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A conserved docking surface on calcineurin mediates interaction with substrates and immunosuppressants

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SUMMARY

The phosphatase calcineurin, target of the immunosuppressants cyclosporin A and FK506, dephosphorylates NFAT transcription factors to promote immune activation and development of the vascular and nervous systems. NFAT interacts with calcineurin through distinct binding motifs: the PxIXIT and LxVP sites. While many calcineurin substrates contain PxIxIT motifs, the generality of LxVP-mediated interactions is unclear. We define critical residues in the LxVP motif, and demonstrate its binding to a hydrophobic pocket at the interface of the two calcineurin subunits. Mutations in this region disrupt binding of mammalian calcineurin to NFATc1, and interaction of yeast calcineurin with substrates including Rcn1, which contains an LxVP motif. These mutations also interfere with calcineurin-immunosuppressant binding, and an LxVP-based peptide competes with immunosuppressant-immunophilin complexes for binding to calcineurin. These studies suggest that LxVP-type sites are a common feature of calcineurin substrates and that immunosuppressant-immunophilin complexes inhibit calcineurin by interfering with this mode of substrate recognition.

INTRODUCTION

Calcineurin (CN), or protein phosphatase 2B (PP2B), is a highly conserved eukaryotic calcium-calmodulin-activated protein phosphatase that couples calcium-mobilizing signals to cell responses (Aramburu et al., 2000; Rusnak and Mertz, 2000). CN is the target of the immunosuppressant drugs cyclosporin A (CsA) and FK506, which as suppressors of graft rejection are important treatments for post transplant recovery (Friedman and Weissman, 1991; Liu et al., 1991). As investigative tools, CsA and FK506 have enabled roles of CN to be

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identified in diverse biological processes, including cell-stress responses and drug tolerance in fungi, and immune responses, pancreatic β-cell function, early vascular development, neurological functions, ion homeostasis, and muscle differentiation in mammals. In addition to their use in preventing transplant rejection, CN inhibitors have been successfully used to treat atopic dermatitis, severe asthma, segmental glomerulosclerosis and refractory rheumatoid arthritis (Crabtree and Olson, 2002; Cyert, 2003; Hogan et al., 2003).

CN is a heterodimer comprising a catalytic subunit A (CnA) and a regulatory subunit B (CnB). CnA contains the phosphatase domain and a regulatory region, which are connected by a 13-amino acid linker sequence. The regulatory region contains a CnB-binding domain (BBD), a calmodulin binding domain and an auto-inhibitory domain (AID) (Klee et al., 1998). CsA and FK506 each form complexes with a specific immunophilin binding protein (cyclophilin – Cyp, and FK506 Binding Protein – FKBP, respectively), and it is these complexes that inhibit CN activity (Cardenas et al., 1995; Liu et al., 1991). Immunophilins are prolyl isomerases, but this activity appears unrelated to immunosuppression. Immmunophilins bind CN only in the presence of immunosuppressants. Structural, biochemical and genetic studies show that immunosuppressant-immunophilin (IS-IP) complexes interact with a hydrophobic groove formed at the junction of the CnA and CnB subunits (Griffith et al., 1995; Huai et al., 2002; Jin and Harrison, 2002; Kissinger et al., 1995). A discrete set of amino acids in CN is involved in the recognition of both IS-IP complexes (Ke and Huai, 2003). Since CsA (a cyclic peptide) and FK506 (a macrolide) are structurally unrelated, their interaction with the same residues in a common site in CN suggests that this site is a recognition region for other CN-binding proteins.

Many CN-dependent processes in mammals involve the regulation of the nuclear factor of activated T-cells (NFAT) family of transcription factors (Crabtree and Olson, 2002; Hogan et al., 2003). In resting cells, NFAT proteins are phosphorylated and reside in the cytoplasm. Upon stimulation, NFATs are dephosphorylated by CN, triggering their translocation to the nucleus and transcription of target genes (Hogan et al., 2003). NFAT dephosphorylation requires a docking interaction between NFAT and CN, and two CN-interacting sequences in the regulatory domain of NFAT have been identified. The main docking site, located at the regulatory domain N terminus, has the consensus sequence PxIxIT (Aramburu et al., 1998). A high-affinity 14mer peptide derived from this sequence, PVIVIT, forms contacts along the edge of strands β-11 and β-14 of the CnA phosphatase domain (Aramburu et al., 1999; Li et al., 2007). Other CN substrates and regulators also contain PxIxIT motifs, and the binding affinities of peptides spanning these sequences have been compared (Roy et al., 2007). In the yeast *Saccharomyces cerevisiae*, the PxIxIT interaction modulates the CN-substrate affinities of several substrates and determines the output of in vivo signaling (Bultynck et al., 2006; Heath et al., 2004) (Hilioti and Cunningham, 2003; Roy et al., 2007). Furthermore, the transcriptional activity of the yeast CN substrate Crz1 is regulated by CN-dependent dephosphorylation and nuclear localization in a manner analogous to mammalian NFAT (Cyert, 2003).

NFAT also interacts with CN through a second binding site called LxVP (Liu et al., 2001; Liu et al., 1999; Martínez-Martínez et al., 2006). The LxVP motif, located at the C-terminus of the regulatory domain, is only moderately conserved. This motif is less understood than the PxIxIT motif and it is unclear if other CN substrates contain an equivalent CN-binding motif. We recently showed that a peptide based on the LxVP motif of NFATc1 (the LxVPc1 peptide) interferes with the interaction of NFAT with activated CN and, unlike the PVIVIT peptide, inhibits CN-phosphatase activity (Martínez-Martínez et al., 2006). Here we characterize the LxVP-CN interaction and identify a hydrophobic pocket at the junction of the CnA and CnB subunits that mediates binding to LxVPc1, NFAT, and several yeast CN substrates. Specifically, we identify an LxVP motif in Rcn1, the yeast RCAN (Davies et al., 2007) (Hilioti

and Cunningham, 2003; Roy et al., 2007), and show that it is required for CN-binding. Since the CN hydrophobic docking surface is also essential for binding IS-IP (CsA and FK506) complexes, we propose that these drugs block the interaction of CN with LxVP-type motifs common to CN substrates, thereby inhibiting CN-dependent signaling.

RESULTS

LxVPc1 peptide inhibits NFAT-dependent transcription and modifies the enzymatic activity of calcineurin

CsA and FK506, complexed with their corresponding immunophilins, inhibit immune responses by blocking CN-dependent dephosphorylation and nuclear translocation of NFAT transcription factors (Liu et al., 1991). To investigate whether similar effects are produced by LxVPc1, a 15 amino-acid synthetic peptide corresponding to the LxVP motif in NFATc1 (Fig. 1A), we infected Jurkat T cells with a lentivirus encoding a GFP-fusion of LxVPc1. Consistent with earlier experiments in transiently-transfected HeLa cells (Martínez-Martínez et al., 2006), expression of LxVPc1, but not LxVPc1mut, inhibited NFAT dephosphorylation induced by phorbol ester (PMA) plus Ca^{2+} ionophore (P+Io) in Jurkat cells (Fig. 1B, C). Examination of NFAT downstream responses showed that infection with LxVPc1 lentivirus also diminished P+Io–induced mRNA expression of the NFAT-dependent genes RCAN1 and IL-2 (Fig. 1D). This correlated with inhibited transcription from the RCAN1 and IL-2 promoters (Fig. 1E). Expression of the NFAT-dependent interleukins IL-3, IL-8 and IL-13, normally induced during lymphocyte activation, was impaired by the LxVPc1 peptide, in a similar though less pronounced manner as seen with CsA (Fig 1F).

We showed previously that LxVPc1 inhibits CN activity towards the 19 amino acid phosphopeptide RIIp (Martínez-Martínez et al., 2006). Here we show that LxVPc1 inhibits CN with ~10-fold greater efficacy than a peptide corresponding to the CN auto-inhibitory domain (Fig. 2A). As expected, a control PxIxIT peptide failed to affect CN activity. A commonly used alternative to peptide substrates for investigation of phosphatase reaction mechanisms is the small phosphorylated compound p-nitrophenylphosphate (pNPP). Paradoxically, the immunosuppressants CsA and FK506 increase CN-mediated pNPP dephosphorylation, probably by easing access of this small molecule to the CN active site and increasing its affinity (Swanson et al., 1992; Yin and Ochs, 2003). Addition of either LxVPc1 or CsA-Cyp A increased the activity of CN toward pNPP, although LxVPc1 was a less potent activator (Fig. 2B and supplemental table 1). Thus, like CsA and FK506, LxVPc1 modifies the activity of CN through an interaction with a site distinct from the active site.

Contribution of LxVP motif residues to the interaction with calcineurin

The 15mer LxVP motif sequence is only moderately conserved among NFAT regulatory domains (Fig. 1A), and this discrepancy may account for differences in interaction with CN: the LxVP motifs of NFATc1, c3 and c4 bind CN strongly whereas that from NFATc2 barely interacts (Park et al., 2000). To assess the importance of individual residues, we first pulled down activated CN with GST-LxVPc1 fusions containing a point mutation at each position. This showed that the sequence YLxVP is essential for binding CN and that residues in the C-terminal portion of LxVPc1 also contribute to the interaction strength (not shown). For a quantitative analysis, we determined the dose-response profiles and IC50 values of the mutant LxVPc1 peptides for inhibition of CN RIIp phosphatase activity. This analysis confirmed the importance of the YLxVP sequence and showed that the W13A mutation also impaired inhibition (Fig. 2C and supplemental table S2). Consistently, these mutations impaired the LxVPc1-mediated activation of CN activity toward pNPP (supplemental Fig S1).

Alignment of the LxVPc1 and LxVPc2 sequences reveals differences at the amino- and carboxy-ends that may account for their different CN binding capacities (Fig. 2D). For example, LxVPc2 has isoleucine at position 3, whereas LxVPs from other NFATs have an aromatic residue (Tyr or Phe), which in LxVPc1 is essential for its binding to CN. Also, LxVPc2 contains adjacent prolines that in other NFAT LxVP sequences are separated by one or two residues (Fig. 1A). This juxtaposition may misplace residues important for CN interaction. We substituted LxVPc2 with residues from the equivalent positions in LxVPc1 (Fig. 2D). Individual I3Y and P13W substitutions increased CN binding, and the combination of both further increased the amount of CN bound (Fig. 2D, lanes 1, 3 and 4), indicating the importance of both residues. To examine the influence of carboxy-end residues, we made an LxVPc2:LxVPc1 chimera containing the LxVPc2 core sequence and the C-terminal region of LxVPc1. By itself, this alteration only weakly affected CN binding; however, introduction of the I3Y substitution into the LxVPc2:LxVPc1 chimera increased CN binding to a level similar to that of wild-type (wt) LxVPc1 (Fig. 2D and supplemental Fig. S2).

CnA and CnB subunits are both required for LxVPc1 binding

To identify CN regions required for LxVPc1 binding, we tested the interaction of glutathione S-transferase (GST)-LxVPc1 with CN containing flag-tagged truncated CnA (Fig. 3A). Deletion of the CnB-binding domain (BBD) of CnA completely abolished binding (Fig. 3B). Interaction of GST-LxVPc1 with CnA371 (the shortest truncated CN that contains the BBD) was specific, since it was competed by free LxVPc1 but not by control peptide.

Requirement for the BBD in the CN–LxVPc1 interaction suggests either that LxVPc1 obstructs the interaction of CN subunits (by binding to the BBD of CnA or to CnB) or that it binds to a composite site present only in the CnA-CnB heterodimer. To test the first possibility, we pulled down CnB with GST-CnA in the presence of increasing concentrations of LxVPc1 peptide, and found that LxVPc1 did not compete the binding of CnB to CnA (data not shown). To test the possibility of LxVPc1 binding to a composite site at the CnA and CnB interface, we expressed each subunit separately and used them in pull-down experiments. GST-LxVPc1 pulled down CnA only when the CnB subunit was included in the binding reaction (Fig. 3C); in contrast, GST-PxIxITc2 bound all CnA truncated forms independently of the presence of CnB (Fig. 3D and data not shown). The LxVPc1 peptide competes effectively for activated CN (Martínez-Martínez et al., 2006); we therefore tested whether the CN activation state affects the binding to LxVPc1. We found that binding of LxVPc1 requires the presence of Ca^{2+} and calmodulin, showing that interaction takes place only with activated CN (Figure 3E).

LxVPc1 blocks binding of immunosuppressant-immunophilin complexes to calcineurin in vitro

The data shown above suggested that LxVPc1 might share a similar mechanism of action with immunosuppressant–immunophilin complexes. We therefore pulled down CN with GST-CypA or GST-FKBP12 in the presence of the corresponding immunosuppressant and either LxVPc1 or LxVPc1mut. Wild-type LxVPc1 competed binding to the GST-immunophilin fusion proteins but LxVPc1mut had no effect (Fig. 4A, B), suggesting that LxVPc1 and immunosuppressant-immunophilin complexes recognise a shared docking surface.

We next used Autodock software (Morris et al., 1998) to predict the structure of a CN-LxVPc1 complex, and identified a hydrophobic pocket formed at the CnA–CnB interface as a possible binding surface. This pocket includes three CnA residues (W352, S353 and F356) and two from CnB (M118 and V119) (Figure 4C). To examine the contribution of each to LxVPc1 binding, we generated alanine substitution mutants. These mutations did not affect the CnA–CnB interaction or the overall CN secondary structure; and all mutant CN proteins retained phosphatase activity, though the phosphatase activity of the W352A mutant toward pNPP was

reduced (Supplemental Fig. S3–6). In binding experiments with GST-LxVPc1, only mutations W352A and F356A, in the BBD of CnA, severely hindered interaction. None of the mutations affected the interaction of CN with GST-PxIxITc2 (Fig. 4D).

The crystal structures of CN-bound IS-IP complexes reveal that W352 and F356 from CnA and M118 and V119 from CnB are also key residues for CN interaction (Ke and Huai, 2003). Also, cysteine substitution of the conserved tryptophan (W352 in human CnA or W388C in yeast Cna1) renders CN resistant to FK506 (Cardenas et al., 1995; Zhu et al., 1996). We therefore analyzed the binding of the CnA alanine-substitution mutants to GST-CypA plus CsA or GST-FKBP12 plus FK506. Only the W352A mutation severely impaired CN interaction with IS-IP complexes (Fig 4E and Supplemental Fig. S7A). However, unlike LxVPc1 peptide, both IS-IP complexes interacted efficiently with F356A CN. As with LxVPc1, the CnB mutants had no detectable effect on binding to either IS-IP complex. We next found that the W352A and F356A CN mutations strongly inhibited CN-NFATc1 interaction (Fig 4F and supplemental Fig S7B). Thus, LxVPc1, NFATc1 and the immunosuppressants CsA and FK506 bind to a similar region of CN.

Mutations in the BBD of CnA interfere with calcineurin signaling in yeast

Yeast calcineurin is similar to the mammalian enzyme, and is also inhibited by FK506 and CysA. Several yeast CN substrates have been identified, including Crz1, Hph1, Slm1 and Slm2. Each of these proteins contains a PxIxIT-related sequence that fails to match the consensus defined for NFAT, but does mediate interaction with CN (Roy et al., 2007). Similarly, while none of these proteins contains LxVP, they may possess a functionally equivalent motif. We mutated the *Saccharomyces cerevisiae* gene *CNA1* (one of two yeast CnA homologs), changing either W388 or F392 to alanine. These positions correspond to W352 and F356 in mammalian CnA (Fig 5A). Using the yeast two hybrid assay, we tested the interaction of Cna1, Cna1W388A, and Cna1F356A with substrates Hph1, Slm1 and Slm2, the regulator Rcn1, and the CnB homolog Cnb1 (Fig 5B). Neither mutation affected interaction with Cnb1; however, both reduced the interaction with Slm1 and Slm2, with the effect of the F392A mutation being considerably more severe than W388A in both cases. Interactions of Cna1 with Hph1 and Rcn1 were unaffected by the W388A mutation, whereas the F392A mutation reduced both substantially. Thus the same mutations that reduce CnA binding to LxVPc1 and NFATc1 impair the interaction of yeast CN with substrates and regulators.

Next, the mutant proteins were expressed in a strain lacking endogenous CN (*cna1*Δ *cna2*Δ) at levels comparable to those in wt cells (data not shown), and their ability to activate the transcription factor Crz1 was measured using a reporter construct bearing 4 tandem copies of the Crz1 DNA binding site fused to β-galactosidase (4X-CDRE-*LacZ*) (Stathopoulos and Cyert, 1997). This approach, rather than the yeast two hybrid assay, was used to assess Cna1-Crz1 interaction because expression of the Crz1-Gal4AD (activation domain) fusion is toxic to yeast. Cells lacking CnA expressed little or no β-galactosidase, because Crz1 is active only when dephosphorylated by CN (Fig 5C). Cells transformed with wt Cna1 showed low basal Crz1 activity that increased upon CN activation by addition of extracellular Ca^{2+}. Basal and Ca^{2+}-activated Crz1 activity was substantially reduced in strains expressing Cna1W388A or Cna1F392A, suggesting impaired dephosphorylation of Crz1. Moreover, FK506 reduced Ca^{2+}-induced CDRE-lacZ expression to ≤ 10% of that observed without the drug in cells expressing Cna1 or Cna1F392A. In contrast, cells expressing Cna1W388A expressed CDRE-lacZ at higher levels in the presence of FK506 (30% of that observed in non drug-treated cells), suggesting that this mutant CnA is partially resistant to the inhibitor.

We next examined the effect of these mutations on the ability of yeast cells to survive environmental stress (Fig. 5D). CN is not required for growth of yeast in rich medium, but is essential when cells are exposed to extra-cellular stresses such as osmotic, salt and alkaline

stresses or metal ions (Cyert, 2003). Thus, the ability of yeast to grow under these conditions reflects dephosphorylation of the full complement of CN substrates *in vivo*. Cells expressing Cna1W388A and Cna1F392A were more sensitive than cells expressing wt Cna1 to manganese ions and salt stress (Fig. 5D), and were also more sensitive to alkaline stress and zinc ions (data not shown), establishing that CN function was impaired. In addition, cells expressing Cna1W388A grew better than Cna1 or Cna1F392A-expressing cells on media containing NaCl and FK506 (Fig 5D), consistent with previous work showing that mutation of this residue (W388C) confers FK506 resistance (Cardenas et al., 1995). Expression of either Cna1W388A or Cna1W388C results in similar growth phenotypes (supplemental Fig S8). Thus, mutation of this conserved tryptophan residue in yeast or mammalian CnA disrupts FK506-FKBP binding. Together, these findings suggest that the participation of residues in the hydrophobic pocket between CnA and CnB in binding to substrates and immunosuppressants is broadly conserved.

To confirm that LxVP-type docking sites mediate interaction of yeast substrates with CN, we sought to identify and mutate one such site. Rcn1, a conserved regulator and substrate of CN, contains a close match to the LxVP sequence: KQYLKVPESEKVF (aa 98–110). Cna1 and Cna2 co-purified with Gst-Rcn1 expressed in yeast (Fig 6A and data supplemental Fig. S9). Addition of LxVPc1 peptide, but not LxVPc1mut, to the Gst-Rcn1-CN complexes greatly decreased the amount of bound CN, suggesting that an LxVP-type motif in Rcn1 mediates the interaction. A 16mer peptide based on the YLKPV site in Rcn1 inhibited CN phosphatase activity, and a GST fusion (GST-YLKVP) bound to CN (Fig 6B). This interaction was competed by the CypA-CsA complex and by LxVPc1 (fig 6C). Thus the Rcn1 YLKPV site interacts with CN in a manner analogous to LxVPc1. Finally, when expressed in yeast, GST-Rcn1YaKaa, in which YLKVP is mutated to YAKAA, did not co-purify with Cna1 or Cna2 (Fig 6D and data not shown). These mutations also disrupted Rcn1-Cna1 interaction in the two hybrid assay (Fig 6E) and decreased the ability of Rcn1 to inhibit CN signaling (supplemental Fig S9), indicating that the YLKVP site is required for interaction of Rcn1 with yeast CN *in vivo*.

DISCUSSION

In this study we have defined the contribution of specific residues of the NFAT-derived LxVP binding site to CN binding and have identified a hydrophobic pocket in CN where LxVP binds. This pocket also mediates the interaction of several yeast CN targets, including Rcn1. Our results thus suggest that partners interact with CN through two important routes, the PxIxT motif and the LxVP motif, which appears to be a conserved mode of substrate-CN interaction. Our analyses also provide insights into the mechanism by which immunosuppressants inhibit CN, showing that IS-IP complexes compete for binding to the same docking surface in CN that mediates LxVP-type interactions with natural substrates.

In yeast, PxIxIt motifs regulate the affinities of several CN substrates, thereby determining the calcium concentration dependence and in vivo signaling output of CN (Roy et al., 2007). The availability of CN in yeast cells is limiting, so substrate selection and the profile of signaling pathways activated downstream of CN are determined by relative binding affinities (Roy et al., 2007). Our data show that conserved CN residues essential for LxVP-binding are required for interaction of yeast CN with substrates and Rcn1, Ca^{2+}-activated transcription of Crz1, and the ability of yeast cells to survive stress. Thus, we propose that CN-interacting proteins contain regions with LxVP-type activity of varying affinities for CN, which, in cooperation with PxIxIT motifs, establish the overall affinity of each substrate or regulator for CN, and determine calcium dependence and signaling output.

Among yeast CN partners, only Rcn1, a member of the RCAN family of CN regulators, contains a readily identifiable LxVP motif (Görlach et al., 2000; Hogan et al., 2003; Kingsbury and Cunningham, 2000). This may indicate degeneracy in the sequence requirements for binding to the BBD-CnB composite site, so that CN-binding motifs are not easily detected by sequence comparison. Data presented here, together with earlier work, identify key components of the LxVP motif in NFAT, and show that amino acids flanking the LxVP core, including Y3, are critical for CN binding (Park et al., 2000). Since all known functional LxVP motifs have an aromatic residue at this position, we can redefine an extended core sequence, ΦLxVP, where x cannot be glycine. These insights will be critical in identifying functional ΦLxVP motifs in other CN partners, both mammalian and fungal.

It is possible that the CN-binding surface on some substrates is formed by conformational association of separate regions of primary sequence. This scenario is reminiscent of immunosuppressant-immunophilin complexes, in which three dimensional structures that specifically bind to the BBD-CnB composite site are created fortuitously by the association of distinct immunophilin residues with the structurally unrelated FK506 and CsA molecules (Ke and Huai, 2003). This raises the possibility that the BBD-CnB composite site acts as a shared docking surface for substrates with dissimilar sequences. Further analysis is needed to identify the motifs or tertiary structures in these substrates that confer LxVP-like functions.

In mammals, NFATs mediate transcriptional activation in many CN-dependent processes (Crabtree and Olson, 2002; Hogan et al., 2003), and there is strong evidence that LxVP and PxIxIT motifs in NFAT proteins cooperate to determine their overall affinity for CN. The PxIxIT motif plays a major role in determining NFAT-CN interaction (Aramburu et al., 1998), and binds to the phosphatase domain of inactive CN at a region distinct from the active site (Aramburu et al., 1998; Li et al., 2007). Our earlier work showed that the LxVP motif also contributes to docking, since its deletion reduces NFATc1 binding to CN (Martínez-Martínez et al., 2006), and here we show that NFATc1 binding is inhibited by mutations in the CN hydrophobic pocket. In vitro, NFAT binding to CN increases substantially upon CN activation, an effect that is independent of phosphatase activity and is inhibited by the FK506-FKBP12 complex (Garcia-Cozar et al., 1998). These findings suggested the presence of PxIxIT-independent contacts that might increase affinity or restrict the orientation of the substrate relative to CN (Li et al., 2007). Indeed, it has been proposed that a docking site near the CN active site is unmasked when CN is activated (Garcia-Cozar et al., 1998). The docking surface we identify here fulfils the requirements of this proposed site, since LxVPc1 binds near the active site and does not bind inactive CN. Our results thus support the idea that the CnA-CnB composite surface is responsible for the increased binding of NFAT to active CN. Unlike protein kinases, phosphatases do not target stringent sequence motifs (Agostinis et al., 1990). The activity of CN on short peptides is very low, and addition of residues N-terminal to the phospho-site increases the dephosphorylation rate (Blumenthal et al., 1986; Donella-Deana et al., 1994), suggesting that substrate motifs which neighbor target phospho-residues can increase interaction efficiency. Substrates and regulators of protein phosphatase 1 including DARPP32 and inhibitor 1 require two distinct interaction domains to bind this phosphatase (Kwon et al., 1997). Our studies of calcineurin suggest recognition of substrates through multiple interaction domains may be a general feature of protein phosphatases.

Since no data are available on the structure of LxVP-CN, we modeled possible structural configurations with the HADDOCK program. Molecular dynamic (MD) simulations were performed on the most likely docking structures, estimating the relative free energies of binding (details in Supplemental Data). LxVPc1 is almost parallel to the CnB-binding α-helix of CnA, with the YLAVP core sits in the hydrophobic pocket formed by W352, P355 and F356 (Fig 7). The MD simulation suggests that these residues are responsible for the electrostatic and van der Waals interactions between LxVPc1 and CN. This study predicts that the proline of

LxVP interacts with aromatic residues in CN, a common feature in proline-binding domains (Zarrinpar et al., 2003). Such proline-based interactions are weak but specific, and almost all have additional binding sites for recognizing substrates. Thus the docking surface in CN may act as a proline-recognition domain important for specific substrate recognition.

IP-IS complexes are large structures and since they bind to a CN region close to the active site, inhibition was first proposed to involve occlusion of the active site by regions not directly involved in CN binding (Griffith et al., 1995; Kissinger et al., 1995). The hydrophobic pocket we identify at the interface of the CnA and CnB subunits mediates binding not only to CN substrates but also to CsA and FK506 immunophilin complexes. These complexes compete with LxVP for CN, suggesting that they bind to the same docking surface on CN. Although we cannot discard the occlusion model, our results indicate that blockade of the interaction between CN and LxVP-type structures in substrates is sufficient to explain immunosuppressant inhibition of CN.

Many of the severe side effects of immunosuppressants (such as neurotoxicity, diabetes, nephrotoxicity and hypertension) are at least partly independent of CN (Kiani et al., 2000; Martínez-Martínez and Redondo, 2004). These actions preclude use of these drugs for chronic inflammatory conditions and other diseases. Therefore identifying selective CN inhibitors that avoid these secondary effects is of high interest. The common CN docking pocket for substrates, regulators and IP-IS complexes presents a target for the development of immunophilin-independent immunosuppressive drugs that would avoid some side effects associated with the FK506 and CsA. Such drugs would also be valuable for exploring important CN-regulated processes such as cell-stress responses, immune activation and cardiac hypertrophy.

EXPERIMENTAL PROCEDURES

Plasmids, cells, peptides, antibodies, cell culture conditions, reporter assays and circular dichroism

See Supplemental Data.

In vitro calcineurin phosphatase assays

Phosphatase assays with *p*-nitrophenyl phosphate (pNPP) were performed as described (Chan et al., 2005) with minor modifications detailed in Supplemental Data. CN activity on its specific substrate RII phosphopeptide was measured as described (Martínez-Martínez et al., 2006). Data are shown as a percentage of the activity of the uninhibited control reaction.

Lentiviral production, titration and infection

Lentiviruses expressing GFP-peptide fusion proteins were obtained by transient calcium phosphate transfection of HEK-293 cells using a three plasmid HIV-derived and VSV pseudotyped lentiviral system (details in Supplemental Data).

RNA extraction and real time PCR

Total RNA was extracted with TRIZOL (Sigma) and 400ng was reverse transcribed to cDNA with MMLV-RT (Invitrogen). Real-time PCR was carried out on 20 ng cDNA using TaqMan probes (Applied Biosystems). PCR reactions were run in triplicate in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). TaqMan probes are listed in Supplemental Data.

GST-fusion protein expression, pull-down assays and western blot

GST-fusion proteins were expressed as described (Rodríguez et al., 2005). Proteins were recovered by PreScission protease treatment (GE Healthcare Life Sciences) and purified GST proteins were quantified by densitometry of Coomassie-stained SDS-PAGE gels (Quantity One software, BioRad). Pull down of FLAG-tagged CnA proteins expressed in HEK-293 cells and western blot were as described (Rodríguez et al. 2005). Pull down of fully activated bovine brain CN (Sigma) was as described (Martínez-Martínez et al., 2006). Unless specified, all pull down experiments were in the presence of 1.5 mM $CaCl_2$, and experiments with full length CN included 600 nM calmodulin.

In silico docking and modeling

QYLAVPQH peptide was in silico docked to the CnA-CnB dimer with the AutoDock algorithm (details in Supplemental Data). HADDOCK, free energy binding calculations and molecular dynamic simulations are detailed in Supplemental Data.

Yeast manipulations and experimental procedures

Yeast strains used in this study and generation of *CNA1* and *RCN1* mutations, Gal4 plasmids and two-hybrid analysis are detailed in Supplemental Data.

CDRE-*LacZ* activity was measured as in Roy et al., 2007. FK506 (LC Laboratories, Woburn, MA), dissolved in 90% ethanol and 10% Tween-20 buffer (ET), was added to cultures at 2 μg/ml. $CaCl_2$ (200 mM) was added where indicated at least 2 h after addition of FK506/ET. Values are means of ≥3 independent extracts measured in triplicate.

Growth of yeast under stress conditions was scored by dilution plating on YDP-media containing 4mM $MnCl_2$, 800 mM NaCl + 0.1% ET or 800 mM NaCl + 2 μg/ml FK506.

Association of yeast CNA proteins with GST-tagged wt or mutant Rcn1 proteins *in vivo* was examined by pull-down assays of yeast S100 extracts. LxVPc1 or LxVPc1mut peptides were added to wash buffers where indicated. Details are provided in Supplemental Data.

Statistical analysis

Statistical analysis was performed by employing the GRAPHPAD PRISM software version 5.01, San Diego. When indicated statistics were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Statistical significance was assigned at $p < 5\%$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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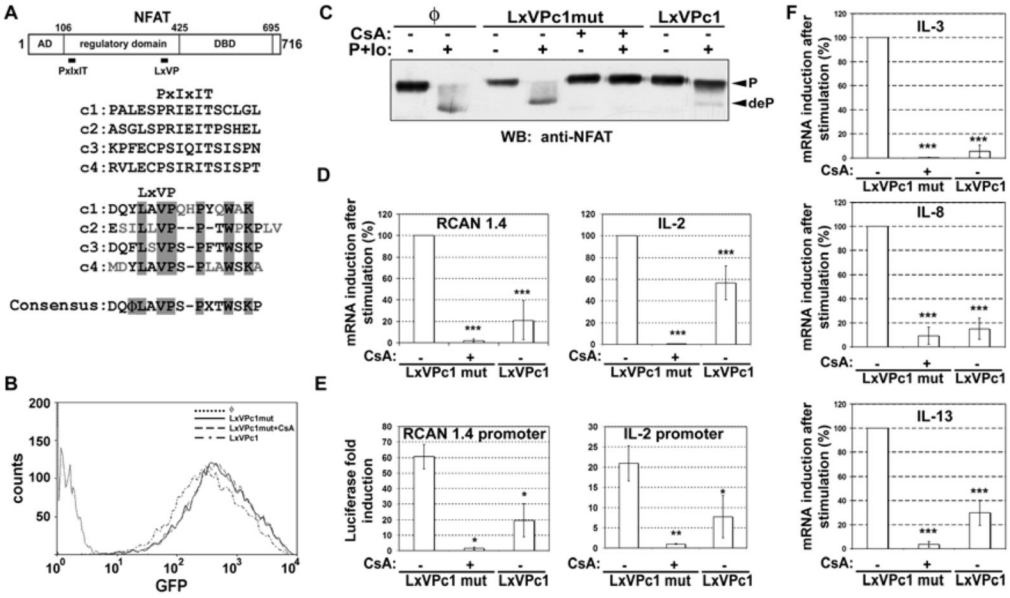


Figure 1. LxVPc1 blocks NFAT-dependent signaling

(A) Schematic representation of the N-terminal regulatory and CN-interacting domain of NFAT proteins, based on the structure of human NFATc1. Conserved regions among NFAT members (c1–c4) are indicated: AD = activation domain; DBD = DNA binding domain. The PxIxIT and LxVP sequences from NFAT members are shown. For LxVP motifs, sequences are aligned to show conserved residues (black); identical residues are shaded. The consensus reference LxVP motif is shown below. Φ, aromatic residue.

(B) Jurkat T cells were infected with lentivirus encoding a GFP fusion of either wt LxVPc1 or LxVPc1mut (control) peptide. Infection efficiency was monitored by flow cytometry. Plots show cell number versus GFP fluorescence. Non-infected cells were used as negative control (dotted line). Viruses encoding LxVPc1 (dotdashed line) or LxVPc1mut (solid line) infected the whole population, and CsA treatment (200 ng/ml) did not alter GFP expression (dashed line).

(C) Western blot showing the effect of LxVPc1 on NFAT activation. Infected cells were stimulated (30 min) with PMA (P, 10 ng/ml) plus Ca^{2+} ionophore (Io, 1μM). As a positive control of NFAT-pathway inhibition, cells infected with LxVPc1mut were treated with CsA (200 ng/ml). The blot shows expression of NFATc2 in total cell lysates, and the positions of phosphorylated (P) and dephosphorylated (deP) proteins are indicated. Φ, non-infected cells.

(D and F) Expression of NFAT-regulated genes. Total mRNA was extracted after P+Io stimulation (4h), and mRNA levels of RCAN1.4 and IL-2 (D), and IL-3, IL-8 and IL-13 (F) were quantified by Q-PCR. Rq values were calculated by comparing the amount of mRNA post stimulation with that obtained with corresponding non-stimulated cells. 100% corresponds to the amount of mRNA induced in cells infected with control virus (LxVPc1mut). Data are means ± sd of three independent experiments. ***, $p<0.0001$ vs. LxVPc1 mut.

(E) NFAT-dependent transcription. Infected cells were transiently transfected with plasmids encoding luciferase under control of the RCAN1.4- or the IL-2 promoter. After 24h, cells were pretreated (30min) with 200 ng/ml CsA or vehicle and stimulated with P+Io (4h). Luciferase activity is expressed as fold induction over baseline levels in transfected unstimulated cells (means ± sd; n=3). *, $p<0.05$ vs. LxVPc1 mut; **, $p<0.001$ vs. LxVPc1 mut.

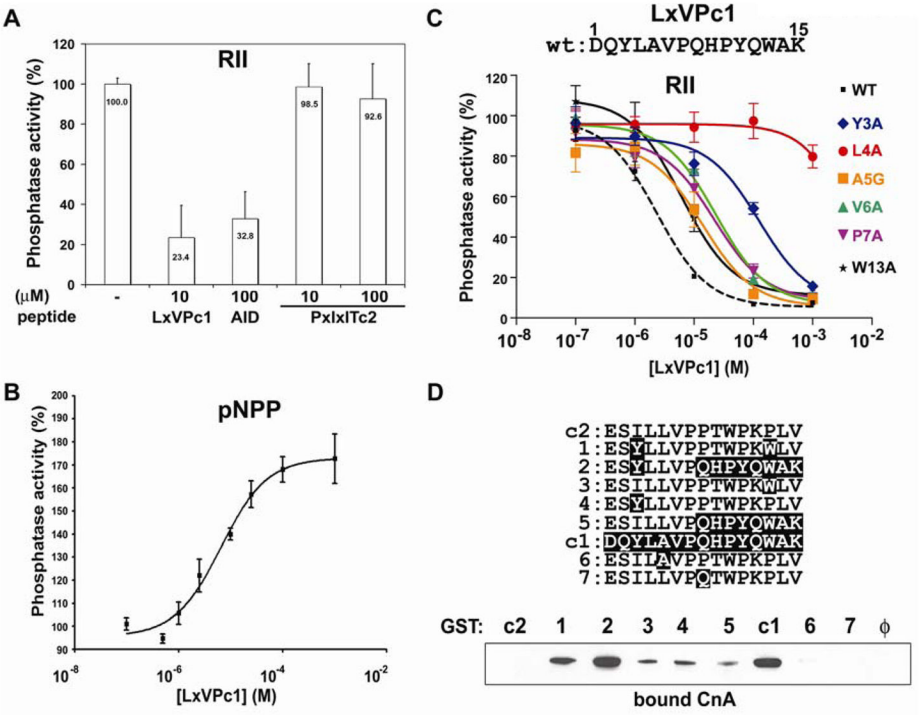


Figure 2. Inhibition of calcineurin phosphatase activity by LxVPc1 peptide and contribution of LxVP-motif residues to calcineurin binding

(A) LxVPc1 inhibits CN phosphatase activity on the 19mer phosphopeptide RIIp. Inorganic phosphate release in the presence of the indicated synthetic peptides (AID=auto-inhibitory domain of CN) was determined spectrophotometrically. Maximal activity was defined as phosphate release in the absence of synthetic peptide. Data are from a representative experiment performed in triplicate (means ± sd).

(B) LxVPc1 increases CN phosphatase activity on the small compound pNPP. Each LxVPc1 concentration was assayed three times and the corresponding values (means ± sd) plotted as percentages of phosphate release in the presence of mutant LxVPc1 peptide (LxVPc1mut: LxVP>AxAA).

(C) LxVPc1 mutant peptides were generated in which each residue of the 15mer wt sequence was replaced in turn by alanine or glycine. The impact of each mutation was tested on the inhibition of CN phosphatase activity toward RIIp. The graph shows plots for wt LxVPc1 (dashed line) and mutant peptides that strongly reduced inhibitory activity; n≥3 for each peptide concentration.

(D) CN pull-down with GST-fusions of the LxVPc2 sequence (NFATc2) bearing individual or grouped substitutions based on LxVPc1. Peptide sequences are shown with LxVPc1-directed mutations shaded. c2 and c1 are the wt LxVPc2 and LxVPc1 sequences. The blot shows a representative pull-down experiment of four.

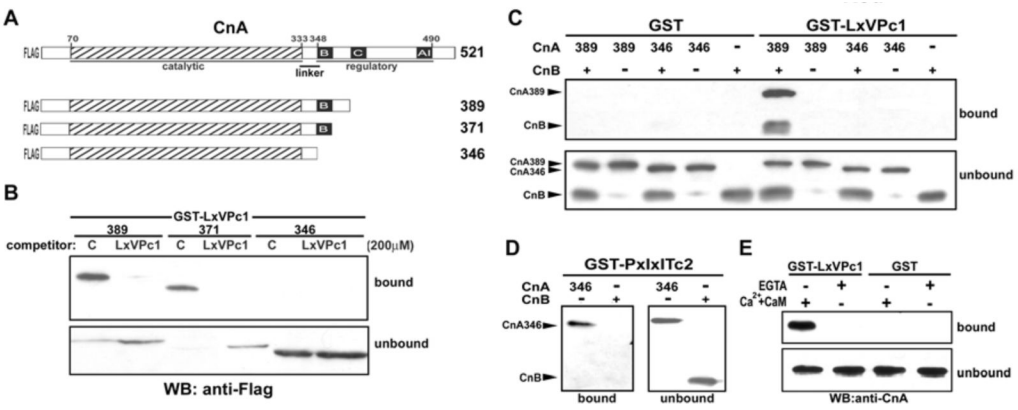


Figure 3. Calcineurin binding by LxVPc1 requires CnA and CnB subunits

(A) Flag-tagged full-length (521) and deletion-mutant versions of human CnA-α. Functional regions are underlined and conserved domains indicated: B = CnB binding domain; C = calmodulin binding domain; AI = auto-inhibitory domain.

(B) HEK cells were co-transfected with CnB and the indicated flag-tagged CnA construct. CN was pulled down from lysates with GST-LxVPc1 in the presence of 200 μM LxVPc1 or control peptide. Bound and unbound CN was detected with anti-Flag.

(C–E) Pull-down experiments with (C,E) GST-LxVPc1 or GST or (D) GST-PxIxITc2. (C,D) CnB and CnA deletion mutants were expressed in bacteria, purified and used in pull-down experiments with (C) GST-LxVPc1 or GST or (D) GST-PxIxITc2. (E) Binding of bovine CN to GST-LxVPc1 in the presence of Ca^{2+} (1.5mM) and calmodulin (600nM) or EGTA (5mM). Bound and unbound CN subunits were detected by western blot.

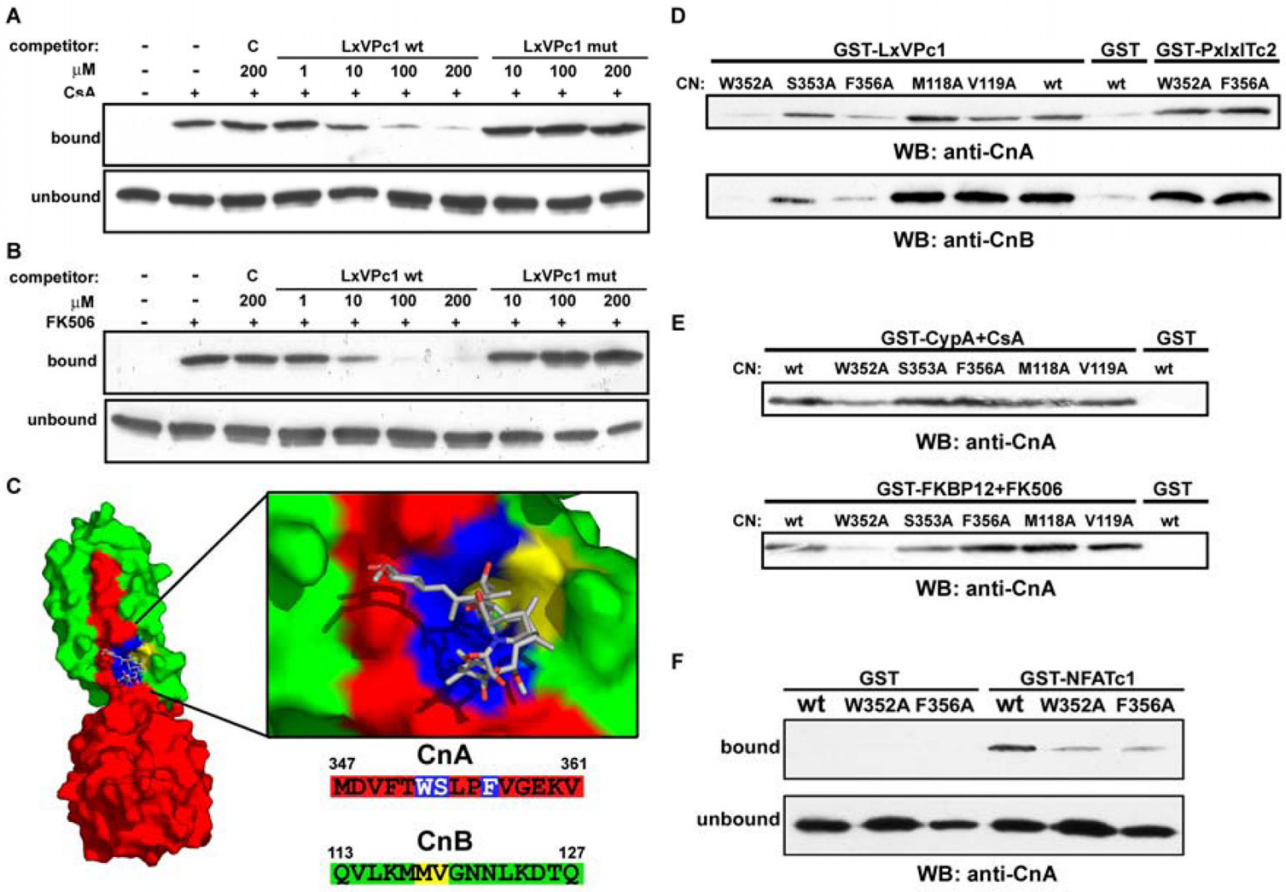


Figure 4. LxVPc1 peptide and immunosuppressant drugs dock at a common region in calcineurin

(A–B) CN was pulled-down with immunosuppressant-immunophilin complexes formed between (A) CsA and GST-CypA and (B) FK506 and GST-FKBP12. Binding was competed with LxVPc1 synthetic peptide. Scrambled (C) and mutant LxVP (LxVPc1mut) peptides were used as controls. Bound CN was detected by western blot. CN did not bind GST-immunophilins in the absence of the corresponding immunosuppressant (first lanes).

(C) Identification of CN residues putatively involved in interaction with LxVPc1 peptide by in silico docking (AutoDock). A surface representation of CnA (red) bound to CnB (green) is shown. The in silico modeling locates the LxVPc1 peptide (sticks) in a hydrophobic groove comprising amino acids from both subunits. The positions of the identified amino acids are shown in the primary structures of CnA (blue) and CnB (yellow).

(D–F) CN subunits carrying individual alanine substitutions of the residues identified by in silico analysis were used in pull-down experiments with (D) GST-LxVPc1 (right) or GST-PxIxITc2 (left), (E) GST-immunophilins, or (F) GST-NFATc1. Bound CN was eluted and detected by western blot with anti-CnA or anti-CnB mAb. A representative experiment is shown.

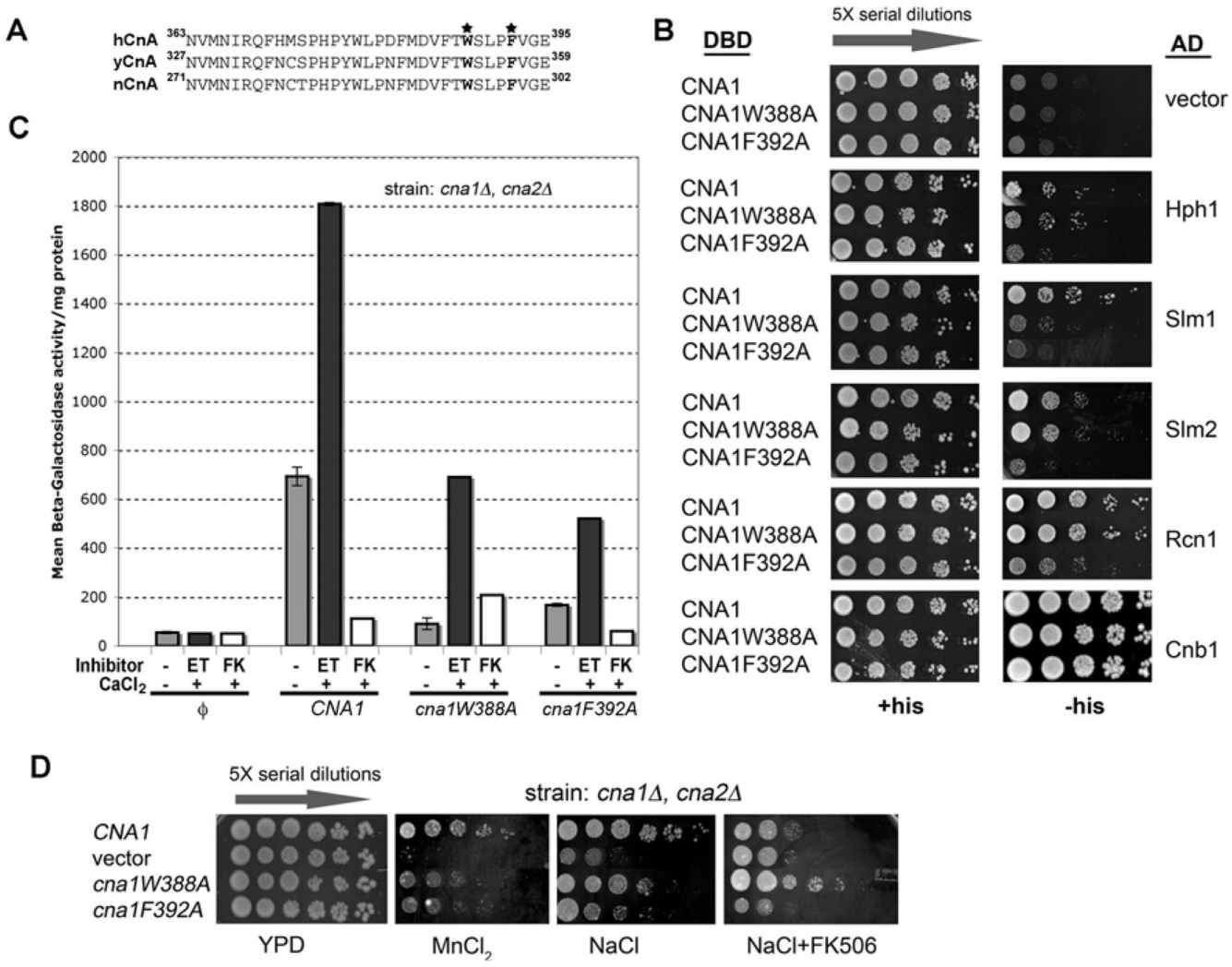


Figure 5. Mutations of key calcineurin residues involved in interaction with LxVPc1 impair signaling in yeast

(A) Alignment of human calcineurin Aα (hCnA), *Saccharomyces cerevisiae* calcineurin (yCnA) and *Neurospora crassa* calcineurin A (nCnA). The conserved yCnA residues W388 and F392 (equivalent to W352 and F356 in human CnA) are indicated.

(B) Two-hybrid assay assessing interaction between wt or mutant Cna1 proteins and the substrates Hph1, Slm1 and Slm2, the regulator Rcn1 (homolog of mammalian RCAN1), and the CN B subunit (Cnb1). Growth was assayed by spotting serial dilutions of yeast strains on medium lacking tryptophan and leucine to select for both Gal4-fusion plasmids, and where indicated on medium lacking histidine to assay reporter activation. Interactions with Hph1, Slm1 and Slm2 were assessed using histidine deficient medium supplemented with 3mM 3-AT (see Experimental Procedures). DBD: Gal4–DNA-binding-domain fusion of wt or mutant CNA1. AD: Gal4–activation-domain fusions containing the indicated open reading frames. All plasmids were transformed into strain pJ69-4A (*GAL1-HIS3*). Cell growth was scored after 4–5 days at 30°C.

(C) Crz1 transcriptional activity. β-galactosidase activity was measured in strain BY1001 (*Δcna1 Δcna2* 4X-CDRE-*lacZ*) transformed with vector pRS314 encoding wt or mutant *CNA1*. Cells were grown in the presence of FK506 (FK) or vehicle (ET), and where indicated 200 mM $CaCl_2$ was added 2 h before harvest.

(D) Growth of cells expressing wt or mutant CNA alleles under environmental stress. Cells of strain *cna1*Δ, *cna2*Δ were transformed with empty vector, *CNA1*, *cna1W388A* or *cna1F392A* and scored by spotting serial dilutions on rich YPD medium or YPD containing 4mM $MnCl_2$, 800 mM NaCl + ET solvent, or NaCl + 2 μg/ml FK506. Cell growth was scored after 3 days at 30°C.

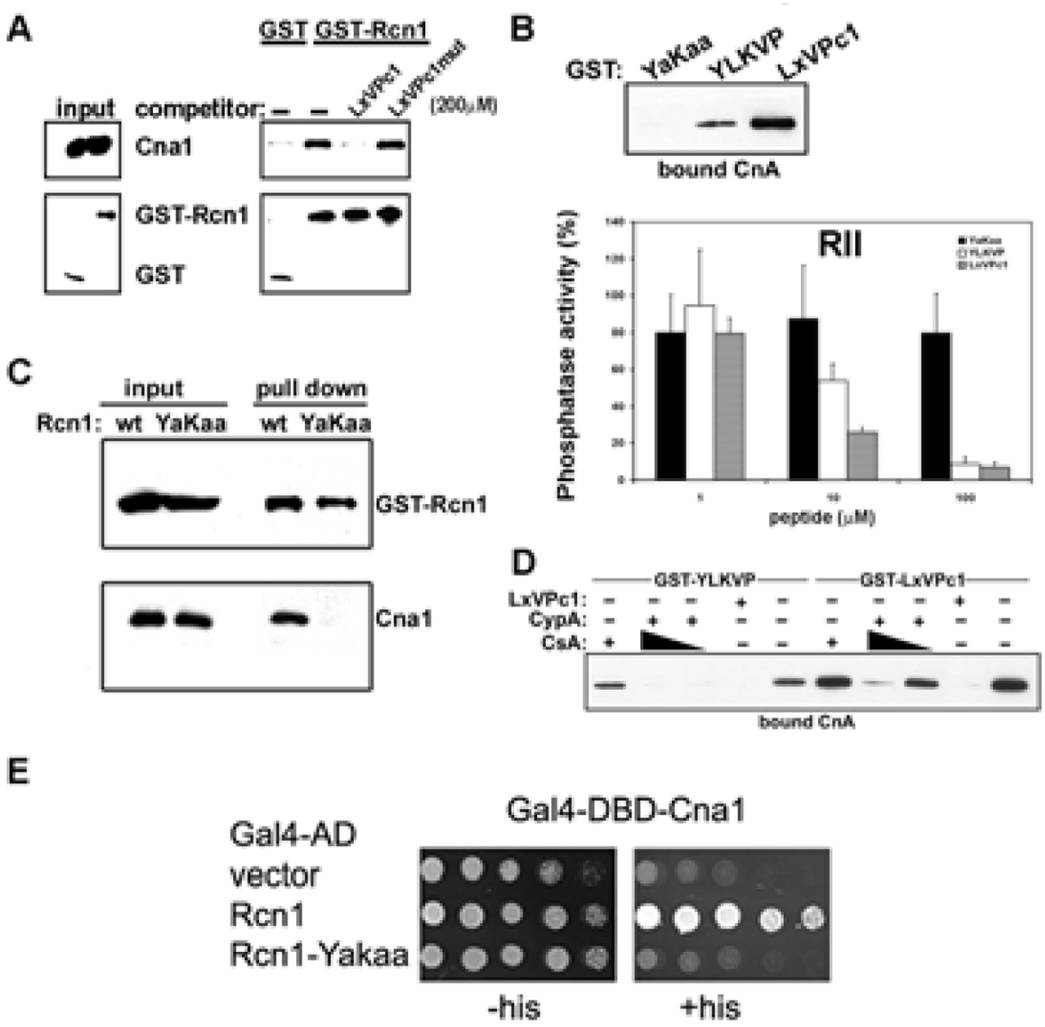


Figure 6. Rcn1 interacts with Cna1 *in vivo* via an LxVP motif

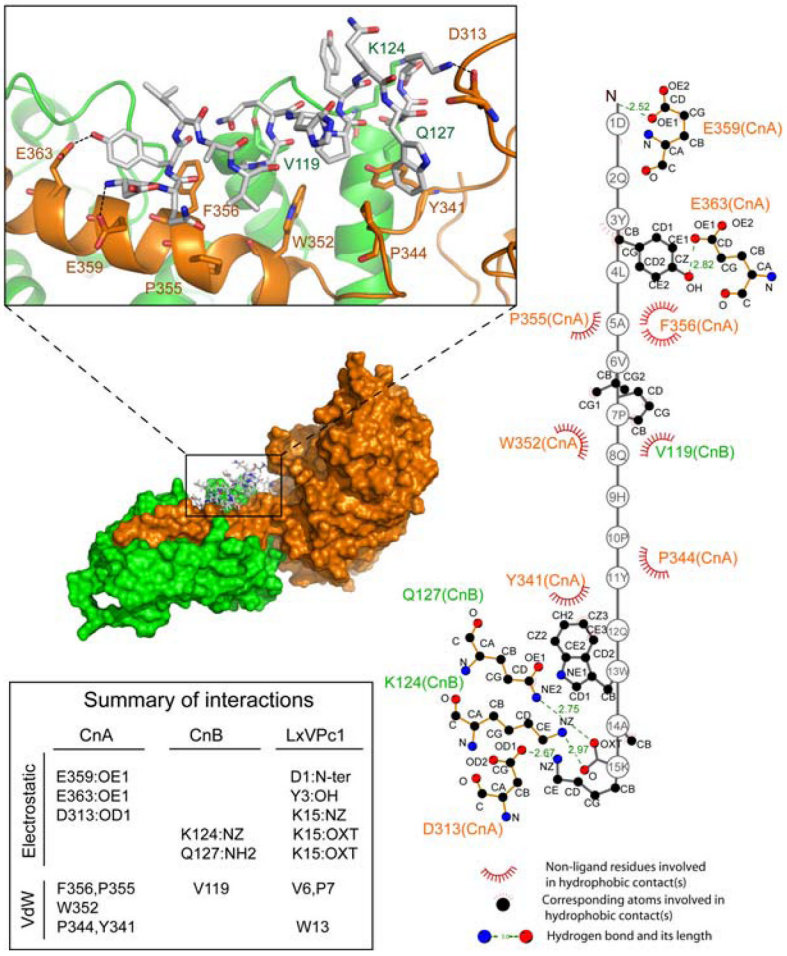
(A) GST or GST-Rcn1 was expressed in yeast strain Y258 and purified. Co-purification of Cna1 with GST-tagged proteins was assessed by western blot. Where indicated, Rcn1–Cna1 interaction was competed *in vitro* with LxVPc1 or LxVPc1mut peptides.

(B) *Left.* The Rcn1 YLKVP motif inhibits mammalian CN phosphatase activity toward RIIp. Inorganic phosphate release in the presence of YLKVP, YaKaa or LxVPc1 synthetic peptides was determined spectrophotometrically. 100% activity was defined as phosphate release in the absence of peptide. Data are from a representative experiment performed in triplicate (means ± sd). *Right.* Binding of CN to GST fusions of the Rcn1 YLKVP motif, its mutated form YaKaa or LxVPc1. Pull down experiments were conducted in the presence of Ca^{2+} (1.5mM) and calmodulin (600nM), and bound CN detected by western blot.

(C) Binding of mammalian CN to GST-YLKVP or GST-LxVPc1 was competed by adding either LxVPc1 peptide or increasing concentrations of CsA-CypA complex (10 and 100μM). CsA alone was used as negative control.

(D,E) The Rcn1-LxVP sequence is required for interaction with CN *in vivo.* (D) GST-Rcn1 or GST-Rcn1-YaKaa was expressed in yeast strain Y258 and purified. Co-purification of Cna1 was analyzed as in (A). (E) Two-Hybrid interaction between the Cna1-Gal4-DNA binding domain fusion and Gal4 activation domain fusions with Rcn1 or Rcn1-YaKaa was assayed in strain PJ69-4A (*GAL1 HIS3*). Cells were analyzed by dilution plating on medium lacking

tryptophan and leucine for selection of plasmids and, where indicated, lacking histidine to assay reporter construct activation. Growth was scored after 4 days at 30°C.



Summary of interactions

	CnA	CnB	LxVPc1
Electrostatic	E359:OE1		D1:N-ter
	E363:OE1		Y3:OH
	D313:OD1		K15:NZ
		K124:NZ	K15:OXT
		Q127:NH2	K15:OXT
VdW	F356,P355	V119	V6,P7
	W352		
	P344,Y341		W13

Figure 7. Predicted binding mode of LxVP based on docking and molecular dynamic simulations

The LxVP peptide (ball and sticks) is positioned parallel to the B-subunit-binding helix of CnA (orange), and forms additional contacts with residues in CnB (green). In the zoom view (top), hydrogen bonds and salt bridges are shown as dashed lines. CN residues predicted by MD simulation to be involved in ligand binding are labeled and numbered. The interactions are detailed in 2D (right) and summarized in a table (bottom): vdW; van der Waals.