The unique C terminus of the calcineurin isoform CNAβ1 confers non-canonical regulation of enzyme activity by Ca\(^{2+}\) and calmodulin

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Calcineurin, the conserved Ca\(^{2+}\)/calmodulin-regulated phosphatase and target of immunosuppressants, plays important roles in the circulatory, nervous, and immune systems. Calcineurin activity strictly depends on Ca\(^{2+}\) and Ca\(^{2+}\)-bound calmodulin (Ca\(^{2+}\)/CaM) to relieve autoinhibition of the catalytic subunit (CNA) by its C terminus. The C terminus contains two regulatory domains, the autoinhibitory domain (AID) and calmodulin-binding domain (CBD), which block the catalytic center and a conserved substrate-binding groove, respectively. However, this mechanism cannot apply to CNA\(^{1}\), an atypical CNA isoform generated by alternative 3′-end processing, whose divergent C terminus shares the CBD common to all isoforms, but lacks the AID. We present the first biochemical characterization of CNAβ1, which is ubiquitously expressed and conserved in vertebrates. We identify a distinct C-terminal autoinhibitory four-residue sequence in CNAβ1, 462-LAVP465, which competitively inhibits substrate dephosphorylation. In vitro and cell-based assays revealed that the CNAβ1-containing holoenzyme, CNB1, is autoinhibited at a single site by either of two inhibitory regions, CBD and LAVP, which block substrate access to the substrate-binding groove. We found that the autoinhibitory segment (AIS), located within the CBD, is progressively removed by Ca\(^{2+}\) and Ca\(^{2+}\)/CaM, whereas LAVP remains engaged. This regulatory strategy conferred higher basal and Ca\(^{2+}\)-dependent activity to CNB1, decreasing its dependence on CaM, but also limited maximal enzyme activity through persistence of LAVP-mediated autoinhibition during Ca\(^{2+}\)/CaM stimulation. These regulatory properties may mandate observed differences between the biological activities of CNB1 and canonical CNB2. Our insights laid the groundwork for further studies of CNB1, whose physiological substrates are currently unknown.

Calcineurin, the Ca\(^{2+}\)/calmodulin (CaM)\(^4\) regulated serine/threonine-protein phosphatase, transduces Ca\(^{2+}\) signals to regulate diverse physiological functions. Calcineurin is a key signaling enzyme in eukaryotic cells, from yeast, where it regulates survival during environmental stress (1), to mammals, where it plays well-established roles in synaptic plasticity (2), heart development (3), and adaptive immunity (4). Signaling by calcineurin through its best characterized substrates, the nuclear factor of activated T-cells (NFAT) family of transcription factors, is critical for T-cell activation, and for this reason the calcineurin inhibitors FK506 and cyclosporin A are in wide clinical use as immunosuppressants (5). Calcineurin, a heterodimer of catalytic (CNA) and regulatory (CNB) subunits, is ubiquitously expressed in humans, but encoded by several genes whose expression is tissue-dependent. In mammals, three genes encode isoforms of CNA (α, β, and γ), which are highly homologous, differing mostly at their N and C termini, and display a domain architecture and activation mechanism that are conserved throughout eukaryotes (4). An exception is CNAβ1, a transcript variant of the CNAβ gene that, due to alternative 3′-processing, lacks the C-terminal autoinhibitory domain (AID) present in all other forms of calcineurin (Fig. 1A) (6, 7). Understanding the distinct mechanisms by which this isoform is regulated may provide novel insights into the full range of calcineurin functions and activities.

Calcineurin utilizes evolutionarily conserved surfaces to recognize substrates containing short linear motifs (SLiMs), conserved peptides found in disordered regions (8–10). Calcineurin relies on SLiM interaction at docking surfaces distinct from the catalytic center to achieve substrate specificity, rather than recognition of a phosphosite motif (11). One such surface along β sheet β14 of CNA binds SLiMs with the consensus sequence PXIIT (8, 12). This docking site anchors substrates as well as scaffolds and regulators. A second surface, formed at the interface between CNA and its obligate regulatory subunit CNB, is available only in the Ca\(^{2+}\)/CaM-activated enzyme and binds SLiMs with the consensus sequence LAVP (13, 14). This interaction is required for dephosphorylation, and is

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This article contains supplemental Tables S1 and S2 and Figs. S1–S4.

4 The abbreviations used are: CaM, calmodulin; NFAT, nuclear factor of activated T-cells; SLiM, short linear motif; AIS, autoinhibitory segment; BBH, CNB-binding helix; CBD, CaM-binding domain; mTOR, mechanistic target of rapamycin; MBP, maltose-binding protein; CNA, calcineurin catalytic subunit; CNB, calcineurin regulatory subunit; pNP, p-nitrophenyl phosphate; AID, autoinhibitory domain; Nt-NTA, nickel-nitrilotriacetic acid; SC, synthetic complete; hCNB, human CNB; sCaM, sea urchin CaM.
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blocked by inhibitors, including the immunosuppressant-immunophilin complexes, FK506-FKBP and cyclosporin A-cyclophilin, as well as the viral protein, A238L (15). Furthermore, a sequence within the regulatory domain of CNA termed the autoinhibitory segment (AIS) contributes to autoinhibition by occupying the LXVP-docking groove in the unstimulated enzyme (16). Thus, recognition of LVP SLiMs is an essential step in substrate interaction with and dephosphorylation by calcineurin.

Calcineurin is activated by a well-documented mechanism that is shared between fungal and animal kingdoms, because of the high degree of sequence and structural conservation among homologs. As shown in Fig. 1A, CNA contains an N-terminal catalytic domain followed by a regulatory domain consisting of a CNB-binding helix (BBH), CaM-binding domain (CBD), which includes the AIS mentioned above, and the C-terminal AID. When cellular Ca2+ levels are low, the inactive enzyme adopts a closed conformation that shields substrate access to the active site. Upon cellular stimulation that gives rise to elevated Ca2+ levels, Ca2+ binding to CNB and to CaM results in a series of conformational changes that release the regulatory domain and reveal both the LXVP substrate-binding pocket and the catalytic center, allowing substrate dephosphorylation (16–20).

In contrast to the well-studied cytosolic CNAβ2 isoform, the non-canonical CNAβ1 variant lacks the C-terminal AID and instead contains unique sequences that share no homology with AID (Fig. 1, A and C). This alternative C terminus confers distinct protein–protein interactions and contains a membrane localization sequence that targets CNAβ1 to the Golgi apparatus (21, 22). CNAβ1 has been reported to perform functions distinct from canonical calcineurin isoforms. In mouse, CNAβ1 is highly expressed in regenerating muscle tissue and stem cells, is cardioprotective rather than pro-hypertrophic, and regulates signaling pathways that are unaffected by CNAβ2 (7, 21–23). Surprisingly, the biochemical properties of this interesting isozyme, referred to here as CNβ1, have not been studied.

Because it lacks the AID, CNβ1 was originally thought to be constitutively active. However, the failure of CNAβ1 overexpression to yield cardiac hypertrophy, an outcome observed for expression of constitutively active calcineurin, challenged the idea that CNβ1 activity was unregulated (22, 24). Additionally, unlike CNB2, CNβ1 does not dephosphorylate NFAT or impact NFAT-responsive gene expression (22). In fact, although dozens of substrates of calcineurin have been identified, which, if any, are dephosphorylated by CNβ1 is unknown.

Here, we investigate the mechanism of CNβ1 activation. Using in vitro and cell-based assays, we identify a conserved substrate-like LXVP SLiM within the CNAβ1 C terminus, and demonstrate that it behaves as a pseudosubstrate to block substrate recognition. Thus, the atypical domain structure of CNAβ1 gives rise to Ca2+ /CaM-dependent regulation of CNβ1 phosphatase activity through a non-canonical mechanism that confers unique functional characteristics to this isoform.

Results

The CNAβ1 transcript is ubiquitously distributed in human tissues

CNAβ1 and CNAβ2 were the first calcineurin catalytic subunits to be cloned, and were identified as variant isoforms present in human brain mRNA (6). Differential 3’ processing, carried out in part by the splicing and polyadenylation factor muscle-blind-like 1, produces the shorter CNAβ1 transcript, which lacks exons 13 and 14 found in CNAβ2 (21). In addition to the brain, CNAβ1 transcripts are present in mouse heart, testis, and spleen (7), but neither the tissue distribution of this transcript nor its abundance relative to canonical CNAα and CNAβ2 isoforms has been systematically studied. Therefore, we analyzed RNAseq datasets from a panel of adult human tissues (25) and determined the relative levels of CNAα, CNAβ2, and CNAβ1 mRNA. These analyses revealed that the CNAβ1 transcript was present in all tissues examined at a consistently low level, in contrast to CNAα and CNAβ2, whose expression levels were more variable between tissues (Fig. 1B). Thus, CNAβ1 protein is likely found throughout the body, and may not predominate, but could contribute distinctly to calcineurin-dependent regulation of cellular processes.

CNB1 and CNB2 display different activities in vivo

We sought to compare the biochemical properties of CNAβ1- or CNB2-containing isozymes in vivo, but were challenged by the lack of known substrates for CNβ1 in human cells. Therefore, we took advantage of the yeast Saccharomyces cerevisiae, where calcineurin promotes survival during exposure to metal ions and other types of environmental stress, in part by dephosphorylating and activating the Crz1 transcription factor (1, 26–28). Because mechanisms of calcineurin substrate recognition are highly conserved (8), we reasoned that human calcineurin would likely dephosphorylate yeast substrates, and thus provide a method to compare, directly, the activity of CNβ2 and CNβ1 in a simple in vivo system. In the absence of stress, calcineurin-deficient yeast (cnalΔ cnb2Δ cnb1Δ) transformed with human CNB (hCNB) and either CNAβ2 or an empty vector grew identically (Fig. 2A). Under stress conditions neither of these strains survived, suggesting failure of CNAβ2 to function in yeast. However, expression of a constitutively active form of human CNAβ, which lacks the CBD and C-terminal regulatory sequences, did complement the growth defects of yeast calcineurin mutants when co-expressed with hCNB (data not shown). Therefore, we considered whether endogenous yeast CaM, which is only 59% identical to human calmodulin (29), might be unable to activate human calcineurin. Indeed, co-expression of CNAβ2 and hCNB with sea urchin CaM (sCaM), which is 93% identical to human CaM, supported yeast growth under stress conditions (Fig. 2A). Surprisingly, when CNAβ1 was co-expressed with hCNB, cells displayed modest growth under stress in the absence of sCaM (Fig. 2A). Growth of CNAβ1-expressing cells improved upon co-expression of sCaM, but was less robust than that of CNAβ2-expressing cells, despite the identical CBD sequences and similar expression levels for both isoforms (Fig. 2B). Both isoforms were sensitive to FK506, as yeast expressing either CNAβ1 or CNAβ2
failed to grow under stress conditions in the presence of this inhibitor (Fig. 2A). These results suggest that CNAβ1- and CNAβ2-containing isoforms both dephosphorylate yeast substrates, but may differ in their maximal activity and dependence on CaM.

In light of these findings, we examined the distinct C termini of CNAβ1 and CNAβ2 for possible regulatory sequences. The CNAβ1 C terminus shares no homology with the AID, but does contain a 4-residue sequence, LAVP<sup>462-465</sup>, which is well-conserved across vertebrate CNA<sub>β</sub> homologs, and contains the core residues found in LVVP motifs, which mediate substrate binding to calcineurin (Fig. 1C) (8, 13, 14). We hypothesized that this LAVP sequence might block substrate access to the conserved hydrophobic substrate-docking groove of CNβ1, and thus inhibit its activity.

The LAVP sequence from CNAβ1 blocks substrate engagement at the substrate-recognition groove

To compare the regulatory properties of the different CNAβ C termini directly, we synthesized peptides derived from these sequences or from the calcineurin substrate NFATç1 (Table 1) and added them in trans to CNAβ<sub>trunc</sub>, a heterodimer composed of hCNB with a truncated catalytic subunit (residues 1–400), which lacks all sequences C-terminal to the BBH, and is thus constitutively active. Notably, both the catalytic center and substrate-binding pocket are exposed in this form. The activity of this recombiniant, purified human CNβ<sub>trunc</sub> was assessed in vitro using two different substrates, para-nitrophenyl phosphate (pNPP) and RII phosphopeptide, which probe the availability of the catalytic center and substrate-docking site, respectively (Fig. 3, A and C).

First, we analyzed calcineurin activity toward pNPP, a small molecule that interacts only with the catalytic center of the phosphatase and whose hydrolysis is blocked by inhibitors such as inorganic phosphate or the AID, which directly occlude the active site (30–33). As expected, the AID peptide derived from CNAβ2 (β2-AID) inhibited hydrolysis of pNPP by CNβ<sub>trunc</sub> with an apparent Km<sub>(app)</sub> of 56.4 ± 3.3 μM (Fig. 3B). In contrast, NFATç1-LAVP<sub>wt</sub>, an LVVP-containing peptide derived from the well-characterized calcineurin substrate NFATç1 (13, 14), did not inhibit, but rather enhanced pNPP hydrolysis (K<sub>M</sub><sub>(app)</sub> 5.1 ± 0.4 μM, Fig. 3B), a response that was noted previously for molecules that engage this substrate-docking groove, i.e. LVVP-containing peptides (13, 15) and immunosuppressant-immunophilin complexes (5, 34, 35). Stimulation of pNPP hydrolysis was abolished when key residues within the LVVP motif were mutated to alanine (NFATç1-LAVP<sub>mut</sub>). Similarly, the β1-LAVP<sub>wt</sub> peptide stimulated pNPP hydrolysis (K<sub>M</sub><sub>(app)</sub> 14.5 ± 3.1 μM), as did a mutant peptide retaining the LAVP sequence (β1-DWGT<sub>mut</sub>, K<sub>M</sub><sub>(app)</sub> 20.2 ± 2.6 μM) (Fig. 3B). In contrast, the β1-LAVP<sub>mut</sub> peptide, in
which the Leu, Val, and Pro residues were substituted by alanine, had no effect on activity. Together, these findings suggest that the LAVP sequence from CNA/H9252 engages the substrate-docking cleft of calcineurin, and does not block the catalytic site.

In contrast to pNPP, the 19-aa RII phosphopeptide is a calcineurin-specific substrate derived from the regulatory subunit of PKA that contains an N-terminal LXVP motif, LDVP, and must dock at the substrate-binding pocket of calcineurin to be efficiently dephosphorylated (15, 36). As expected, the β2-AID peptide inhibited dephosphorylation of RII phosphopeptide by CNtrunc, presumably by preventing access to the active site (K_{D(app)} 30.4 ± 4.8 μM, Fig. 3D). The NFATc1-LAVP_wt peptide, but not NFATc1-LAVP_mut, also effectively blocked RII phosphopeptide dephosphorylation (K_{D(app)} 4.9 ± 0.6 μM), demonstrating that inhibition depended on the integrity of the LAVP motif (Fig. 3D). Similarly, the β1-LAVP_wt and β1-DWGT_mut peptides, but not β1-LAVP_mut inhibited phosphatase activity toward RII phosphopeptide (K_{D(app)} 11.4 ± 1.0 and 19.2 ± 1.9 μM, respectively, Fig. 3D).

We predicted that peptides containing an LAVP sequence would directly compete with RII phosphopeptide at the substrate-docking site. To test this, we measured CNtrunc activity with RII while varying the substrate and inhibitor peptide concentrations. Indeed, we observed that LAVP-containing peptides NFATc1-LAVP_wt, β1-LAVP_wt, and β1-DWGT_mut were all competitive inhibitors (Fig. 3E). In contrast, the β2-AID peptide inhibited RII dephosphorylation noncompetitively, in agreement with previous reports (16, 18, 19), and suggesting that RII and AID may bind CN simultaneously.

CNtrunc activity was not inhibited in the presence of up to 100 μM NFATc1-LAVP_mut or β1-LAVP_mut peptide. Taken together, results of these analyses suggest that the LAVP sequence in CNA/H9252 is a bona fide LXVP motif that binds calcineu-
rin with similar affinity to the LXVP motif of NFATc1, and can occlude substrate binding. We also note that a peptide encoding the AIS (419FSVL422), an LXVP-like motif within the CBD of CNA (16), failed to affect dephosphorylation in these analyses (supplemental Fig. S1), suggesting that this sequence has very low affinity for calcineurin when isolated from the rest of the CBD.

**CNβ1 is autoinhibited in vitro by the LXVP site within its C terminus**

Next, we investigated potential autoinhibition of intact CNβ1 and its regulation by Ca\(^2+\) and CaM in vitro. To this end we expressed and purified, from yeast, recombinant human CNβ2, CNβ1wt, and mutants CNβ1\(^{LAVPwt,AlSmut}\), CNβ1\(^{LAVPmut,AlSmut}\), and CNβ1\(^{LAVPwt,AlSmut}\), and CNβ1\(^{LAVPmut,AlSmut}\).
and CNβ1_LAVPmut,AISmut in which 462LAVP465 and/or 419FSVL422 was mutated to AAAA or ASAA, respectively. Initial attempts to purify the CNβ1 heterodimers were unsuccessful due to aggregation, which was alleviated by deleting the highly hydrophobic C-terminal 23 aa of CNA. This small truncation did not perturb enzyme expression and had little effect on function, as assessed under calcineurin-activating conditions in yeast (supplemental Fig. S2). Therefore, these forms of CNβ1 were purified to homogeneity and analyzed below (supplemental Fig. S3).

Phosphatase activities of purified CNβ isozymes were first measured in vitro using pNPP under three conditions: 1) in the presence of excess Ca²⁺ chelator (EGTA), 2) with Ca²⁺, or 3) in the presence of saturating levels of Ca²⁺-bound CaM. As shown in Fig. 4, in the absence of Ca²⁺/CaM, CNβ2 displayed low basal activity toward pNPP. Addition of Ca²⁺ increased activity 5-fold, reflecting the elevated rate of hydrolysis that has been previously observed when all four Ca²⁺-binding sites of CNB are occupied (37). Finally, the binding of Ca²⁺/CaM to CNβ2 increased activity an additional 4.5-fold, because of structural changes that release the AID from the active site upon CaM binding (38, 39).

CNβ1wt differed from CNβ2 in exhibiting an increased rate of pNPP hydrolysis in EGTA, and maximal stimulation by Ca²⁺ in the absence of calmodulin (Fig. 4). This is consistent with the catalytic center of CNβ1wt being open, i.e. not blocked by an AID that requires Ca²⁺/calmodulin for removal. The mutant enzymes, CNβ1_LAVPwt,AISmut, CNβ1_LAVPmut,AISwt, and CNβ1_LAVPmut,AISmut, displayed similar regulatory properties as CNβ1wt, but had slightly higher maximal activities.

To investigate the regulation of calcineurin isozymes in the context of a peptide substrate, we performed in vitro phosphatase assays with RII phosphopeptide in the absence or presence of Ca²⁺ and Ca²⁺/CaM (Fig. 5). As expected, CNβ2 was inactive in the absence of Ca²⁺ or CaM and exhibited a steep dependence on Ca²⁺/CaM, which stimulated activity 20-fold relative to Ca²⁺ alone. Dephosphorylation of RII phosphopeptide by CNβ1wt was similarly very low in EGTA. Activity was stimulated 13-fold by Ca²⁺, but in contrast to CNβ2, Ca²⁺/CaM only modestly raised the activity of CNβ1wt, and maximal activity was significantly lower than for CNβ2 under the same conditions. Mutating the AIS (CNβ1_LAVPwt,AISmut) resulted in no significant differences in enzyme activity or regulation. However, mutation of the LAVP sequence (CNβ1_LAVPmut,AISwt) caused a significant increase in activity both in the presence of Ca²⁺ and Ca²⁺/calmodulin. Thus, LAVP is responsible for the low phosphatase activity observed for CNβ1 under stimulated conditions, and this sequence, which is unique to the C terminus of CNA, acts as an autoinhibitory domain by blocking substrate engagement, not the active site. Analysis of the double mutant, CNβ1_LAVPmut,AISmut, revealed a significant (18-fold) increase in phosphatase activity in EGTA compared with CNβ1wt.

Figure 4. Phosphatase activities of wild-type and mutant CNβ isoforms. Purified recombinant CNβ isozymes were assayed for activity toward 20 μM pNPP in the presence of 2 mM EGTA, 0.4 mM CaCl₂, or 0.4 mM CaCl₂ + 0.25 μM CaM. The assays were repeated 2–3 times with one (CNβ2) or two (CNβ1wt, CNβ1_LAVPmut,AISwt, CNβ1_LAVPmut,AISmut) independent preparations of pure enzyme, and the data shown are the mean ± S.D. (n = 5 or 6).
AIS, other elements within the regulatory domain contribute to autoinhibition.

**CNβ1 is autoinhibited in vivo by both the AIS and the LXVP motif**

Having analyzed calcineurin phosphatase activity in vitro, we sought to investigate how CNβ1 activity was regulated in cells, especially in the context of protein substrates that include both PXIIXIT and LXVP calcineurin interaction motifs. Thus, we re-examined the activity of human calcineurin in yeast, and measured the ability of human calcineurin to specifically dephosphorylate and activate the Crz1 transcription factor. To assay calcineurin-dependent Crz1 activity quantitatively, we incorporated a reporter gene consisting of four tandem repeats of the DNA sequence that Crz1 binds (CDRE) placed upstream of the -galactosidase gene (4X-CDRE-lacZ) (26). The amount of -galactosidase activity in extracts of yeast carrying this reporter gene reflects the activity of calcineurin in the cell.

![Figure 5. Phosphatase activities of wild-type and mutant CNβ isoforms.](image)

| Substrate: RII phosphopeptide | CNβ2 wt | CNβ1 wt | CNβ1LAVPwt,AISmut | CNβ1LAVPmut,AISwt | CNβ1LAVPmut,AISmut |
|-----------------------------|---------|---------|--------------------|-------------------|-------------------|
| EGTA                        | ++      | ++      | ++                 | ++                | ++                |
| CaCl2                      | ++      | ++      | ++                 | ++                | ++                |
| CaM                        | ++      | ++      | ++                 | ++                | ++                |

Expression of human calcineurin in yeast was assessed under three conditions: 1) unstimulated: growth in standard medium, which contains low amounts of Ca2+; 2) Ca2+-stimulated: following exposure to metal ion stress which induces Ca2+ signaling; 3) Ca2+/CaM-stimulated: following stress treatment of cells co-expressing sCaM. In the absence of stress, expression of sCaM did not significantly affect observed CDRE-lacZ activity (data not shown).

Expression of CNβ2 failed to generate detectable CDRE-lacZ activity under unstimulated or Ca2+-stimulated (stress) conditions (Fig. 6). However, statistically significant amounts of -galactosidase were produced under stress when sCaM was co-expressed. These results mirror the initial growth assays (Fig. 2A) and confirm that canonical calcineurin absolutely requires both Ca2+ and CaM for activation in vivo (Fig. 6). In cells expressing CNβ1wt, CDRE-lacZ activity was low but detectable under unstimulated conditions and progressively increased upon stress treatment and co-expression of sCaM, suggesting that Ca2+ and Ca2+/CaM each contributed to enzyme activation. By contrast, in cells expressing CNβ1LAVPwt,AISmut, CDRE-lacZ activity was low but detectable under unstimulated conditions and progressively increased upon stress treatment and co-expression of sCaM, suggesting that Ca2+ and Ca2+/CaM each contributed to enzyme activation. However, mutation of the LAVP (CNβ1LAVPwt,AISwt and CNβ1LAVPmut,AISmut) resulted in high CDRE-lacZ activity under all conditions tested. This suggests that these enzymes are active even under standard growth conditions, causing -galactosidase to continually accumulate regardless of stimulation conditions (supplemental Fig. S4). Expression levels of hCNB and sCaM were similar between strains and reproducible in experimental replicates (data not shown). Each CNβ1 isoform was also comparatively expressed, with the exception of...
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CNβ1LAVPmut,AISmut, whose levels were reduced by ~50% (data not shown). Together, these findings suggest that for activation of a protein substrate (the yeast Crz1 transcription factor), both the AIS and the LXVP motif in CNAβ1 contribute to autoinhibition, with loss of the LXVP motif resulting in high levels of activity.

Discussion

These studies establish the biochemical mechanisms that regulate phosphatase activity of CNAβ1, a conserved isoform that is generated through alternative 3’ end mRNA processing and consequently contains unique C-terminal regulatory sequences. Both in vitro and when expressed in yeast, CNβ1 differs from CNβ2 by exhibiting significant CaM independence, a more restricted activity range, and lower maximal activity in the presence of Ca\(^{2+}\)/CaM. Our data show that the conserved LAVP sequence in the C-terminal domain of CNAβ1 is an LXVP SLiM that competitively inhibits substrate dephosphorylation in trans, and serves as a cis-autoinhibitory sequence, with mutation of key motif residues abolishing inhibition. These distinct regulatory properties likely contribute to the difference in biological functions observed for CNβ1 and CNβ2.

Elegant studies by Klee and others (4, 17–20) have elucidated the complex mechanism of canonical calcineurin activation, which is consistent with our results for CNβ2 and is described below. In Fig. 7A, we present a model, based on this body of work, in which a snapshot of possible conformations for each signaling condition is shown, with thicker lines surrounding forms proposed to predominate in the ensemble. Under basal Ca\(^{2+}\) conditions (EGTA), the enzyme is in an inactive, closed conformation (form I). Only two of the four Ca\(^{2+}\) sites in CNβ1 become occupied, promoting a conformational change that disrupts the BBH-CBD interaction (20). Release of the CBD (including the AIS) increases accessibility of the substrate-binding pocket. Meanwhile, Ca\(^{2+}\) also alters the structure of the LXVP-binding surface and the catalytic center to enhance dephosphorylation (17, 37). The inactive conformation, I-Ca\(^{2+}\), still predominates, but the modest increase in activity observed upon Ca\(^{2+}\) addition (Fig. 5) suggests some availability of the catalytically active
forms (II-IV-Ca\(^{2+}\)). Ca\(^{2+}\)/CaM-bound calcineurin is fully active and cannot be further activated except through proteolysis that removes the regulatory domain (37). Ca\(^{2+}\) binding to CNB is essential for CaM stimulation of calcineurin and Ca\(^{2+}\)-bound CaM binds calcineurin at very high affinity (\(K_D \leq 0.1\) nM) (17, 40). Upon CaM binding, the CBD adopts an \(\alpha\)-helical structure that results in the full removal of the domain, including the AIS, from the LXVP pocket, and triggers a conformational change that displaces, but may not fully remove, the AID from the catalytic center (16, 39). In summary, most calcineurin isoforms, including CNA\(\beta\)2, form a heterodimer with CNB whose activity is strictly controlled by Ca\(^{2+}\)/CaM. Both Ca\(^{2+}\) and CaM are required in concert to relieve autoinhibition at the catalytic site and CaM.

In contrast to CNA\(\beta\)2, CNA\(\beta\)1 lacks the AID and instead possesses a unique C-terminal regulatory domain. Based on our results, we propose a model for the multistep regulation of this isoform (Fig. 7B). In the presence of EGTA, the catalytic center of CNA\(\beta\)1 is open, as demonstrated by the elevated level of pNPP hydrolysis by CNA\(\beta\)1 compared with CNA\(\beta\)2 (Fig. 4), and the enzyme exists as an ensemble of inactive (forms I and II) and active (form III) conformations. In forms I and II, the substrate-binding pocket is engaged by the LXVP motif or AIS, respectively. Mutation of either (Fig. 6) or both sequences (Figs. 5 and 6) significantly increased basal enzyme activity suggesting that both forms are present, but the overall low amount of RII phosphopeptide dephosphorylation suggests that a minor fraction of the enzyme adopts the fully active form III (Fig. 5). By analogy with CNA\(\beta\)2, Ca\(^{2+}\) weakens the BBH/CBD interaction (20), thus shifting the equilibrium away from the CBD-bound form (II-Ca\(^{2+}\)) and toward forms I-Ca\(^{2+}\) and III-Ca\(^{2+}\). This aspect of the model is supported by the modest activation observed for CNA\(\beta\)1LAVPwt,AISmut in vivo in the presence of stress (Fig. 6), compared with the dramatic activity increase observed for CNA\(\beta\)1LAVPmut,AISwt both in vivo and in vitro (Figs. 5 and 6). The effects of the AIS mutation in CNA\(\beta\)1LAVPwt,AISmut may be more apparent in vivo due to the high effective concentration of an endogenous substrate containing both calcineurin interaction motifs (PXIXIT and LXVP). The presence of the active form III-Ca\(^{2+}\) in the ensemble explains the ability of CNA\(\beta\)1-expressing yeast to grow under stress in the absence of sCaM compared with yeast expressing CNA\(\beta\)2 (Fig. 2A). Ca\(^{2+}\)/CaM fully dissociates the CBD, including the AIS, from the substrate-binding pocket. In contrast to CNA\(\beta\)2, however, the LXVP motif in CNA\(\beta\)1 limits activity even in the presence of Ca\(^{2+}\)/CaM. This is apparent in vitro, where Ca\(^{2+}\)/CaM-stimulated CNA\(\beta\)1 activity never reaches the level of CNA\(\beta\)2, versus CNA\(\beta\)1LAVPmut,AISwt and CNA\(\beta\)1LAVPmut,AISmut which are highly active (Fig. 5). Indeed, this evidence suggests that of the two CaM-bound states (forms I-CaM and II-CaM), the inactive, LXVP-bound form I-CaM may predominate.

In conclusion, we propose that CNA\(\beta\)1 exhibits a unique mechanism of regulation by Ca\(^{2+}\)/CaM. In contrast to CNA\(\beta\)2, CNA\(\beta\)1 is autoinhibited at a single site, but by two inhibitory regions that compete with each other and with substrates. The first, the AIS-containing CBD, is progressively removed by the addition of Ca\(^{2+}\) and CaM, but the second, LXVP, remains bound. This confers higher basal and Ca\(^{2+}\)-dependent activity, but less dependence on CaM, and limits the maximum activity achievable by this isoform.

**Figure 7. Model describing regulation of CNA\(\beta\) isoforms by Ca\(^{2+}\) and calmodulin.** Each panel represents a snapshot of possible enzyme conformations for different signaling conditions. Thicker lines surround forms proposed to predominate in the ensemble. See text for details. CNA, gray, with catalytic center marked by notch; CNB, orange; Ca\(^{2+}\), white circles; LXVP-binding pocket, green. A, CNA\(\beta\)2 model. B, CNA\(\beta\)1 model.
Mechanism of CNAβ1 autoinhibition

These unique biochemical properties may allow CNAβ1 to play distinct regulatory roles in vivo. In vitro, autoinhibition of CNAβ1 by its C-terminal LXVP motif limits its activity toward the RII phosphopeptide. However, in vivo, this LAVP sequence may rather serve as a filter that restricts CNAβ1 substrate specificity. Our model suggests that CNAβ1 will selectively dephosphorylate substrates whose high-affinity LXVP SLiMs can outcompete the C-terminal LAVP sequence for binding to the substrate docking groove on calcineurin. SLiMs do vary, as NFAT family members contain LXVP motifs with differing affinities for calcineurin (41). Indeed, altering PXIXIT affinity in either Crz1 or NFAT modulates signaling strength (42, 43), and the same is likely to apply to LXVP motifs. An unexpected finding is that CNAβ1 activity in vitro and in vivo is lower compared with CNAβ2 in the presence of maximal Ca2+/CaM stimulation. This limited activity range might be biologically advantageous in vivo under extreme Ca2+ conditions, such as in microdomains near Ca2+ entry sites, or during sustained signaling (44). We also show that CNAβ1 has significant CaM-independent activity, suggesting that it can signal under CaM-limiting conditions (45). Finally, in vivo, the C-terminal 23 aa of CNAβ1, deleted in our studies, directs the enzyme to the Golgi, in contrast to the cytosolic localization of CNAβ2 (21). This membrane association potentially targets CNAβ1 to a unique pool of substrates, including those regulating mechanistic target of rapamycin (mTOR) and AKT signaling (21). The CNAβ1 C terminus also mediates unique protein-protein interactions, including binding to Rictor, a subunit of mTORC2 (22). Thus, in vivo, interaction of this C-terminal tail with membranes and/or proteins might relieve autoinhibition by the LAVP pseudosubstrate sequence to activate the enzyme in a spatially regulated manner. Overexpression and depletion studies have clearly indicated that CNAβ1 has unique physiological functions that are distinct from CNAβ2 (7, 21–23). The biological mechanisms underlying these effects remain to be elucidated as CNAβ1 substrates are identified and their unique regulation by CNAβ1 can be studied in vivo.

Protein expression and purification

For MBP-CNBβtrunc-His (MBP, maltose-binding protein), CNA and CNB subunits were expressed in tandem in Escherichia coli BL21(DE3) RIL cells (Invitrogen) and cultured in LB containing chloramphenicol (34 μg/ml) and ampicillin (50 μg/ml) at 37 °C to mid-log phase. Expression was induced for 2 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation (4,000 × g, 20 min) and frozen at −80 °C. For purification, all steps were performed at 4 °C. Cells were resuspended in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM DTT, protease inhibitors) and supplemented with 2 μg/ml of DNase. Cells were lysed by two passages through a French press at 35,000 psi. Crude extract was clarified by three rounds of centrifuging (15,000 × g, 30 min). MBP-CNBβtrunc-His heterodimers were bound in batch to amylose resin (New England Biolabs) for 2 h, washed with lysis buffer, and eluted with lysis buffer containing 20 mM maltose. MBP-CNBβtrunc-His was then affinity purified with Ni-NTA-agarose (Invitrogen) in the presence of 15 mM imidazole for 1 h, washed with lysis buffer containing 15 mM imidazole, and eluted with lysis buffer containing 300 mM imidazole. Pure MBP-CNBβtrunc-His was brought to 20% glycerol, aliquoted, and stored at −80 °C.

Recombinant CNAβ2, CNAβ1wt, and CNAβ1 mutant isoforms were expressed and purified as follows. Plasmids for CNA (pCuGST-CNAβ2-His, pCuGST-CNAβ1ΔC-His, pCuGST-CNAβ1LAVPwt,AISmutΔC-His, pCuGST-CNAβ1LAVPmut,AISmutΔC-His), pCuGST-CNAβ1LAVPmut,AISmutΔC-His, pCuGST-CNAβ1LAVPmut,AISmutΔC-His), pCuGST-CNAβ1LAVPmut,AISmutΔC-His, and pCuGST-CNAβ1LAVPmut,AISmutΔC-His) were co-transformed with pCu425-StrepII-CNβ into the protease-deficient, CNB1-lacking Saccharomyces cerevisiae strain yHX-1. Cultures were grown at 30 °C to mid-log phase in selective media, and protein expression was induced for 2 h with 500 μM CuSO4. Cells were harvested (4,000 × g, 30 min) and resuspended in yeast lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, protease inhibitors) and frozen in liquid N2. Frozen cell droplets were lysed with a Retsch MM301 Ball Mill (5 × 3 min, 20 Hz). All following steps were performed at 4 °C. Extracts were thawed and clarified by centrifugation (10,000 × g, 10 min; 15,000 × g, 30 min × 2). CNβ isoforms were bound in batch to glutathione-Sepharose (GE Healthcare) for 2 h, then the CaCl2 concentration was raised to 5 mM, and beads were washed on column with glutathione wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 0.5 mM CaCl2, 0.5 mM DTT) and eluted with glutathione elution buffer (glutathione wash buffer containing 40 μM reduced l-glutathione, pH 7.0). The GST tag was removed by overnight cleavage with thrombin (Calbiochem). The sample was brought to 10 mM imidazole, and CNβ was next affinity purified with Ni-NTA-agarose (Invitrogen) for 2 h, washed in Ni-NTA wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mM CaCl2, 0.5 mM DTT) and eluted with Ni-NTA elution buffer (Ni-NTA wash buffer with 300 mM imidazole). Uncleaved protein was removed by subtraction purification with glutathione-Sepharose. Calcineurin was further purified by size exclusion chromatography (Superose 6 (GE Healthcare) equilibrated in 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT).

Experimental procedures

Growth media and general methods

Yeast media and culturing methods were followed as described in synthetic complete (SC) medium (46) except twice the amount of amino acids and nucleotides were used. Yeast transformations were performed by the lithium acetate method (47). Plasmids and yeast strains used in this study are listed in supplemental Tables S1 and S2, respectively.

RNaseq analysis

FASTQ files from the ArrayExpress database at EMBL-EBI under accession number E-MTAB-1733 were downloaded and paired reads were aligned to unique sequence regions of CNAα, CNAβ2, CNAβ1, and TATA box-binding protein using Bowtie2 version 2.2.3. Reads were filtered for quality with SAMtools version 1.1. Raw counts for each CNA variant were divided by the length of the reference sequence and then normalized to TATA box-binding protein, whose expression variation is low across most tissues (48).
some cases the full-length protein required further purification by Ni-NTA affinity resin and SEC to remove partially degraded forms. Pure calcineurin was adjusted to 20% glycerol, aliquoted, and stored at −80 °C. Two independent purifications of CNβ1 wild-type and all mutant isozymes were prepared and used for the phosphatase assays in Figs. 4 and 5. Protein concentration was determined by Coomassie staining using bovine serum albumin as standard. Enzyme purity was visualized by Silver Stain Plus (Bio-Rad).

**Enzyme activity assays**

The rate of RII dephosphorylation was determined by measuring continuous PO₄ release detected with the phosphate-binding fluorophore MDCC-PBP (Invitrogen). Reactions were performed in assay buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 100 μg/ml of BSA, 1 mM DTT, 0.4 mM CaCl₂, 0.25 mM MDCC-PBP). For peptide trans-inhibition assays, reactions contained 5 nM MBP-CNβ₁ trunc, 50 μM RII phosphopeptide substrate, and 0–100 μM peptide. For assays to determine the mode of inhibition, reactions contained 5 nM MBP-CNβ₁ trunc, 100–1000 μM RII phosphopeptide substrate, and 0–100 μM peptide. The mode of inhibition was determined by comparing alternative models with Akaike Information Criterion using Prism software (GraphPad) and selecting the more statistically probable model, which was always favored by >90%.

For assays with calcineurin isozymes (CNβ2, CNβ1wt, CNβ1 LAVPmut, AISwt, CNβ1 LAVPwt, AISmut) and CNβ1 LAVPmut, AISmut reactions contained 2.5 mM CN, 50 μM RII phosphopeptide substrate, and 2 mM EGTA, 0.4 mM CaCl₂, or 0.4 mM CaCl₂ + 0.25 μM bovine CaM (Calbiochem). Experiments were performed in black low-binding half-area 96-well plates in a Biotek Neo plate reader (BioTEK, Winooski, VT) with continuous shaking at room temperature. Fluorescence was measured at 425 nm excitation/530 nm emission. Reaction rates were linear and constituted less than 10% of product formation. Phosphate standards were used to convert fluorescence signal to PO₄ concentration. Enzyme rate is reported as v/[E], where v is initial velocity and [E] is total enzyme concentration.

The rate of pNPP hydrolysis was measured in a continuous assay by monitoring the production of pNP at 405 nm. Reactions were performed in assay buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 100 μg/ml of BSA, 1 mM DTT, 0.4 mM CaCl₂, 0.25 mM MDCC-PBP). For peptide trans-inhibition assays, reactions contained 5 nM MBP-CNβ₁ trunc, 50 μM RII phosphopeptide substrate, and 0–100 μM peptide. For assays to determine the mode of inhibition, reactions contained 5 nM MBP-CNβ₁ trunc, 100–1000 μM RII phosphopeptide substrate, and 0–100 μM peptide. The mode of inhibition was determined by comparing alternative models with Akaike Information Criterion using Prism software (GraphPad) and selecting the more statistically probable model, which was always favored by >90%.

Peptides

Peptides were synthesized and purified by the Tufts University Core Facility (Boston, MA) or by Peptide 2.0 (Chantilly, VA). The amino acid sequences of peptides used in the trans-inhibition assay are described in Table 1. Sequences of peptides used in supplemental Fig. S1 are as follows: AIS-FSVLmut peptide, ARVSFLREESVL; AIS-FSVLmut peptide, ARVFSVLREESVL; AIS-FSVLmut peptide, ARVFSVLREESVL and RII phosphopeptide, DLDVPVGRFDRVSp VAAE. Peptides were dissolved in 10 mM Tris, pH 8.0.

**β-Galactosidase assay**

Yeast strains (RBY05-14) were grown in SC lacking leucine, uracil, and tryptophan, and were supplemented with 80 μg/ml of adenine and 2% dextrose to mid-log phase. Calcineurin expression was induced with 100 μM CuSO₄ for 30 min prior to the 1.5-h treatment in 5 mM MnCl₂. Cultures were transferred to 96-well plate for lacZ analysis, whereas 1 ml was reserved to confirm even protein expression (see below). Calcineurin-dependent activity was determined using substrate fluorescein di-β-D-galactopyranoside (F-1179, Invitrogen) as previously described (49). Fluorescence emission at 530 nm was detected using a Biotek Neo plate reader and normalized to A₆₀₀. Activity of each strain was measured in triplicate, and at least 3 independent transformants were evaluated per strain. Under standard growth conditions without CuSO₄ induction or stress treatment, calcineurin subunits were expressed at a low level (supplemental Fig. S4A) yet CNβ₁ LAVPmut, AISmut remained highly active (supplemental Fig. S4B).

Protein expression levels were verified by Western blot. Proteins were extracted by NaOH lysis/TCA precipitation. Cells were resuspended in 500 μl of water, then lysed 10 min on ice with 50 μl of 1.85 M NaOH with 300 mM β-mercaptoethanol. Proteins were precipitated on ice with 50 μl of 50% TCA for 30 min, then pelleted 12,000 × g for 10 min at 4 °C. The pellet was dissolved in 40 μl of urea/SDS buffer (8 M urea, 5% SDS, 200 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, pH 8.0, 0.1% bromphenol blue, 100 mM DTT, 100 mM Tris base). Samples were analyzed by SDS-PAGE and Western blot using standard procedures. Immunoblots were probed with rabbit polyclonal anti-CNβ1 (C0581, Sigma), mouse monoclonal anti-CNB (CN-B1, Sigma), rabbit monoclonal anti-calmodulin (EPR5028, Xbcam), and/or mouse monoclonal anti-PGK (459250, Invitrogen). Primary antibodies were detected by IR fluorescence with secondary anti-rabbit conjugated to Alexa 790 (A11369, Invitrogen) or anti-mouse conjugated to Alexa 680 (A-21058, Invitrogen) and visualized with an Odyssey scanner (Li-Cor Bioscience). Band intensity was quantified using Image Studio (Li-Cor Bioscience).

**Yeast growth assay**

Yeast strains (RBY01-06) were grown in selective media to mid-log phase, then 0.2 A₆₀₀ units of cells from each culture were 5-fold serially diluted in water and spotted onto plates. Plates contained SC lacking leucine, uracil, and tryptophan, and were supplemented with 80 μg/ml of adenine, 2% dextrose, and 0 or 10 mM MnCl₂. Where noted, plates were supplemented with 1 μg/ml of FK506 (LC Laboratories) dissolved in 90% eth-
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anol, 10% Tween-20 vehicle, or vehicle alone. Plates were incubated at room temperature for 6 days.

Author contributions—R. B., N. L. and M. S. C. designed the experiments; R. B. and N. L. performed research and analyzed the results; R. B. and M. S. C. wrote the manuscript. All authors read and approved the final manuscript.

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