calcineurin signaling levels will be similar in both cases.

To discriminate the two possibilities, we created a *cdc4-1 rcn1* double mutant and compared its ability to induce *CDRE-lacZ* to those of isogenic *cdc4-1* and *rcn1* single mutants and wild-type cells. Calcineurin signaling levels were equivalent in both *cdc4-1 rcn1* double mutants and *cdc4-1* single mutants at the nonpermissive temperature of 37°C (Fig. 6A). At the semipermissive temperature of 30°C, calcineurin signaling was increased by the presence of Rcn1 in these same *cdc4-1* mutant strains as well as in wild-type cells (Fig. 6B). These experiments

are consistent with the hypothesis that SCF^{Cdc4} is necessary for the stimulatory activity of Rcn1, but they do not exclude a special case where Rcn1 would stimulate calcineurin normally in *cdc4-1* mutants but the stimulation would be exactly counterbalanced by increased inhibition due to Rcn1 overaccumulation in the mutant. Arguing against this possibility, Rcn1 was found to underaccumulate in *cdc4* mutants at the nonpermissive temperature (39) and it also underaccumulated in *mck1* mutants (30). Furthermore, Rcn1 inhibited calcineurin signaling in *mck1* mutants only after strong overexpression (Fig. 4C). The simplest interpretation of all these findings is that the TxxP motif, the ExxP motif, GSK-3, SCF^{Cdc4}, and the proteasome all act in a common pathway that specifically stimulates calcineurin signaling. A model where the binding, phosphorylation, and degradation of Rcn1 are necessary for calcineurin reactivation is discussed more fully below.

Divergent RCANs in yeasts. In the same overexpression screen used to identify *RCN1*, a second uncharacterized gene (*YOR220w*) was isolated (38). Because of several functional and structural similarities to Rcn1 and other RCANs, we have renamed this gene product Rcn2. BLAST searches revealed homologs of Rcn2 in 22 other Hemiascomycetes (budding yeasts) but no obvious similarity to RCANs or other proteins in other species. Iterative PSI-BLAST searches of protein sequence databanks and PHYRE searches of protein structure databanks (4, 9) both identified the RRM domain of canonical RCANs as the best hit (data not shown). To study the evolutionary origin of the Rcn2 family of proteins, a multiple-sequence alignment of all Rcn2 and Rcn1 homologs was generated using CLUSTAL W (74) and analyzed by the neighbor-joining method (see Materials and Methods). The resulting phylogenetic tree is consistent with the view that Rcn2 arose via duplication of Rcn1 in an ancestral species of yeast and then rapidly diverged into its present forms in the various modern yeasts (Fig. 7).

Multiple-sequence alignment of 24 Rcn2 orthologs (see Fig. S2 in the supplemental material) revealed weak conservation within the putative RRM domains located at their N termini. At their C termini, a strongly conserved P[ST]IT[IVL]N sequence matched the consensus sequence for PxIxIT-like motifs ([PG]x[IV]x[IVL][EDNHT]) derived from canonical RCANs. PPKSPSITVNEFFH, a synthetic peptide derived from the C terminus of yeast Rcn2, was able to compete with the PVIVIT peptide for binding to human calcineurin ~40-fold more effectively than the corresponding peptide from yeast Rcn1 (Fig. 1C). As with Rcn1, truncation or substitution mutations that removed or destroyed the PxIxIT-like motif of Rcn2 abolished its ability to inhibit calcineurin signaling, and conversion to PVIVIT enhanced its ability to inhibit calcineurin signaling (Fig. 8A). Also as with Rcn1, an N-terminal truncation for removal of the RRM domain of Rcn2 had no effect on inhibitory activity (Fig. 8A).

All the Rcn2 orthologs lack the C-terminal TxxP motif of canonical RCANs that was required for calcineurin stimulation. The SP domain of canonical RCANs appears to have degenerated in Rcn2 and its orthologs, as no conserved LxxP motif, GSK-3 phosphorylation site, or ExxP motif could be identified. Nevertheless, Rcn2 can be phosphorylated in vivo at two serine residues within the degenerated SP domain (22). To test if Rcn2 retains any ability to stimulate calcineurin, *CDRE-lacZ* expression in *rcn2* knockout strains with and without *rcn1* knockout mutations was monitored. Rcn2 failed to alter the calcineurin-dependent induction of *CDRE-lacZ* in these experiments (Fig. 8C). Additionally, expression of Rcn2 at a variety of different levels from the methionine-repressible promoter (Fig. 2C) failed to stimulate calcineurin signaling in *rcn2* mutants or *rcn1 rcn2* double mutants (Fig. 8D). The loss of Rcn2 also had no effect on the growth of *pmc1* mutants and *rcn1 pmc1* double mutants in high-calcium-concentration environments (data not shown). Therefore, Rcn2 lacked the ability to stimulate calcineurin signaling but appeared to retain an ability to compete with other substrates when overexpressed.

DNA microarray experiments showed that expression of the *RCN2* genes in *S. cerevisiae* (30, 81) and *C. albicans* (37) was strongly induced by Ca^{2+}-, calcineurin-, and Crz1-dependent processes. To confirm that calcineurin signaling regulates Rcn2 protein levels, a PCR fragment encoding a triple-HA epitope tag was integrated at the 3′ end of the chromosomal *RCN2* gene and the product was examined by Western blotting under a variety of conditions. In wild-type cells, the tagged Rcn2 protein migrated as three discrete bands, corresponding to molecular masses of approximately 44 kDa, 38 kDa, and 36 kDa (Fig. 8B). Incubation in a high-calcium-concentration environment strongly induced all three bands in wild-type cells (Fig. 2C and 8B), but this effect was completely absent in mutants lacking calmodulin, calcineurin, or Crz1 (Fig. 8B). In the mutant strains, the intensity and mobility of each band was unaffected by the presence or absence of calcium, suggesting little or no modification by calcineurin or other calcium-dependent enzymes. Interestingly, the loss of either A or B subunits of calcineurin resulted in a selective loss of the fastest-migrating band, independent of calcium and calmodulin (Fig. 8B). A similar effect was noted previously for Rcn1 (38). The simplest model for explaining these phenomena is one where inactive calcineurin A/B heterodimers bind and stabilize a pool of Rcn2 molecules under nonsignaling conditions and activated calcineurin induces Rcn2 accumulation, which might interfere with the docking of substrates onto calcineurin, as well as having other, unknown consequences.

Human RCAN1 function in yeast. Previous studies have demonstrated that human RCAN1 can stimulate calcineurin signaling in yeast by a process that depends on GSK-3 and the GSK-3 phosphorylation site (serine-108 of RCAN1) (30). A structure-function analysis similar to the one described above for Rcn1 was performed on RCAN1 to determine if the two proteins operate by similar mechanisms. As with Rcn1, we found that a truncation removing the RRM domain of RCAN1 had no effect on stimulatory activity but that a truncation removing the TxxP motif abolished the stimulatory activity (Fig. 9A). Similarly, alanine substitutions in the ExxP motif, the LxxP motif, or the PxIxIT-like motif of RCAN1 also resulted in the loss of stimulatory activity (Fig. 9B). Therefore, human RCAN1 largely employs the same conserved motifs as yeast Rcn1 to stimulate calcineurin signaling.

Similar to Rcn1-S113A, the RCAN1-S108A mutant inhibited calcineurin signaling even at low levels of expression, and this effect depended on the PxIxIT-like motif and the RRM domain but not the TxxP motif (Fig. 10A). Wild-type RCAN1 expressed at low levels also exhibited a low but detectable level of inhibitory activity that depended on the RRM domain (Fig.

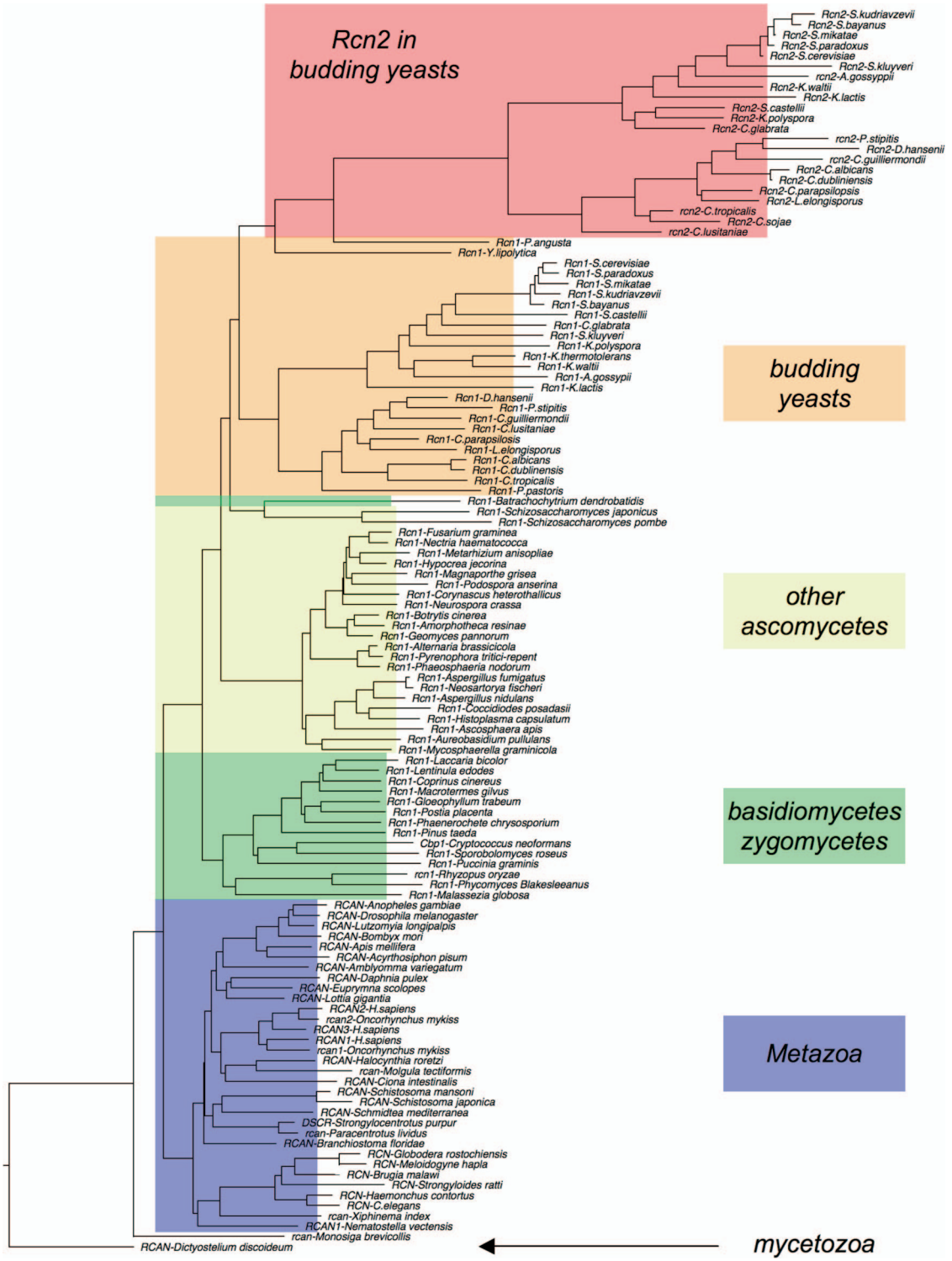

FIG. 7. Evolutionary relationship of canonical and noncanonical RCANs. A multiple-sequence alignment of Rcn2, Rcn1, RCAN1 and homologs in fungi and animals was generated using CLUSTALW and analyzed using the neighbor-joining method (see Materials and Methods). The resulting phylogenetic tree supports the hypothesis that Rcn2s (red box) arose from the duplication of Rcn1 in an ancestor of budding yeasts and subsequently diverged. Orange, yellow, green, and blue boxes depict the average sequence distances of canonical RCANs from budding yeasts (orange box), filamentous fungi and fission yeasts (yellow box), nonascomycetes fungi (green box), and animals (blue box), relative to the level for the hypothetical ancestral RCAN.

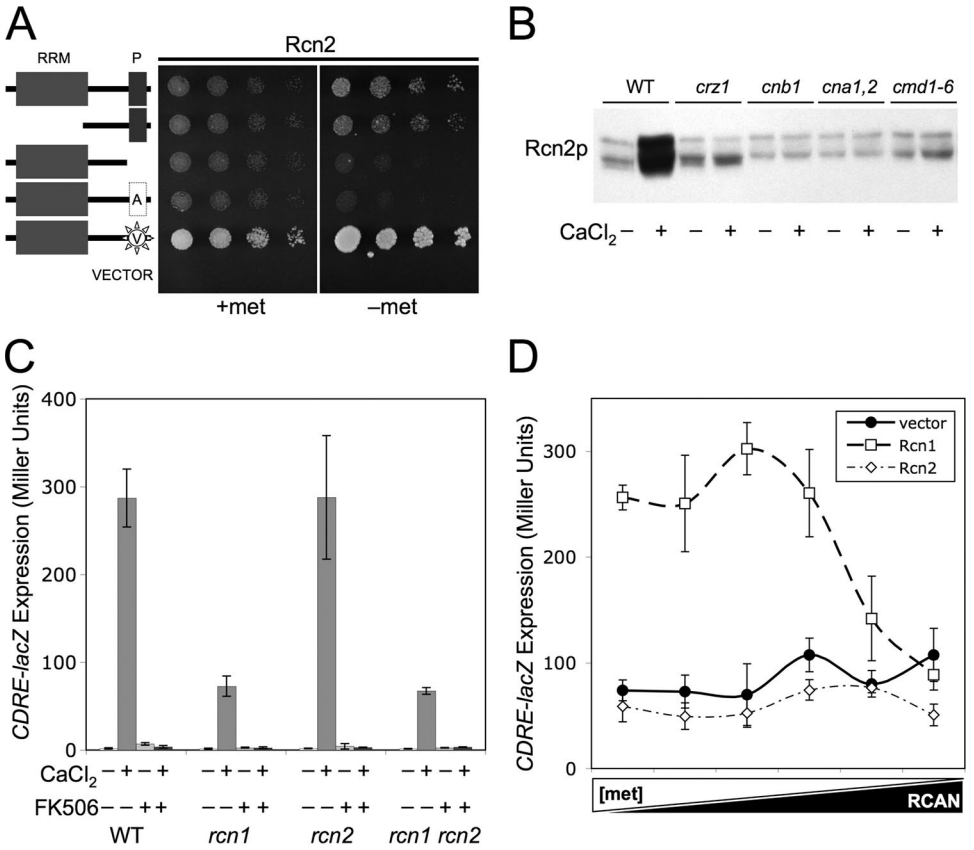

FIG. 8. Characterization of a divergent yeast RCAN, Rcn2. (A) Feedback inhibition of calcineurin signaling by Rcn2. Rcn2 and derivatives lacking the RRM domain or the PxIxIT-like motif were expressed at low and high levels in strain DDY156 (*pmc1 crz1* double knockout mutant) and grown in medium supplemented with 300 mM $CaCl_2$ as described in the legend to Fig. 3. (B) Calcineurin/Crz1-dependent induction of Rcn2. Expression of chromosomally tagged Rcn2-HA_3 in the indicated yeast strains was monitored by Western blotting after 4 hours of growth in medium with or without 100 mM $CaCl_2$ added. WT, wild type. (C and D) Calcineurin signaling is not stimulated by yeast Rcn2. (C) Expression of the *CDRE-lacZ* reporter gene in wild-type, *rcn1* (RBY255), *rcn2* (RBY19), and *rcn1 rcn2* (RBY257) knockout mutant strains was measured after 4 hours of growth in medium containing or lacking 100 mM $CaCl_2$ and 0.3 μM FK506, as indicated. Bars show the averages for three replicates (± SD). (D) Expression of the *CDRE-lacZ* reporter gene in strain SM005 (*rcn1 rcn2 vcx1* triple knockout mutant) bearing Rcn1 and Rcn2 expression plasmids was measured after 4 hours of growth in medium containing 100 mM $CaCl_2$ and various concentrations of L-methionine as described in the legend to Fig. 4.

10B). Therefore, the RRM domain may stabilize RCAN1, similar to its role in Rcn1. With this in mind, the truncated RCAN1 and RCAN1-S108A proteins lacking their RRM domains still inhibited calcineurin signaling, similar to the truncated Rcn1 and Rcn2 proteins (Fig. 10). The inhibitory activities of RCAN1-S108A and RCAN1 were blocked by substitutions that destroyed the PxIxIT-like motif (Fig. 10). However, substitutions that destroyed the LxxP motif had little or no effect on inhibitory activity in either case. The higher affinity of the PxIxIT-like motif in RCAN1 (Fig. 2) may obviate the LxxP motif in inhibitory interactions with calcineurin. Consistent with this view, replacing the PxIxIT-like motif with the very-high-affinity PVIVIT sequence did not detectably increase inhibitory activity under these conditions. In summary, human RCAN1 appeared to stimulate and inhibit calcineurin signaling in yeast in much the same way as Rcn1. Therefore, canonical RCANs may also function in humans primarily as stimulators of calcineurin signaling and secondarily as inhibitors of calcineurin signaling.

DISCUSSION

Inhibition of calcineurin by RCANs. The structure-function experiments described above indicate that overexpressed RCANs inhibit signaling by docking onto calcineurin and interfering with the docking of other substrates. After RCAN sequences from many fungal and animal species were aligned, a conserved motif that resembled the PxIxIT motifs of NFAT, Crz1, and other substrates of calcineurin was found (5, 11, 20, 28, 63). Synthetic peptides from Rcn1 and RCAN1 containing these PxIxIT-like motifs can bind the substrate-docking cleft on the surface of calcineurin. Mutations known to increase and decrease the affinity of PxIxIT motifs for calcineurin were found to increase and decrease the inhibition of calcineurin signaling in yeast when introduced into Rcn1 or RCAN1. Therefore, overexpressed RCANs likely compete with other substrates that utilize PxIxIT motifs for productive binding to calcineurin.

Truncation analysis showed that the PxIxIT-like motifs of

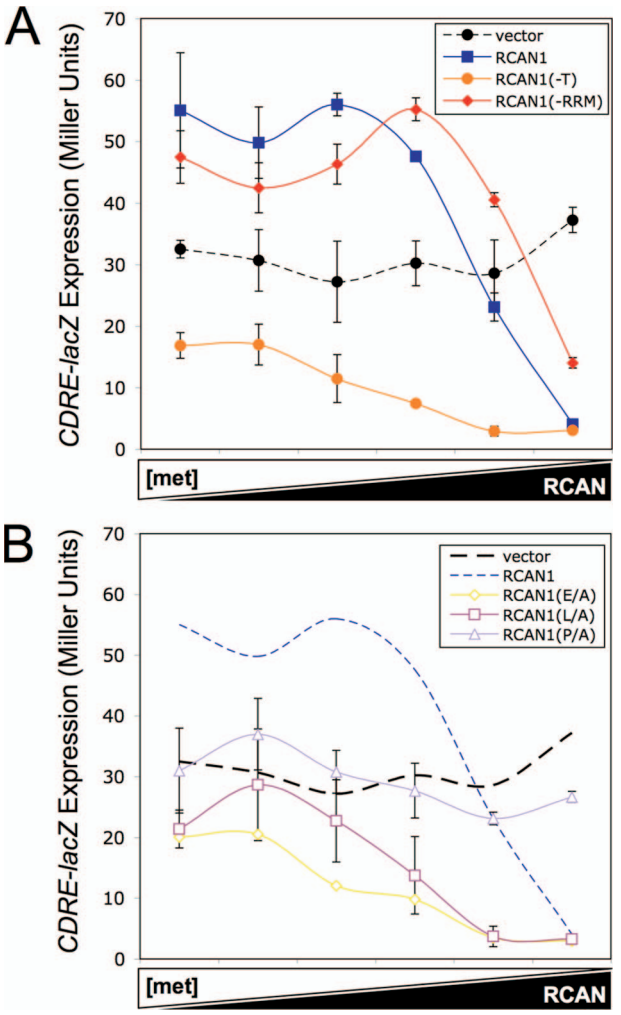

FIG. 9. Domains required for calcineurin stimulation and inhibition by RCAN1. RCAN1 and derivatives were expressed at various levels in yeast strain K1373 (*rcn1 vcx1* double knockout mutant) bearing a *CDRE-lacZ* reporter gene and examined for effects on calcineurin signaling as described in the legend to Fig. 4. Dashed black and blue lines in panel B were copied from panel A.

Rcn1 and RCAN1 were not sufficient to inhibit calcineurin signaling in yeast at the levels of expression achieved and that the SP domain was also required. Within this domain, a conserved LxxP motif also proved necessary for the inhibitory effects of even the full-length proteins, except when the PxIxIT-like motif of Rcn1 or RCAN1 was mutated to have higher affinity for calcineurin. A peptide containing the LxxP motif of RCAN1 (residues 95 to 118) was able to inhibit both the RII phosphatase and the pNP phosphatase activities of calcineurin (H. Li and P. G. Hogan, unpublished), whereas a smaller peptide lacking this motif (residues 103 to 120) could inhibit the former activity but not the latter (27). Therefore, the LxxP motif may interact with the active site in this context. Interestingly, peptides containing an LxVP motif derived from NFAT proteins have been shown to bind an unknown site on calcineurin and inhibit its RII phosphatase activity and to inhibit calcineurin signaling when overexpressed in cultured cells (45, 51, 57). It is not yet clear whether LxVP motifs are important for NFAT recognition or docking by calcineurin. The LxxP motifs of Rcn1 and RCAN1 seemed to be important for both stimulatory and inhibitory effects on calcineurin signaling in yeast, so they may function in conjunction with the PxIxIT-like motifs at an early step in the interaction with calcineurin.

Truncations that eliminate both the RRM and the SP domains were sometimes inhibitory when overexpressed in mammalian cell lines (8, 12, 15, 25, 61, 76) though not when overexpressed in yeast. Several factors may contribute to this difference. First, the truncated proteins may be better expressed in mammalian cells than in yeast. Truncated Rcn1 was expressed at lower levels in yeast than in other derivatives due to the apparent acquisition of a degron that did not depend on the GSK-3 phosphorylation site. Second, the PxIxIT-like motif of RCAN1 may bind more tightly to human calcineurin than to yeast calcineurin. The dissociation constant measured for a synthetic peptide containing the PVIVIT motif was approximately fourfold higher with yeast calcineurin than with human calcineurin (5, 11, 20, 28, 63). Third, the calcineurin-RCAN interactions in mammals may involve additional sites of interaction that are not present in yeast. In support of this view, a

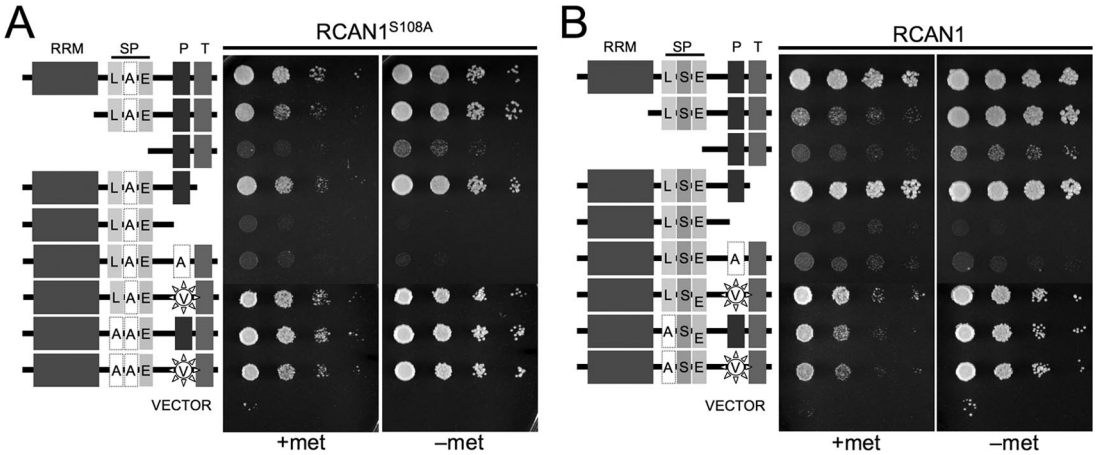

FIG. 10. Calcineurin inhibition by human RCAN1. The growth of strain DDY156 (*pmc1 crz1* double knockout mutant) expressing low and high levels of nonphosphorylatable human RCAN1-S108A (A) and wild-type human RCAN1 (B) and their derivatives on high-calcium-concentration medium is shown as in Fig. 3.

C-terminal fragment of RCAN1 (residues 141 to 197) containing the PxIxIT-like motif and the TxxP motif could competitively inhibit the pNP phosphatase activity of recombinant calcineurin (12) whereas the PxIxIT-like motif alone could not (residues 150 to 163; H. Li and P. G. Hogan, unpublished). The conserved TxxP motif (residues 186 to 189) or a poorly conserved ELHA motif (residues 143 to 155) (8) may contribute to these in vitro effects. More work will be necessary to identify all the motifs in RCANs that make contacts with calcineurin.

Several hypotheses have been proposed to explain how phosphorylation of RCANs by GSK-3 might mitigate their inhibitory effects on calcineurin signaling. Phospho-RCANs may possess decreased binding affinity for calcineurin, diminished stability and expression, or some combination of these or other attributes (1, 26, 30, 39, 76). Interestingly, the inhibitory activities of RCANs in yeast did not correlate well with expression. The S-to-A substitutions at the GSK-3 phosphorylation site strongly increased the potency of full-length and ΔTxxP RCANs, while only mildly increasing stability and expression (Fig. 3 and 10). The unstable and poorly expressed ΔRRM RCANs were not detectably modulated by the S-to-A substitution, suggesting that the RRM domain plays a role in the inhibitory effects of nonphosphorylatable RCANs that goes beyond its role in stabilizing the proteins. Indeed, the isolated RRM domain of human RCAN1 interacted with calcineurin and inhibited its signaling in a mammalian cell line (76), though this behavior was not observed in other situations (8). The nonphosphorylatable RCANs may possess a novel manner of interaction with calcineurin, because they were more inhibitory than wild-type RCANs, even in GSK-3-deficient mutants (Fig. 4) where both proteins are stabilized (39). The finding that S-to-A RCANs require functional LxxP motifs and PxIxIT-like motifs for high potency inhibition is consistent with the view that the nonphosphorylatable RCANs engage calcineurin quite normally, perhaps as part of the normal cycle of calcineurin stimulation, but fail to disengage and thereby seq uester calcineurin from all its signaling roles. Despite the unresolved status of these possibilities, the dramatically incre ased inhibitory activity and stability of S-to-A RCANs, coupled with higher affinity PVIVIT substitutions, may prove useful in gene therapies for limiting calcineurin signaling under conditions such as cardiac hypertrophy (62).

Stimulation of calcineurin by RCANs. Previous studies showed that the GSK-3 protein kinase Mck1, the E3 ubiquitin ligase SCF^{Cdc4}, and the proteasome were all required for maximal calcineurin signaling in yeast and that their contributions were not additive, suggesting that they function interdependently in a common regulatory pathway (39). GSK-3 phosphorylates a site in Rcn1 that is conserved in all canonical RCANs (30), and this modification was later shown to produce a binding site for Cdc4, which triggers polyubiquitylation and degradation by the proteasome (39). Because residual calcineurin signaling levels were similar in *mck1 cdc4-1* double mutants and the single mutants, potentially redundant protein kinases, ubiquitin ligases, and proteases seem to be insignificant under the conditions used. However, unexpected side effects of *mck1* and *cdc4-1* mutations on this system have not been ruled out. The idea that Rcn1 overaccumulates in *mck1* and *cdc4* mutants and directly interferes with calcineurin signaling is inconsistent with earlier experimental data showing that Rcn1 levels are lower in *mck1* and *cdc4* mutants than in wild-type yeast cells under both nonsignaling and signaling conditions (30, 39). Also inconsistent with this idea are the observations that the complete loss of Rcn1 did not increase calcineurin signaling in either *mck1* mutants (30) or *cdc4-1* mutants (Fig. 6) and that strong overexpression of Rcn1 was necessary to inhibit calcineurin signaling in *mck1* mutants (Fig. 4). Therefore, the idea that GSK-3 and SCF^{Cdc4} limit the inhibitory effects of Rcn1 on calcineurin in vivo currently has little experimental support. On the other hand, all these findings are compatible with the model in which GSK-3, SCF^{Cdc4}, and the proteasome increase calcineurin signaling by increasing a stimulatory activity of Rcn1.

Rcn1 and other RCANs may stimulate calcineurin signaling through a variety of mechanisms. For instance, phospho-RCAN1 can bind 14-3-3 proteins (1) and potentially diminish a buffering effect of 14-3-3 proteins on phospho-NFAT (16, 44). Several findings reported here argue against a similar mechanism in yeast. First, the structure-function analysis of Rcn1 is difficult to reconcile with this model. Many derivatives of Rcn1 that lacked inhibitory and/or stimulatory functions were efficiently phosphorylated by GSK-3, so they apparently altered calcineurin signaling independent of the potential interaction with 14-3-3 proteins. The 14-3-3 titration mechanism also incorrectly predicts the roles of SCF^{Cdc4} and the proteasome as inhibitors of calcineurin signaling. Several other possible mechanisms of calcineurin stimulation by Rcn1, such as enzyme allostery and enzyme-substrate scaffolding, are also difficult to reconcile with the requirement for Rcn1 proteolysis. The findings that a low expression level of Rcn1 stimulates calcineurin signaling in a docking-dependent and proteolysis-dependent fashion strongly suggest that calcineurin signaling somehow benefits from a transient interaction with Rcn1.

Another mechanism that seems compatible with both the binding and the degradation requirements is one where RCANs function as suicidal molecular chaperones that somehow activate or reactivate calcineurin but require phosphorylation, polyubiquitylation, and degradation to release a fully functional enzyme that is capable of productive interactions with substrates. A similar model was proposed previously for the related type-1 protein phosphatase (PP1) and its regulator known as inhibitor-2 (I-2). In that case, recombinant PP1 was converted to a native-like enzyme after recombinant I-2 was bound, phosphorylated in vitro by GSK-3, and degraded with exogenous proteases (3). Structure-function studies and a recent X-ray crystal structure of I-2–PP1 complexes have suggested that multiple segments of I-2 make independent contacts on the surface of PP1 (see reference 35 and references therein). Remarkably, one conserved motif of I-2 bound to the substrate-docking cleft of PP1 that is homologous to the PxIx IT-binding cleft of calcineurin. Another conserved motif interacted with the active site of PP1, but the GSK-3 phosphorylation site was not located near the active site. Though I-2s and RCANs exhibit no obvious sequence or structural similarity, they both may interact transiently with their respective "clients" in vivo and promote the acquisition of higher activity states through transient intermediates where the client enzyme is sequestered.

Our structure-function analysis of yeast and human RCANs revealed several conserved motifs that were necessary for stim-

ulation of calcineurin signaling in yeast. The PxIxIT-like motif and the LxxP motif within the SP domain appeared to be very important for stimulation at low expression levels, in addition to their important roles in inhibition at high expression levels. Interestingly, the ExxP motif within the SP domains and the TxxP motif near the C termini were not necessary for calcineurin inhibition, but they functioned in the same pathway as GSK-3 for calcineurin stimulation. None of these stimulatory motifs appeared to be necessary for efficient phosphorylation by GSK-3 and subsequent degradation in the absence of calcineurin (Fig. 5), which rules out one possible model of their function. Another possibility is that the TxxP motif promotes signaling by interacting with another site on calcineurin. A 38-residue fragment of RCAN1 containing the TxxP motif without the PxIxIT-like motif (residues 160 to 197) was able to bind calcineurin without inhibiting its signaling when overexpressed in a mammalian cell line (8). Other studies also suggest that the TxxP motif may interact directly with calcineurin (12). The ability of endogenous RCANs to stimulate calcineurin signaling in yeast and mammals (68) may therefore involve multisite interactions similar to that of I-2 and PP1, though the precise modifications of PP1 and calcineurin promoted by I-2 and RCANs have not been elucidated.

Evidence from neurons suggests that calcineurin may normally undergo activity-dependent inactivation (10). Though that phenomenon is not yet understood, it is possible that RCANs facilitate reactivation of the enzyme during prolonged signaling events, such as those employed as described above. Cells may induce RCAN expression in response to calcineurin signaling in order to avoid consumption by the intrinsic phosphorylation-degradation system. The dual phosphorylation of RCANs by GSK-3 and priming kinases that act on a nearby site may link calcineurin function to protein kinase signaling networks (1). Phosphorylation of RCANs may also couple conformational changes to an energy source to help drive modifications of calcineurin. Evidence from yeast suggests that HSP90-type molecular chaperones may also contribute to calcineurin recycling or biogenesis under similar conditions (36), though another study found no role for HSP90 in other calcineurin-dependent processes (21). Finally, mathematical modeling of the calcineurin-RCAN signaling network in cardiac myocytes was consistent with experimental observations only when it was assumed that RCANs increase calcineurin abundance or activity twofold or more (71). Calcineurin abundance in yeast was not affected by the complete loss of Rcn1 (38), and calcineurin abundance in mouse heart, muscle, thymus, and brain was unaltered by the loss of RCAN1 (32, 59, 75) or the further loss of RCAN2 (68). Taken together, these findings indicate a potential need for calcineurin chaperones that could be fulfilled, at least in part, by RCANs.

The ability of RCANs to stimulate calcineurin signaling may not be universal to all cell types or species. For example, RCAN deficiencies in a fruit fly and a nematode did not produce calcineurin-deficient phenotypes and instead resulted in animals with calcineurin-enhanced phenotypes in certain behavioral assays (14, 34, 42, 73). In *Drosophila melanogaster*, the RCAN ortholog was targeted to mitochondria, where it altered a variety of mitochondrial functions independent of calcineurin-interacting motifs (13). In mouse, the loss of RCAN1 increased calcineurin signaling in T lymphocytes and endothelial cells (65, 66). However, the simultaneous loss of RCAN1 and RCAN2 in mouse decreased calcineurin signaling in T lymphocytes and many other cell types (68). In mammals, where RCANs are expressed from three alternatively spliced genes and where GSK-3 can be strongly inhibited by upstream regulators, RCANs may also sequester calcineurin at intermediate stages of stimulation or interfere with the binding of other substrates in certain cell types. Additional work may reveal new functions of RCANs that may or may not depend on calcineurin.

Evolution of RCANs and the noncanonical RCANs in yeasts. By use of PSI-BLAST and other approaches, RCAN orthologs can be found in the genomes of all animals and fungi except for *Encephalitozoon cuniculi*, which also lacks calcineurin orthologs. Most vertebrates, including humans, possess three RCANs that appear to have originated from ancestral gene duplications. As mentioned earlier, RCANs from several yeasts and *Dictyostelium discoideum* appear to be truncated after the PxIxIT-like motif and therefore lack the otherwise well-conserved TxxP motif. These natural truncations reflect several independent evolutionary events, which might alter their abilities to stimulate calcineurin signaling.

Surprisingly, we identified a noncanonical RCAN from *Saccharomyces cerevisiae* in a genetic screen for overexpressed factors that inhibit calcineurin signaling. Though lacking the TxxP motif and containing a highly diverged SP domain, Rcn2 and its orthologs in other budding yeasts retained the RRM domain and a functional PxIxIT-like motif. As expected from our structure-function studies of canonical RCANs, Rcn2 was shown to lack detectable stimulatory activity and to inhibit calcineurin signaling when overexpressed. A phylogenetic tree suggests that Rcn2s may have originated from a gene duplication event in an ancestral species of yeast, followed by rapid subfunctionalization or neofunctionalization in descendant species. After a whole-genome duplication event ~100 million years ago (79), most descendant species lost the duplicates of Rcn1 and Rcn2, though one species, *Kluyveromyces polysporus*, retained both Rcn2 paralogs, one of which became extremely diverged in sequence (69). Because calcineurin-dependent induction of Rcn2s has been observed in both *Saccharomyces cerevisiae* (Fig. 5B) and *Candida albicans* (37), the noncanonical RCANs may simply serve as competitive feedback inhibitors or effectors of calcineurin signaling.

In summary, the findings reported here suggest that canonical RCANs primarily stimulate calcineurin signaling and secondarily interfere with the interactions between calcineurin and other substrates and with its enzymatic activity. Derivatives of RCANs that trap calcineurin during the stimulatory cycle may be significantly more potent as calcineurin inhibitors. A better understanding of how RCANs regulate calcineurin may be useful for development of tissue-specific inhibitors and activators of calcineurin signaling in pathological conditions.

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ADDENDUM IN PROOF

While this article was in press, several of its conclusions were confirmed in two new articles. Mulero et al. (J. Biol. Chem. **284:**9394–9401, 2009) confirmed that synthetic peptides spanning the PxIxIT-like motifs of human RCAN1 and RCAN3 directly bind to the PVIVIT-binding site of calcineurin and not the LxxP-binding site. Additionally, Rodriguez et. al. (Mol. Cell **33:**616–626, 2009) identified a distinct site on calcineurin that binds either the LxxP-containing peptides of human NFAT and yeast Rcn1 or FK506/FKBP-12 complexes, but not simultaneously to both. The earlier finding that FK506 destabilized Rcn1 in nonsignalling yeast cells (38) can now be explained by competition between FK506/FKBP-12 complexes and Rcn1 for the same (LxxP-binding) site.

REFERENCES

1. **Abbasi, S., J. D. Lee, B. Su, X. Chen, J. L. Alcon, J. Yang, R. E. Kellems, and Y. Xia.** 2006. Protein kinase-mediated regulation of calcineurin through the phosphorylation of modulatory calcineurin-interacting protein 1. J. Biol. Chem. **281:**7717–7726.
2. **Agbas, A., D. Hui, X. Wang, V. Tek, A. Zaidi, and E. K. Michaelis.** 2007. Activation of brain calcineurin (Cn) by Cu-Zn superoxide dismutase (SOD1) depends on direct SOD1-Cn protein interactions occurring in vitro and in vivo. Biochem. J. **405:**51–59.
3. **Alessi, D. R., A. J. Street, P. Cohen, and P. T. Cohen.** 1993. Inhibitor-2 functions like a chaperone to fold three expressed isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. Eur. J. Biochem. **213:**1055–1066.
4. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
5. **Aramburu, J., F. Garcia-Cozar, A. Raghavan, H. Okamura, A. Rao, and P. G. Hogan.** 1998. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol. Cell **1:**627–637.
6. **Aramburu, J., M. B. Yaffe, C. Lopez-Rodriguez, L. C. Cantley, P. G. Hogan, and A. Rao.** 1999. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science **285:**2129–2133.
7. **Arron, J. R., M. M. Winslow, A. Polleri, C. P. Chang, H. Wu, X. Gao, J. R. Neilson, L. Chen, J. J. Heit, S. K. Kim, N. Yamasaki, T. Miyakawa, U. Francke, I. A. Graef, and G. R. Crabtree.** 2006. NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature **441:**595–600.
8. **Aubareda, A., M. C. Mulero, and M. Perez-Riba.** 2006. Functional characterization of the calcipressin 1 motif that suppresses calcineurin-mediated NFAT-dependent cytokine gene expression in human T cells. Cell Signal **18:**1430–1438.
9. **Bennett-Lovsey, R. M., A. D. Herbert, M. J. Sternberg, and L. A. Kelley.** 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins **70:**611–625.
10. **Bito, H., K. Deisseroth, and R. W. Tsien.** 1996. CREB phosphorylation and dephosphorylation: a Ca2+- and stimulus duration-dependent switch for hippocampal gene expression. Cell **87:**1203–1214.
11. **Bultynck, G., V. Heath, A. Majeed, J. Galan, R. Haguenauer-Tsapis, and M. Cyert.** 2006. Slm1 and Slm2 are novel substrates of the calcineurin phosphatase required for heat stress-induced endocytosis of the yeast uracil permease. Mol. Cell. Biol. **26:**4729–4745.
12. **Chan, B., G. Greenan, F. McKeon, and T. Ellenberger.** 2005. Identification of a peptide fragment of DSCR1 that competitively inhibits calcineurin activity in vitro and in vivo. Proc. Natl. Acad. Sci. USA **102:**13075–13080.
13. **Chang, K. T., and K. T. Min.** 2005. Drosophila melanogaster homolog of Down syndrome critical region 1 is critical for mitochondrial function. Nat. Neurosci. **8:**1577–1585.
14. **Chang, K. T., Y. J. Shi, and K. T. Min.** 2003. The Drosophila homolog of Down's syndrome critical region 1 gene regulates learning: implications for mental retardation. Proc. Natl. Acad. Sci. USA **100:**15794–15799.
15. **Cho, Y. J., M. Abe, S. Y. Kim, and Y. Sato.** 2005. Raf-1 is a binding partner of DSCR1. Arch. Biochem. Biophys. **439:**121–128.
16. **Chow, C. W., and R. J. Davis.** 2000. Integration of calcium and cyclic AMP signaling pathways by 14-3-3. Mol. Cell. Biol. **20:**702–712.
17. **Crabtree, G. R., and E. N. Olson.** 2002. NFAT signaling: choreographing the social lives of cells. Cell **109**(Suppl.)**:**S67–S79.
18. **Crooks, G. E., G. Hon, J. M. Chandonia, and S. E. Brenner.** 2004. WebLogo: a sequence logo generator. Genome Res. **14:**1188–1190.
19. **Cunningham, K. W., and G. R. Fink.** 1996. Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2226–2237.
20. **Czirjak, G., and P. Enyedi.** 2006. Targeting of calcineurin to an NFAT-like docking site is required for the calcium-dependent activation of the background K+ channel, TRESK. J. Biol. Chem. **281:**14677–14682.
21. **Dudgeon, D. D., N. Zhang, O. O. Ositelu, H. Kim, and K. W. Cunningham.** 2008. Nonapoptotic death of *Saccharomyces cerevisiae* cells that is stimulated by Hsp90 and inhibited by calcineurin and Cmk2 in response to endoplasmic reticulum stresses. Eukaryot. Cell **7:**2037–2051.
22. **Ficarro, S. B., M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt, and F. M. White.** 2002. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat. Biotechnol. **20:**301–305.
23. **Fox, D. S., and J. Heitman.** 2002. Good fungi gone bad: the corruption of calcineurin. Bioessays **24:**894–903.
24. **Fruman, D. A., C. B. Klee, B. E. Bierer, and S. J. Burakoff.** 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc. Natl. Acad. Sci. USA **89:**3686–3690.
25. **Fuentes, J. J., L. Genescà, T. J. Kingsbury, K. W. Cunningham, M. Pérez-Riba, X. Estivill, and S. de la Luna.** 2000. DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum. Mol. Gen. **9:**1681–1690.
26. **Genescà, L., A. Aubareda, J. J. Fuentes, X. Estivill, S. De La Luna, and M. Pérez-Riba.** 2003. Phosphorylation of calcipressin 1 increases its ability to inhibit calcineurin and decreases calcipressin half-life. Biochem. J. **374:**567–575.
27. **Gorlach, J., D. S. Fox, N. S. Cutler, G. M. Cox, J. R. Perfect, and J. Heitman.** 2000. Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in *Cryptococcus neoformans*. EMBO J. **19:**3618–3629.
28. **Heath, V. L., S. L. Shaw, S. Roy, and M. S. Cyert.** 2004. Hph1p and Hph2p, novel components of calcineurin-mediated stress responses in *Saccharomyces cerevisiae*. Eukaryot. Cell **3:**695–704.
29. **Hilioti, Z., and K. W. Cunningham.** 2004. Calcineurin: roles of the Ca2+/calmodulin-dependent protein phosphatase in diverse eukaryotes. Top. Curr. Genet. **5:**73–90.
30. **Hilioti, Z., D. A. Gallagher, S. T. Low-Nam, P. Ramaswamy, P. Gajer, T. J. Kingsbury, C. J. Birchwood, A. Levchenko, and K. W. Cunningham.** 2004. GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. Genes Dev. **18:**35–47.
31. **Hill, J. A., B. Rothermel, K. D. Yoo, B. Cabuay, E. Demetroulis, R. M. Weiss, W. Kutschke, R. Bassel-Duby, and R. S. Williams.** 2002. Targeted inhibition of calcineurin in pressure-overload cardiac hypertrophy. Preservation of systolic function. J. Biol. Chem. **277:**10251–10255.
32. **Hoeffer, C. A., A. Dey, N. Sachan, H. Wong, R. J. Patterson, J. M. Shelton, J. A. Richardson, E. Klann, and B. A. Rothermel.** 2007. The Down syndrome critical region protein RCAN1 regulates long-term potentiation and memory via inhibition of phosphatase signaling. J. Neurosci. **27:**13161–13172.
33. **Hogan, P. G., L. Chen, J. Nardone, and A. Rao.** 2003. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. **17:**2205–2232.
34. **Horner, V. L., A. Czank, J. K. Jang, N. Singh, B. C. Williams, J. Puro, E. Kubli, S. D. Hanes, K. S. McKim, M. F. Wolfner, and M. L. Goldberg.** 2006. The Drosophila calcipressin sarah is required for several aspects of egg activation. Curr. Biol. **16:**1441–1446.
35. **Hurley, T. D., J. Yang, L. Zhang, K. D. Goodwin, Q. Zou, M. Cortese, A. K. Dunker, and A. A. DePaoli-Roach.** 2007. Structural basis for regulation of protein phosphatase 1 by inhibitor-2. J. Biol. Chem. **282:**28874–28883.
36. **Imai, J., and I. Yahara.** 2000. Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. Mol. Cell. Biol. **20:**9262–9270.
37. **Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille, and D. Sanglard.** 2006. CRZ1, a target of the calcineurin pathway in Candida albicans. Mol. Microbiol. **59:**1429–1451.
38. **Kingsbury, T. J., and K. W. Cunningham.** 2000. A conserved family of calcineurin regulators. Genes Dev. **13:**1595–1604.
39. **Kishi, T., A. Ikeda, R. Nagao, and N. Koyama.** 2007. The SCFCdc4 ubiquitin ligase regulates calcineurin signaling through degradation of phosphorylated Rcn1, an inhibitor of calcineurin. Proc. Natl. Acad. Sci. USA **104:**17418–17423.
40. **Klee, C. B., H. Ren, and X. Wang.** 1998. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. **273:**13367–13370.
41. **Ladner, C. J., J. Czech, J. Maurice, S. A. Lorens, and J. M. Lee.** 1996. Reduction of calcineurin enzymatic activity in Alzheimer's disease: correlation with neuropathologic changes. J. Neuropathol. Exp. Neurol. **55:**924–931.
42. **Lee, J. I., B. K. Dhakal, J. Lee, J. Bandyopadhyay, S. Y. Jeong, S. H. Eom, H. Kim do, and J. Ahnn.** 2003. The Caenorhabditis elegans homologue of Down syndrome critical region 1, RCN-1, inhibits multiple functions of the phosphatase calcineurin. J. Mol. Biol. **328:**147–156.
43. **Li, H., A. Rao, and P. G. Hogan.** 2004. Structural delineation of the calcineurin-NFAT interaction and its parallels to PP1 targeting interactions. J. Mol. Biol. **342:**1659–1674.
44. **Liao, W., S. Wang, C. Han, and Y. Zhang.** 2005. 14-3-3 proteins regulate glycogen synthase 3beta phosphorylation and inhibit cardiomyocyte hypertrophy. FEBS J. **272:**1845–1854.
45. **Liu, J., K. Arai, and N. Arai.** 2001. Inhibition of NFATx activation by an oligopeptide: disrupting the interaction of NFATx with calcineurin. J. Immunol. **167:**2677–2687.
46. **Liu, J., J. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber.** 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:**807–815.

47. **Liu, J. O.** 2003. Endogenous protein inhibitors of calcineurin. Biochem. Biophys. Res. Commun. **311:**1103–1109.
48. **Liu, Y. L., C. S. Fann, C. M. Liu, C. C. Chang, W. C. Yang, S. I. Hung, S. L. Yu, T. J. Hwang, M. H. Hsieh, C. C. Liu, M. M. Tsuang, J. Y. Wu, Y. S. Jou, S. V. Faraone, M. T. Tsuang, W. J. Chen, and H. G. Hwu.** 2007. More evidence supports the association of PPP3CC with schizophrenia. Mol. Psychiatry **12:**966–974.
49. **Maesaki, S., P. Marichal, M. A. Hossain, D. Sanglard, H. Vanden Bossche, and S. Kohno.** 1998. Synergic effects of tacrolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. J. Antimicrob. Chemother. **42:**747–753.
50. **Marchetti, O., J. M. Entenza, D. Sanglard, J. Bille, M. P. Glauser, and P. Moreillon.** 2000. Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans*. Antimicrob. Agents Chemother. **44:**2932–2938.
51. **Martinez-Martinez, S., A. Rodriguez, M. D. Lopez-Maderuelo, I. Ortega-Perez, J. Vazquez, and J. M. Redondo.** 2006. Blockade of NFAT activation by the second calcineurin binding site. J. Biol. Chem. **281:**6227–6235.
52. **Matheos, D. P., T. J. Kingsbury, U. S. Ahsan, and K. W. Cunningham.** 1997. Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. Genes Dev. **11:**3445–3458.
53. **Molkentin, J. D., J. R. Lu, C. L. Antos, B. Markham, J. Richardson, J. Robbins, S. R. Grant, and E. N. Olson.** 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell **93:**215–228.
54. **Mumberg, D., R. Muller, and M. Funk.** 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. **22:**5767–5768.
55. **Nikolovska-Coleska, Z., R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P. P. Roller, K. Krajewski, N. G. Saito, J. A. Stuckey, and S. Wang.** 2004. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. Anal. Biochem. **332:**261–273.
56. **Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman.** 1997. Calcineurin is required for virulence of Cryptococcus neoformans. EMBO J. **16:**2576–2589.
57. **Park, S., M. Uesugi, and G. L. Verdine.** 2000. A second calcineurin binding site on the NFAT regulatory domain. Proc. Natl. Acad. Sci. USA **97:**7130–7135.
58. **Patton, E. E., A. R. Willems, D. Sa, L. Kuras, D. Thomas, K. L. Craig, and M. Tyers.** 1998. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box proteincomplexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev. **12:**692–705.
59. **Porta, S., S. A. Serra, M. Huch, M. A. Valverde, F. Llorens, X. Estivill, M. L. Arbones, and E. Marti.** 2007. RCAN1 (DSCR1) increases neuronal susceptibility to oxidative stress: a potential pathogenic process in neurodegeneration. Hum. Mol. Genet. **16:**1039–1050.
60. **Rezzani, R.** 2004. Cyclosporine A and adverse effects on organs: histochemical studies. Prog. Histochem. Cytochem. **39:**85–128.
61. **Rothermel, B., R. B. Vega, J. Yang, H. Wu, R. Bassel-Duby, and R. S. Williams.** 2000. A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J. Biol. Chem. **275:**8719–8725.
62. **Rothermel, B. A., T. A. McKinsey, R. B. Vega, R. L. Nicol, P. Mammen, J. Yang, C. L. Antos, J. M. Shelton, R. Bassel-Duby, E. N. Olson, and R. S. Williams.** 2001. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc. Natl. Acad. Sci. USA **98:**3328–3333.
63. **Roy, J., H. Li, P. Hogan, and M. Cyert.** 2007. A conserved docking site modulates substrate affinity for calcineurin, signaling output, and in vivo function. Mol. Cell **25:**889–901.
64. **Rusnak, F., and P. Mertz.** 2000. Calcineurin: form and function. Physiol. Rev. **80:**1483–1521.
65. **Ryeom, S., K. H. Baek, M. J. Rioth, R. C. Lynch, A. Zaslavsky, A. Birsner, S. S. Yoon, and F. McKeon.** 2008. Targeted deletion of the calcineurin inhibitor DSCR1 suppresses tumor growth. Cancer Cell **13:**420–431.
66. **Ryeom, S., R. J. Greenwald, A. H. Sharpe, and F. McKeon.** 2003. The threshold pattern of calcineurin-dependent gene expression is altered by loss of the endogenous inhibitor calcipressin. Nat. Immunol. **4:**874–881.
67. **Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille.** 2003. Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. **48:**959–976.
68. **Sanna, B., E. B. Brandt, R. A. Kaiser, P. Pfluger, S. A. Witt, T. R. Kimball, E. van Rooij, L. J. De Windt, M. E. Rothenberg, M. H. Tschop, S. C. Benoit, and J. D. Molkentin.** 2006. Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators in vivo. Proc. Natl. Acad. Sci. USA **103:**7327–7332.
69. **Scannell, D. R., A. C. Frank, G. C. Conant, K. P. Byrne, M. Woolfit, and K. H. Wolfe.** 2007. Independent sorting-out of thousands of duplicated gene pairs in two yeast species descended from a whole-genome duplication. Proc. Natl. Acad. Sci. USA **104:**8397–8402.
70. **Sherman, F., J. B. Hicks, and G. R. Fink.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
71. **Shin, S. Y., S. M. Choo, D. Kim, S. J. Baek, O. Wolkenhauer, and K. H. Cho.** 2006. Switching feedback mechanisms realize the dual role of MCIP in the regulation of calcineurin activity. FEBS Lett. **580:**5965–5973.
72. **Stathopoulos, A. M., and M. S. Cyert.** 1997. Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:**3432–3444.
73. **Takeo, S., M. Tsuda, S. Akahori, T. Matsuo, and T. Aigaki.** 2006. The calcineurin regulator sra plays an essential role in female meiosis in Drosophila. Curr. Biol. **16:**1435–1440.
74. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
75. **Vega, R. B., B. A. Rothermel, C. J. Weinheimer, A. Kovacs, R. H. Naseem, R. Bassel-Duby, R. S. Williams, and E. N. Olson.** 2003. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. Proc. Natl. Acad. Sci. USA **100:**669–674.
76. **Vega, R. B., J. Yang, B. A. Rothermel, R. Bassel-Duby, and R. S. Williams.** 2002. Multiple domains of MCIP1 contribute to inhibition of calcineurin activity. J. Biol. Chem. **277:**30401–30407.
77. **Wallis, J. W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein.** 1989. A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. Cell **58:**409–419.
78. **Wang, H., Y. Du, B. Xiang, W. Lin, X. Li, and Q. Wei.** 2008. A renewed model of CNA regulation involving its C-terminal regulatory domain and CaM. Biochemistry **47:**4461–4468.
79. **Wong, S., G. Butler, and K. H. Wolfe.** 2002. Gene order evolution and paleopolyploidy in hemiascomycete yeasts. Proc. Natl. Acad. Sci. USA **99:**9272–9277.
80. **Yang, J., B. Rothermel, R. B. Vega, N. Frey, T. A. McKinsey, E. N. Olson, R. Bassel-Duby, and R. S. Williams.** 2000. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ. Res. **87:**E61–E68.
81. **Yoshimoto, H., K. Saltsman, A. P. Gasch, H. X. Li, N. Ogawa, D. Botstein, P. O. Brown, and M. S. Cyert.** 2002. Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **277:**31079–31088.