The RCAN carboxyl end mediates calcineurin docking-dependent inhibition via a site that dictates binding to substrates and regulators

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Specificity of signaling kinases and phosphatases toward their targets is usually mediated by docking interactions with substrates and regulatory proteins. Here, we characterize the motifs involved in the physical and functional interaction of the phosphatase calcineurin with a group of modulators, the RCAN protein family. Mutation of key residues within the hydrophobic docking-cleft of the calcineurin catalytic domain impairs binding to all human RCAN proteins and to the calcineurin interacting proteins Cabin1 and AKAP79. A valine-rich region within the RCAN carboxyl region is essential for binding to the docking site in calcineurin. Although a peptide containing this sequence compromises NFAT signaling in living cells, it does not inhibit calcineurin catalytic activity directly. Instead, calcineurin catalytic activity is inhibited by a motif at the extreme C-terminal region of RCAN, which acts in *cis* with the docking motif. Our results therefore indicate that the inhibitory action of RCAN on calcineurin-NFAT signaling results not only from the inhibition of phosphatase activity but also from competition between NFAT and RCAN for binding to the same docking site in calcineurin. Thus, competition by substrates and modulators for a common docking site appears to be an essential mechanism in the regulation of Ca²⁺-calcineurin signaling.

docking interaction | NFAT | PxxIT | Cabin1 | phosphatase

Protein kinases and phosphatases are central to many intracellular signal transduction processes. In general, these signaling proteins rely on binding interactions both for interaction with upstream regulators and for enzymatic modification of substrates. In some cases, these interactions are mediated by dedicated modular domains fused to the catalytic domain. In others, the binding surfaces are within the catalytic domain but do not overlap the active site. Both these binding interactions are known as docking interactions and are envisaged principally as a mechanism to increase substrate specificity (1). The serine/threonine protein phosphatase calcineurin (CN, PP2B) uses both of these binding strategies to organize its interactome. CN is a heterodimer composed of a catalytic subunit calcineurin A (CnA) and a regulatory subunit CnB. The phosphatase domain in CnA is connected, through a sequence known as the linker, to a regulatory region that acts as a protein-protein interacting platform and includes binding domains for CnB and calmodulin (CaM) and an autoinhibitory (AI) segment. Increases in intracellular Ca²⁺ allow interaction of CaM with heterodimeric CN, displacing AI from the active site to allow CN to dephosphorylate its substrates (2). The best-characterized CN-substrate interaction is that with members of the nuclear factor of activated T cells (NFAT) family of transcription factors (3). The N-terminal region of NFAT proteins contains 2 CN-binding sites, neither of which contains the substrate motif. These docking motifs are the PxxIT sequence (4) and the LxVP motif (5, 6). Two CnA regions are important for interaction with the NFAT PxxIT sequence: the linker (7) and the cleft formed by β 11 and β 14 strands within the phosphatase domain (8–10).

CN activity is also regulated by interaction with anchoring and regulatory proteins (11); however, little is known about how these proteins form contacts with CN. Among the regulatory proteins, one of the most remarkable families is the recently renamed regulator of calcineurin (RCAN, previously known as DSCR/MCIP/calciressin/Adapt78 in mammals) (12). RCANs bind to and inhibit CN-mediated activities in vitro (13–18). The evidence in these studies comes mostly from overexpression of RCANs, but the CN-inhibitory potential of these proteins is also shown in some mouse models of *Rcan1* loss of function (19–22). However, the phenotypes of null mutants of *Saccharomyces cerevisiae* Rcn1p (23) and *Rcan1*^{−/−} or *Rcan2*^{−/−} mice (19, 24) are compatible with reduced CN activity, which has led to suggestions of a positive action of RCANs on CN. The 3 human RCANs (RCAN1, RCAN2, and RCAN3) have a high amino acid identity in the central and C-terminal regions (12). The CN-binding activity of RCAN1 and RCAN3 is located in the conserved region encoded by their last exon (exon 7 in RCAN1) (14, 18, 25). This region in RCAN1 is thought to contain 2 CN-interacting sites: a PxxIT-like site, which resembles the PxxIT motif from NFAT, located near the C terminus, and the more N-terminal CIC (ELHA-containing calcineurin-inhibitor CALP1) motif (26). The CN-catalytic inhibitory activity maps to the exon 7-region in RCAN1 (25). In addition, a peptide containing the RCAN1 FLISP motif, located in the central part of RCAN proteins and considered the signature of the family, has also been shown to behave as a CN inhibitor in vitro (13, 16, 27).

Here, we show that RCANs interact with CnA via the hydrophobic pocket defined by β -sheets 11 and 14. RCANs and Cabin-1 (CN modulators), NFATs (CN substrates), and AKAP79 (CN scaffold protein) all compete for binding to this docking site in CnA. We further show that docking of RCAN to CN and inhibition of CN catalytic activity are mediated by distinct motifs in the C-terminal region that need to act in *cis* for efficient inhibition.

Results

Hydrophobic Cleft Formed by CnA Strands β 11 and β 14 Is Essential for Interaction with RCAN Proteins. To map sites within CnA that bind RCANs, we performed pulldown assays using the C-terminal

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Fig. 1. Residues from the hydrophobic cleft formed by CnA strands β 11 and β 14 are required for interaction with RCAN in vitro. (A) Lysates of HEK293 cells expressing the indicated FLAG-CnA proteins were assayed in pull-down experiments with GST-RCAN1C; black boxes indicate the CnB-binding domain (B), the CaM-binding domain (C), and the AI domain (AI). CnA proteins that bind RCAN1 are indicated. (B) Lysates of cells expressing the indicated CnA-336 proteins (see scheme) were incubated with GST-RCAN1C. Φ , nontransfected cell extracts. (C) C-terminal regions of RCAN2 and RCAN3 (see Table S1) show similar behavior in CN binding assays. Two amounts of extract from cells expressing either WT or NIR>AAA mutated (NIR) CnA-336 were used. (D) Extracts from cells expressing WT or mutant FLAG-CnA proteins were immunoprecipitated with anti-FLAG, and endogenous RCAN1 (isoform 1.1) detected by Western blot analysis (*, IgG heavy chain). Shown are representative experiments (quantification in Fig. S8).

region (amino acids 170–252) of human RCAN1 (RCAN1C, containing the sequences required for CN binding) as bait and CnA deletion mutants as targets. RCAN1C was able to bind all CnA proteins except for the deletion mutant comprising residues 2–268 (Fig. 1A). This indicates that the CaM-binding, CnB-binding, and linker regions are unnecessary for interaction of CnA with RCAN1C. Moreover, it suggests that the CnA region required for CN-RCAN1C binding is located between amino acids 269 and 336. This region contains β -strands 11 and 14, which together form a hydrophobic cleft identified as the region that interacts with the high-affinity PxIXIT-derived PVIVIT peptide (8–10). We next generated mutant versions of CnA-336 in which β 11 or β 14 residues essential for interaction with the PVIVIT peptide (8) were replaced with alanine (336-YM: mutations on Y288 and M290 from strand- β 11; 336-NIR: mutations on N330, I331, and R332 from strand- β 14). Neither 336-YM nor 336-NIR mutant CnA was able to bind RCAN1C (Fig. 1B). The generality of this behavior among RCAN proteins was confirmed with the equivalent C-terminal regions of human RCAN2 and RCAN3 (Fig. 1C).

Binding to RCAN1C was also impaired by mutation of the NIR site in the context of full-length CnA [supporting information (SI) Fig. S1A]. Consistent with predictions based on published PVIVIT interactions (9, 10), the mutation abrogated CnA interaction with NFATc2 (Fig. S1A), but did not disrupt interaction with CnB (Fig. S1B). Moreover, neither CnA-268 nor the NIR CnA mutants interacted with endogenous full-length RCAN1.1 in cells (Fig. 1D). The NIR residues within CnA β -strand 14 are thus important for CN interaction with human RCANs.

CnA β -Strand 14 Is Required for Binding to the RCAN EV-Motif. The NIR residues in β -strand 14 are important for CnA binding to the high-affinity PVIVIT peptide (8, 9). PVIVIT is based on the PxIXIT CN-docking sequence of NFATs (28), so PxIXIT motifs from NFATs and those described in other CN-interacting proteins should interact with the same CnA region. The C-terminal regions of RCANs contain a putative PxIXIT motif (25, 26). However, we were unable to detect CnA interaction with GST fusions of the putative PxIXIT motifs from RCAN1 (R1Cter: 30 aa) or RCAN3 (R3Cter: 38 aa) (Fig. 2A). Under the same conditions, the NFATc2

PxIXIT site, a low-affinity CN-binding sequence ($K_d = 25 \mu\text{M}$) (9), did bind (Fig. 2A).

Another sequence in the RCAN carboxyl region reported to interact with CnA is the ELHA-containing CIC motif, in which 2 conserved sequence blocks can be identified, one containing the ELHA sequence (26) (labeled E-motif in figures) and the other being the valine-rich sequence PSVVVH (V-motif in figures) (Fig. 2B and E; Fig. S2). We call the full sequence the EV-motif. The EV-motif from RCAN1 or RCAN3 (see Table S1 for residues) pulled down endogenous CnA and CnB (Fig. 2B), indicating that this short region binds heterodimeric CN similarly to the full RCAN C terminus (Fig. S3A). The integrity of the RCAN EV-motif seems to be necessary for targeting RCANs to CN, because peptides of individual E or V motifs did not bind CnA (Fig. S3B).

We next examined whether the pattern of EV-motif binding to CnA is similar to that of the whole RCAN C-terminal domain. The RCAN1 EV-motif interacted effectively with CnA-336 (Fig. 2C), and mutation of the β -strand 14 NIR residues abolished this binding (Fig. 2C). In addition, PVIVIT efficiently and specifically displaced GST-R1EV from CnA in vitro (Fig. 2D). This suggests similar modes of action for the EV-motif and PVIVIT peptide, consistent with the resemblance of the EV-motif PSVVVH sequence to the PVIVIT core (Fig. 2E). The 2 PVIVIT isoleucines accommodate within the CnA hydrophobic docking cleft, with the second one interacting extensively with Ile-331 of the NIR (Fig. S4B), an interaction considered to be essential (8, 10). Replacement of the corresponding hydrophobic residues in the RCAN PSVVVH motif with the polar amino acid glutamine abolished interaction of the EV-motif with CN (Fig. 2F). Alanine substitution of the 2 conserved glutamic acid residues found in the ELHA regions of most RCAN proteins (Fig. 2E and Fig. S2) reduced CN binding but did not abolish it (Fig. 2F), suggesting that although the ELHA motif contributes to binding of the EV region to CN, the major determinant lies within the hydrophobic PSVVVH motif.

EV-Motif Blocks CN/NFAT Signaling but Does Not Inhibit CN Phosphatase Activity. The ability of RCAN EV-motifs to bind CN translated in vivo into an ability to block Ca^{2+} -stimulated NFAT dephosphorylation and nuclear translocation (Fig. 3A and B), agreeing with

Fig. 2. The EV-motif from RCAN interacts with CnA, and the CnA β -strand 14 is required for this interaction. (A) Mouse brain extracts were incubated with GST fusions of either the NFATc2 PxlIT motif or the extreme C-terminal sequences from RCAN1 and RCAN3 (see scheme (A)). Bound CnA and CnB were detected by Western blot analysis. (B) Similar assays performed with GST fusions of the EV-sequences from RCAN1 (R1EV) and RCAN3 (R3EV) (see scheme (B)); underlined sequences correspond to the ELHA (E) and PSVVH (V) motifs). (C) Lysates of cells expressing wt FLAG-CnA-336 or its corresponding NIR mutant were used in pull-down experiments with GST-RCAN1C and GST-R1EV. (D) Binding of FLAG-CnA-336 to GST-R1EV is competed with increasing concentrations of PVIVIT peptide. Quantification for bound CnA as a percentage of binding in the presence of a scrambled peptide (C) is shown. Values are the means $\pm$ SD of 3 independent experiments. (E) Sequence alignment of RCAN EV-motifs. Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Hs, *Homo sapiens* (see also Fig. S2). Strictly conserved amino acids are shown in red; physicochemically conserved amino acids are shown in orange. The core sequence of the PVIVIT peptide is shown. Arrows indicate conserved residues of the ELHA and PSVVH motifs that were substituted in GST-R3EV mutants. (F) These mutations in GST-R3EV affect interaction with CN, as assessed in pull-down assays with mouse brain extracts. Representative experiments are shown (quantification in Fig. S9).

previous work (26). Nonetheless, to rule out the possibility that other RCAN sequences inhibit CN/NFAT signaling independently of the EV-motif, we deleted the PSVVH motif from full-length RCAN1 or RCAN3 (Δ V mutants); the mutant proteins had no effect in preventing NFAT dephosphorylation (Fig. 3C, Figs. S5C and S6A) or nuclear translocation (Fig. S6B).

RCAN1 inhibits CN phosphatase activity, and the C-terminal 57 residues, which include the EV-motif, are sufficient for this

activity (25). Thus, one possible mechanism of the effect of RCAN EV-motifs on CN/NFAT signaling is direct inhibition of CN enzymatic activity. However, a 24-aa peptide spanning the EV-motif did not inhibit CN phosphatase activity against either pNPP (Fig. S7A) or the CN-specific substrate RII peptide (Fig. 3D). Competition experiments confirmed that the EV peptide displaced RCAN3C from CN, suggesting that it adopts the native conformation (Fig. S7B).

Fig. 3. The RCAN EV-motif inhibits CN-induced NFAT dephosphorylation and nuclear translocation, but does not alter CN catalytic activity. (A) Cells expressing GFP-NFATc3 and either GFP or the indicated GFP-tagged RCAN-derived constructs were Ca²⁺ stimulated (ns, nonstimulated) and cell extracts analyzed by Western blot (quantification in Fig. S5A). Pretreatment with FK506 is indicated. The positions of hyper- (P-NFAT) and hypophosphorylated NFAT are indicated. (B) Flag-NFATc2 subcellular localization was determined by indirect immunofluorescence with anti-FLAG in Ca²⁺-stimulated cells coexpressing the RCAN1 or RCAN3 EV-motifs fused to GFP (quantification in Fig. S5B). (C) Cells expressing GFP-NFATc3 and the indicated HA-RCAN1 proteins were analyzed as in A. RCAN1 Δ V indicates deletion of the PSVVH motif (see Table S1) (quantification in Fig. S6A). (D) CN enzymatic activity against phosphopeptide RII in the presence of the EV peptide, AID peptide, or the PxlITc2 peptide, used as a negative control. Values represent percentage activities relative to CN control reactions without added peptide.

Fig. 4. Different CN-interacting proteins compete for a common docking site defined by the CnA NIR motif. (A and B) Increasing concentrations of EV (A) and PSVVVH (B) peptides block the binding of endogenous CnA to the NFATc2 regulatory region in pull-down assays. (C) Lysates of cells expressing WT or NIR-mutated CnA-389 proteins were used in pull-down experiments with GST-NFATc2, GST-AKAP79, or GST-Cabin1. A representative of 4 experiments is shown. (D) Increasing concentrations of PSVVVH and PVIVIT peptides block binding of endogenous CnA from HEK293 cells to GST-Cabin1. The scrambled C peptide was used as a control. Graphs show quantification of 3 independent experiments.

CnA Hydrophobic Cleft Containing the NIR Site Defines a Common Docking Site for CN Regulators. The data presented so far show that the EV-motif does not affect CN catalytic activity, that its interaction with CnA relies on the integrity of the β 14 sheet, that this interaction is blocked by PVIVIT peptide, and that its overexpression blocks CN/NFAT signaling. We therefore hypothesized that the in vivo inhibitory activity of the EV-motif on the CN/NFAT pathway might depend on its ability to disrupt binding of CN to NFAT. Supporting this, the EV peptide competed the binding of NFATc2 regulatory domain to endogenous CnA in a concentration-dependent manner (Fig. 4A). Similarly, a peptide spanning the PSVVVH site had no effect on CN catalytic activity ($107 \pm 5.6\%$ in the presence of 40 μ M PSVVVH peptide), but strongly competed the interaction of NFATc2 with endogenous CnA (Fig. 4B), suggesting that this essential RCAN sequence regulates the Ca^{2+} /CN pathway by competing with NFATs for a common docking site on CN.

Several CN-interacting proteins interact through regions containing a PxIxIT-like site, as in the case of the CN scaffold protein AKAP79 and the negative regulator Cabin1 (Fig. S4A). Interaction of these 2 proteins with CnA was abolished by the NIR mutation (Fig. 4C). Moreover, the binding of Cabin1 to endogenous CnA was competed by increasing amounts of PVIVIT or RCAN PSVVVH peptides (Fig. 4D). These results suggest that all CN target proteins containing PxIxIT-like motifs share the same docking site on CnA and that competition among them might play a role in CN-dependent signaling.

RCAN Inhibition of CN Phosphatase Activity Is Mediated by the Extreme C-Terminal Region. Given that the carboxyl regions of RCAN1 and RCAN3 can directly inhibit CN catalytic activity but that the EV-motif cannot, we reasoned that the inhibitory action of RCANs might be mediated by the extreme C-terminal region. However, a peptide comprising the C-terminal 30 aa of RCAN1C did not block CN phosphatase activity (Fig. 5A), indicating that the full C-terminal region, including the EV-motif, is required. One possible explanation is that the EV-motif functions simply to induce a conformational change in CN that exposes the catalytic site to the

inhibitory RCAN motif. This model would predict that the inhibitory motif acts independently of its physical location within the RCAN protein structure; however, free C-terminal and EV peptides had no inhibitory effect on CN phosphatase activity when added together to the assay (Fig. 5A).

A second possibility is that the binding and inhibitory activities of the RCAN C-terminal region act in cis, and thus need to be physically linked. To test this, we generated a GST fusion containing the last 30 aa of RCAN1 fused to the CN docking peptide PVIVIT (PVIVIT-R1Cter) as a way to tether the C-terminal region to CN. Binding assays confirmed that PVIVIT-R1Cter interacted with CN, in contrast to R1Cter (Fig. 5B). As expected, PVIVIT, although able to interact with CnA, had no effect on CN catalytic activity (Fig. 5B and C). R1Cter likewise did not inhibit CN catalytic activity (Fig. 5C). In contrast, PVIVIT-R1Cter inhibited CN phosphatase activity in a dose-dependent manner (Fig. 5C). This suggests that the CN inhibitory activity lies within the last 30 aa. This region harbors a block of conserved amino acids in all RCAN proteins (see Fig. S2). Substitution of the most conserved residues (Thr-241 and Pro-244; Fig. 5D) with alanine had no apparent effect on binding of RCAN1C to CN (Fig. 5D) or on the ability of RCAN1.1 to inhibit CN-mediated NFAT dephosphorylation in vivo (Fig. 5E); however, the mutation weakened in vitro inhibitory activity toward CN (Fig. 5F). Similar results were obtained when a mutant with a deletion of the conserved stretch (residues 236–244) was used (Fig. S6A–C). Further supporting our proposal that inhibition requires that the RCAN C-terminal domain be recruited to CnA, we found that the EV peptide specifically blocked the inhibition mediated by RCAN1C (Fig. 5G) and that the catalytic activity of the CnA NIR mutant is not inhibited by RCAN1C (Fig. 5H). These results indicate that the RCAN C terminus cannot inhibit CN unless it is tethered to CN via a separate binding interaction at the NIR docking site, provided by the EV-motif.

Discussion

A combination of photoaffinity cross-linking, crystallographic, and NMR analyses have determined that the high-affinity CN-binding

Fig. 5. The extreme C-terminal sequence of RCANs is responsible for their inhibitory action on the catalytic activity of CN when tethered to CnA. (A) CN enzymatic activity was measured by using the phosphopeptide RII in the presence of the R1Cter (250 μ M), the EV (250 μ M), the AID (100 μ M) peptides, or an equimolar combination of R1Cter and EV (125 μ M each). Values are percentage activities relative to CN control reactions without added peptide. (B) R1Cter does not bind CN, but the PVIVIT-R1Cter fusion does. The indicated GST fusion proteins were assessed in CN pulldown assays with mouse brain extract. (C) PVIVIT-R1Cter, but not R1Cter, inhibits CN activity. CN activity against the substrate RII measured in the presence of the indicated GST fusion proteins (0.5 and 1 μ M). (D) Pulldown experiments with WT and C-terminally mutated RCAN1C proteins fused to GST and mouse brain extracts. (E) The TP mutation does not alter RCAN1 ability to inhibit CN-mediated NFAT dephosphorylation. Cells coexpressing GFP-NFATc3 and the indicated HA-RCAN1 proteins were analyzed as in Fig. 3A (quantification in Fig. S6). (F) The TP mutation impairs RCAN CN-inhibitory activity. The indicated GST fusion proteins were assessed in CN phosphatase assays using phosphopeptide RII. Values are percentage activities relative to CN control reactions in the presence of unfused GST. (G) The EV peptide blocks CN inhibition by RCAN. CN was preincubated with EV peptide before GST-RCAN1C was added to the reaction, and phosphatase activity against RII was measured. Values represent percentage activities relative to unfused GST protein in the presence of negative control peptide (C). (H) The CnA NIR mutant is not inhibited by RCAN1C. The phosphatase activity of WT CN or the NIR>AAA mutant was measured in pNPP assays in the presence of unfused GST or GST-RCAN1C (1 μ M). Values (means $\pm$ SD) are percentage activities relative to WT CN control reactions in the presence of unfused GST, of at least 3 independent assays.

peptide PVIVIT occupies a hydrophobic cleft formed by the $\beta 11$ and $\beta 14$ strands within the catalytic domain of CnA, with the NIR group being contact residues (8–10). We have shown here that mutations in β -strand 14 inhibit the interaction of CnA with all human RCANs with other targets such as NFATc2, AKAP79, and Cabin1, suggesting that the hydrophobic cleft is a shared interaction site on CN. The PVIVIT peptide was modeled on the PxIxIT-motif, first characterized as the PRIEIT motif in NFATc2 (4). AKAP79 contains a functional PxIxIT site (29), and Cabin1 contains a PxIxIT site in the CN-interacting region, although it is not known whether it functions as the interacting motif (30). Our findings extend recent results obtained with CN substrates in yeast (31) and highlight the importance of the CnA hydrophobic cleft for the interaction of CN with several of its partners.

Although an earlier study reported interaction between CnA and a PxIxIT-like motif at the C-terminal extreme of RCAN1 (26), our results demonstrate that this motif, from either RCAN1 or 3, is not important for interaction with CN (see summary in [Table S1](#)). The lack of interaction via this motif might be explained by the presence of a polar residue in place of a hydrophobic residue at position 5. The final positions of all published PxIxIT sequences are occupied by a hydrophobic residue followed by a hydrophilic residue ([Fig. S4A](#)). The hydrophobic residue accommodates within the CnA hydrophobic docking cleft, interacting extensively with Ile-331 from the NIR sequence (10) ([Fig. S4B](#)). This hydrophobic interaction is thought to be crucial (8, 10), and the polar residue at this position

in the PxIxIT-like motifs of most RCAN proteins (Fig. S2) may thus preclude interaction with the CnA hydrophobic cleft.

In contrast, the other CN-binding region identified in the carboxyl regions of RCANs, the CIC motif (18, 26) is both necessary and sufficient for CnA binding and is able to stably bind the heterodimeric CN molecule. The term CIC stands for CN-inhibitor calcipressin motif; but given its lack of inhibitory activity, and based on its sequence, we call this region the *EV*-motif. Contrasting the lack of conservation of RCAN PxIxIT-like motifs, the *EV*-motif is highly conserved in vertebrate RCANs (Fig. S2). Importantly, the loss of *EV*-motif binding upon mutation of the CnA NIR residues or the mutation of hydrophobic residues in the PSVVVH sequence, together with the competition of EV/CN interaction by the PVIVIT peptide, suggests that the NIR- and EV-sites are the interacting surfaces involved in the formation of CN/RCAN complexes. We therefore propose that the PSVVVH sequence should be considered the true PxIxIT-motif of RCAN proteins.

The inhibition of CN/NFAT signaling by RCAN1 has been assumed to depend on inhibition of CN phosphatase activity (25). Like full-length RCAN proteins, the EV-motif is able to block CN/NFAT signaling in cells. However, unlike full-length RCAN proteins, the EV peptide does not block CN catalytic activity in vitro. Our results show that both the EV peptide and its component peptide PSVVVH compete for interaction of CN with NFAT. This activity could account for the blockade of CN-dependent NFAT dephosphorylation by RCANs in cells. Given that binding of one of

the molecules would exclude binding of the other, their relative concentrations and respective binding affinities will be key factors in determining the signaling outcome. This competition model has profound implications for the interpretation of RCAN action in vivo: RCANs can inhibit CN catalytic activity directly in vitro, but evidence for this activity in intact cells has been based on impaired NFAT signaling, which may instead reflect the binding competition effect. Nonetheless, our results do not rule out that RCAN phosphatase inhibitory activity could contribute to the inhibition of CN-dependent pathways in defined physiological situations.

Our results, summarized in Table S1, lead us to propose that RCAN proteins have 2 distinct activities: They specifically block the binding and subsequent dephosphorylation of substrates that interact with CN through a functional PxIxIT docking site, as in the case of NFAT proteins, and they act as direct inhibitors of the CN catalytic activity. These 2 activities, although structurally linked, do not overlap: The EV-motif appears to mediate CN binding and competition with PxIxIT-containing substrates, whereas the extreme C terminus accounts for the direct inhibitory activity. Such a mechanism would be reminiscent of some inhibitors of the phosphatase PP1, such as Inhibitor-1 and DARPP-32. These molecules bind PP1 through an RVXF targeting motif that fits into a groove distinct from the active site of the enzyme, whereas a second sequence docks within the active site (32, 33). This mechanism, a combination of a docking site with a *cis*-regulatory element, could be a feature of the phosphatase family. Docking interactions are widely used in the regulation of phosphorylation signaling networks (1). In protein kinases, these interactions are not only a way to recruit substrates and regulators, but also to confer allosteric regulation (34). In the case of phosphatases, allosteric effects have not been found yet (35), and the role of docking could just be that of tethering the regulatory domains.

The findings presented here provide a molecular explanation for RCAN inhibition of CN catalytic activity. They also identify a molecular mechanism for RCAN-mediated inhibition of CN/

NFAT signaling, involving RCAN-dependent displacement of NFAT from CN. Moreover, our results highlight the importance of the NIR sequence in CN for this protein's interactions with regulatory proteins and substrates. The hydrophobic cleft is thus emerging as a key element in the regulation of CN-dependent signaling pathways, and our findings suggest avenues for pharmacological intervention directed at modulating the phosphatase protein-protein interacting network rather than CN catalytic activity.

Materials and Methods

Plasmids, Peptides, and Antibodies, Cell Culture, Transfections and Immunofluorescence, and GST Fusion Protein Expression and Protein Binding Assays. Full information is provided in *SI Text*. For nomenclature and amino acid sequences of the expression plasmids used, see Table S2, and for oligonucleotides and backbone vectors used to generate the pGEX-derived expression vectors, see Table S3.

In Vitro CN Assay. CN activity toward its specific RII phosphopeptide substrate was measured with a colorimetric assay (Biomol). Phosphatase assays using pNPP as substrate were performed as described (25) with minor modifications detailed in *SI Text*.

Statistical Analysis. Data are presented as means $\pm$ SD and were analyzed with GRAPHPAD PRISM software (v3.02; GraphPad). In peptide competition assays, statistics were analyzed by 1-way ANOVA, followed by Neuman-Keuls multiple comparison test. In all other experiments, data were evaluated with the 2-tailed Student's test for independent samples. Differences were taken to be statistical significant at *P* values of 5% or below (*, *P* $\leq$ 0.05; **, *P* $\leq$ 0.01; ***, *P* $\leq$ 0.001).

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