Calcineurin Undergoes a Conformational Switch Evoked via Peptidyl-Prolyl Isomerization

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Abstract

A limited repertoire of PPP family of serine/threonine phosphatases with a highly conserved catalytic domain acts on thousands of protein targets to orchestrate myriad central biological roles. A major structural reorganization of human calcineurin, a ubiquitous Ser/Thr PPP regulated by calcium and calmodulin and targeted by immunosuppressant drugs cyclosporin A and FK506, is unveiled here. The new conformation involves trans- to cis- isomerization of proline in the SAPNY sequence, highly conserved across PPPs, and remodels the main regulatory site where NFATc transcription factors bind. Transitions between cis- and trans- conformations may involve peptidyl prolyl isomerases such as cyclophilin A and FKBP12, which are known to physically interact with and modulate calcineurin even in the absence of immunosuppressant drugs. Alternative conformations in PPPs provide a new perspective on interactions with substrates and other protein partners and may foster development of more specific inhibitors as drug candidates.

Introduction

Reversible phosphorylation, orchestrated by the opposing activities of kinases and phosphatases, is estimated to occur in about one-third of proteins and is responsible for modulating many cellular functions including cell growth, proliferation and differentiation [1, 2]. Phosphoserine and phosphothreonine, which account for over 98% of protein-bound phosphate in eukaryotic cells, are regulated by a large number of Ser/Thr protein kinases and, surprisingly, far fewer Ser/Thr phosphatases including, in particular, the widely distributed family of
phosphoprotein phosphatases (PPPs), encoded by a relatively small number of genes in the human genome[3]. Protein phosphatase 1 (PP1), with more than two hundred confirmed targeting proteins, and calcineurin (PPP3C, formerly PP2B) are the most abundant and extensively studied PPPs [4].

Calcineurin (CN), ubiquitously expressed and highly conserved from yeast to humans, plays a critical role coupling Ca^{2+} signals to different gene expression patterns and cellular responses[5]. CN was first identified as the target of the immunosuppressants cyclosporine A (CsA) and FK506[6, 7], which are the cornerstone of current immunosuppressive therapy. These drugs bind to the endogenous immunophilins cyclophilin A (CypA) and FKBP12[8], respectively, and the corresponding complexes bind to CN inhibiting phosphatase activity for all CN substrates resulting in both desired therapeutic outcomes and in some cases severe side-effects[9]. CN functions as an heterodimer with a large catalytic subunit (CNA) (59 kDa for the human α isoform) interacting with two Ca^{2+} binding proteins, calmodulin and the small regulatory CN subunit (CNB) (19 kDa for the type 1 isoform)[5]. CNA is organized as an N-terminal catalytic domain, of about three hundred residues, and a C-terminal regulatory domain comprising the CNB and the calmodulin binding regions followed by an autoinhibitory domain (AID) that, under specific conditions, binds into the active site blocking the access of substrates [10]. The catalytic domain of CNA presents a high sequence and structural homology to other PPP catalytic domains [11, 12]. The active site contains two metal ions located between two helical domains and a characteristic central sandwich of two β-sheets that are interconnected between strands β12 (from sheet I) and β13 (from sheet II) by a highly conserved sequence (PSAPNYxxxxNx) (Fig 1a)[13]. The β12-β13 connection, also called loop 7 in CNA, is one of the key regulatory elements in PPPs [14, 15].

The cytosolic Nuclear Factor of Activated T cells (NFATc) transcription factors, critical in many cellular processes, bind to CN through two binding sequences, the PxIxIT and LxVP motifs[16, 17], and at least one of them is present in all the endogenous CN modulators identified so far[18]. The structures of a diversity of CN constructs alone or in complex with FK506/FKBP12[11, 12], CsA/CypA[19, 20], or with different peptides, have been determined[18, 21–23]. Regulatory PxIxIT-containing peptides add a strand at the β14 edge of β-sheet II. Similarly, PP1 phosphatase recognizes an RVxF motif in its targeting subunits through binding at a site cognate to the PxIxIT-binding site [1, 24].

Materials and Methods

Materials

Peptides were purchased from the Peptide 2.0 Company (Chantilly, VA) and synthesized as acetylated N-terminal and C-terminal amides for the unlabeled peptides and N-carboxyfluorescein (CF) and C-terminal amide for the labelled peptide. The sequences of the peptides used are the following: KYELHAGTESTPSVVVHVCES for the RCAN183–203 peptide; NNKAAVLKYE for the cis-CNA derived peptide; and ASGLSPRIEITPSHEL for the NFATc2–SPIEIT (SPIEIT) peptide. All peptides were resuspended in 100% DMSO at 10 mM. Cyclosporin A (CsA) was obtained from Sandoz. Ionomycin (Io) sodium salt and Phorbol 12-myristate 13-acetate sodium salt (PMA) were obtained from Sigma. The anti-calcineurin A antibody was purchased from BD Biosciences and the anti-Flag M2 antibody was purchased from Sigma. The protease and phosphatase inhibitor cocktails were from Calbiochem.

Methods

Protein expression and purification. Large scale production of the catalytic domain of CNA was achieved with the pGEX-6P-1-CNAα plasmid construct, kindly provided by Patrick
Hogan, that encodes the Glutathione S-transferase protein linked to the human CNAα isoform (NCBI NP_000935.1) catalytic domain (residues 2–347). Expression, purification and isolation of the CNAα domain was performed as previously described[25]. The isolated CNAα catalytic domain was further purified using a Superdex 75 size-exclusion column equilibrated at 0.4 ml/min with 100 mM NaCl and 50 mM Tris pH 8.0, where the protein eluted as a unique peak corresponding to 40 kDa. The purified protein was kept at 4°C for short periods of time.

Fig 1. Sequence and structural alignments. (a) The catalytic domain of CNA is highly conserved around the β12 and β13 connection in PPPs. Sequence alignments around the β12 and β13 connection of the catalytic domains of human PPPs. PPP1 to PPP7, protein phosphatases 1 to 7; PPEF1 and 2, protein phosphatase EF-hand calcium binding domain 1 and 2; and CPPED1, calcineurin-like phosphoesterase domain containing 1. A, B and C correspond to α, β and γ isozymes, respectively. Alignment were performed using MAFFT v.7 online version (http://mafft.cbrc.jp/alignment/software)[38] using default parameters and subsequently edited using Jalview software v.2.8[39]. (b) Structural alignment of cis-CNA (brown) and trans-CNA (blue) showing the sequence shifts and the secondary structural elements correspondence. Small letters indicate amino acid residues not modeled in the structure. Residues from loops 6 and 7 are shadowed in the trans-CNA conformation.

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Protein crystallization and structure determination. Hexagonal crystals were obtained by vapour diffusion, using the human CNAα catalytic domain a final protein concentration of 7.3 mg/ml. Crystals (with a reservoir buffer of 0.1M Hepes—pH 7.5 -, 26% PEG 3350 and 4% PGA) are in space group P622 with cell parameters of a = b = 185.01 Å, c = 106.74 Å and α = β = 90°, γ = 120°. Crystals were cryoprotected using reservoir solution supplemented with 20% (v/v) glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data, collected at 100K on beam line PROXIMA-1 (SOLEIL synchrotron, France) with a Dectris Pilatus 6M detector and oscillation angles of 0.2° per frame, were processed using the interactive iMOSFLM package at 3.35 Å resolution[26]. Crystals, with similar unit cell parameters, have also been obtained in the absence of inhibitory peptides, though diffraction from these crystals was always below 3.5 Å resolution.

The structure was solved by molecular replacement using MOLREP[27] and the coordinates of human CN (PDB entry 1AUI) as search model. Refinement was performed with REFMAC5 [28] and the molecular-graphics program COOT[29] (Table 1). Non-crystallographic restraints together with the restraints of the trans-CNA subunit towards high resolution information were also applied.

Fluorescence polarization. Competition assays were performed as described[30]. Briefly, carboxyfluorescein (CF)-SPRIEIT-CNA complex was performed using 10 nM of CF-labeled SPRIEIT peptide and 10 μM CNA. Unlabeled competitor peptides were pre-incubated with CNA at increasing concentrations for 15 min before adding the fluorescence labeled peptide. Experiments were performed in a OptiPlate black 384-well-flat-bottom plates (PerkinElmer Life Sciences, Waltham, MA) and measured using a Wallac VICTOR (TM) X5 2030 Multilabel Reader (PerkinElmer Life Sciences) with excitation and emission wavelengths of 485 nm and 535 nm respectively. All assays were performed for 15 min at room temperature. All data were obtained from at least three independent experiments performed in triplicates.

Table 1. X-ray Data and Refinement statistics.

| A | X-ray Data | CNA |
|---|---|---|
| Resolution limits (Å) | 50.0–3.35 (3.53–3.35) | |
| Space group | P62 2 2 | |
| Unit cell parameters (Å, °) | 185.01, 185.01, 106.74 90, 90, 120 | |
| R pim (%) | 5.2 (33.6) | |
| Completeness (%) | 100 (100) | |
| <I/σ(I)> | 13.0 (2.7) | |
|Multiplicity | 17.1 (15.7) | |
| N° of unique reflections | 15.994 (2.270) | |
| Total number of observations | 272.976 (33.646) | |

| B | Refinement | CNA |
|---|---|---|
| Resolution limits (Å) | 50.0–3.35 | |
| Rwork (%) | 21.56 | |
| Rfree (%) | 24.91 | |
| Rms bond lengths (Å) | 0.017 | |
| Rms bond angles (°) | 2.1 | |
| N° of protein atoms | 5.064 | |
| Protein mean B-factor (Å²) | 48 | |

* In brackets for the last resolution shell.
GST-pull-down competition assays. In GST-RCAN3 pull-down competition assays, HEK 293T cells were lysed in co-immunoprecipitation buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM CaCl2, 5 mM MgCl2, 1% IGEPAL, 1 mM DTT, 2 mM PMSF and protease and phosphatase inhibitor cocktails) and the CNA- and the RCAN3-derived peptides containing a PxIxIT sequence were added to the extracts at the indicated concentrations for 30 min. Then, the soluble extracts were incubated with GST–RCAN3 bound to Glutathione Sepharose beads for 90 min at 4°C. After extensive washing, co-precipitated proteins were eluted by resuspension in 2× Laemmli buffer and boiled for 10 min. For GST–NFATc2 pull-down competition assays, HEK 293T cells transfected with FLAG-CNA (2–389) were lysed in a buffer containing 50 mM Tris–HCl, pH 8, 100 mM NaCl, 1.5 mM CaCl2, 6 mM MgCl2, 0.2% TX-100, 1 mM PMSF and protease and phosphatase inhibitor cocktails. Soluble extracts were incubated with GST–NFATc2 bound to Glutathione Sepharose beads for 60 min at 4°C.

NFATc-luciferase reporter gene assay. Flag-hCNAα mutants were performed by PCR using CN specific primers (Table 2). ΔNIR mutant lacks the CNA PXIXIT binding region VMNIR ranging from amino acids 328 to 332. All Flag-CNA constructs bear the Y341F mutation to achieve CsA resistance unless for the specified wt. Luciferase gene assays were performed in HEK 293T cells transfected with FLAG–CNA (2–389) were lysed in a buffer containing 50 mM Tris–HCl, pH 8, 100 mM NaCl, 1.5 mM CaCl2, 6 mM MgCl2, 0.2% TX-100, 1 mM PMSF and protease and phosphatase inhibitor cocktails. Soluble extracts were incubated with GST–NFATc2 bound to Glutathione Sepharose beads for 60 min at 4°C.

Results and Discussion

The structure of the catalytic domain of human CNAα (residues 2–347) has been determined from hexagonal crystals. The two subunits of the catalytic domain contained in the asymmetric unit present important structural differences (Table 1, Fig 2). The first subunit in the crystal shows the essentially invariant conformation (herein referred to as *trans*-CNA) found in all available structures of CN and other PPP phosphatases. The second subunit in the crystal
shows an alternative new conformation (herein referred to as cis-CNA) (Fig 2a). The two subunits in the asymmetric unit are related by a non-crystallographic quasi-two-fold rotation (172°) plus a 3.7 Å translation, about an axis roughly in the middle of the β14 strands that interact with each other extending sheet II across both subunits (Fig 2b). The thirteen residues long connection (Phe306-Lys318) joining strands β12 and β13 in trans-CNA are shown with a different color (green) to emphasize that they differ from the ones in trans-CNA. The thirteen residues long loop 7 joining strands β12 and β13 in trans-CNA is reduced to a tight β-turn with only four residues (SAP309N) in cis-CNA.
Fig 3. Complete reorganization of the Pro309 environment between the trans- and cis- conformations of CNA. Stereo views showing the large structural differences observed in the Pro309 environments between the (a) trans-CNA and (b) cis-CNA conformations. The (2Fo-Fc) electron density map, contoured at 1σ and depicted in dark grey, and the (Fo-Fc) maps, contoured at 3σ (positive in green and negative in red), are also
shown for both CNA conformations. Residues from Phe299 to Asn326 and from Phe299 to Tyr315 are shown for trans-CNA (blue) and cis-CNA (brown), respectively. The corresponding molecular models are represented as thick rods while the superimposed structures of the alternative conformations are depicted as thin rods. Both conformations are essentially identical till residue Phe306, where only side chains differ significantly. Beyond Phe306 also main chains separate completely. (c) Superimposition of the structures of the Ser-Ala-Pro-Asn-fragment forming the turn (within the SAPNY sequence), as found in cis-CNA (brown) and in the enzyme family 5 xyloglucanase (yellow) where the cis conformation adopted by the proline is accurately defined at 1.4 Å resolution (PDB code 2JEP). The 2Fo-Fc electron density corresponding to the boxes in panel b is also shown to emphasize the quality of the fitting of cis-CNA residues around Pro309 (from Phe306 to Tyr311).

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present in cis-CNA and superimpose structurally well with the same strands of trans-CNA, but the amino acid residues within these strands differ between trans-CNA and cis-CNA (Figs 1b, 3a and 3b). Strand β13 presents a ten residue shift between the two conformations with Asn310 in cis-CNA being structurally equivalent to Ala320 in trans-CNA. For the β14 strand the structurally equivalent residues present a nine residue shift with Ala319 in cis-CNA being structurally equivalent to Val328 in trans-CNA. Therefore, the nine residue shift increases the number of residues after β14 in cis-CNA with respect to trans-CNA. This region loops around the active site in cis-CNA, with side chains poorly defined, interacting also with neighboring subunits in the crystal. This region would correspond to the linker connecting the catalytic domain and the CNB binding region in full-length CNA. In trans-CNA, loop 7 and strand β13 interact with loop 6, which corresponds to residues from Ala280 to Gly287 between strands β10 and β11 including the catalytically essential residue His281[2] (Fig 1b). The decrease in the number of residues for the β12–β13 connection together with the amino acid sequence changes in strand β13, can explain the destabilization of loop 6 and of strand β11 that in cis-CNA are disordered from Ala283 until Arg289.

Differences between trans-CNA and cis-CNA have major implications both on the active site organization and on the crucial docking site for PxIxIT-containing proteins. Concerning the active site, PP1 phosphatases have been described as containing two metal ions at the intersection of three putative substrate binding grooves, referred to as hydrophobic, acidic and C-terminal grooves[24]. Following on this description for CN (Fig 4a), loops 6 and 7 are located between the acidic and the C-terminal grooves forming the internal walls of these grooves in trans-CNA (Fig 4b). Instead, in cis-CNA the acidic and C-terminal binding grooves merge into a continuous surface, due to the reduction of loop 7 and the flexibility of loop 6, rendering the catalytic metal ions accessible to substrates with wider binding surfaces than for trans-CNA (Fig 4c). Moreover, in cis-CNA disordered residues from loop 6 and the enlarged linker define a new environment around the active site (Fig 4d). Residues from loop 7 (in particular Tyr311 and Tyr315) in trans-CNA participate in AID binding and consequently the absence of this loop 7 in cis-CNA should weaken or prevent AID binding and the corresponding inhibitory effects. Given that in cis-CNA the active site is more accessible than in trans-CNA, no significant differences in phosphatase activity are expected for the standard small substrates pNPP and RII peptide (from the regulatory RII subunit of cAMP-dependent protein kinase[32]). Concerning the PxIxIT binding site, in trans-CNA strand β14 defines the central structural element of the site with binding affinities that are finely tuned from both consensus and non-consensus positions in the motif[18, 21]. Accordingly, affinity for the PxIxIT binding site is redefined by the sequence shift between β14 strands in trans-CNA and cis-CNA (Figs 1b and 2b). Mutating residue Tyr288, a residue next to loop 6 that is disordered in cis-CNA and in trans-CNA interacts with Lys323 from β-13 and with Ile331 from β-14, to alanine or to asparagine decreases sharply NFATc activity (Fig 5). In turn, mutant of Tyr288 to phenylalanine, which can retain the hydrophobic interactions with Ile331, also retains a significant NFATc
Fig 4. Reorganization of the active site between trans- and cis- CNA. (a) Surface representation of a CNA subunit (grey), truncated after the CNB binding region, in complex with a CNB subunit (pink), with the binding sites for proteins containing LxVP and for PxIxIT (green) motifs [18]. A bound autoinhibitory Calcineurin Conformational Change
Therefore, altering loop 6 results in the destabilization of trans-CNA and consequently of the CN PxIxIT binding site.

In all the reported structures of catalytic domains from PPPs the central proline in the highly conserved SAPNY sequence shows the peptidyl trans isomer found also in the trans-CNA subunit in this work [21, 23, 33]. Strikingly, in the only available structure of a protein unrelated to PPP phosphatases containing a SAPNY sequence (the prokaryotic enzyme family 5-xyloglucanase, PDB codes 2JEP and 2JEQ), the peptide forms a solvent exposed turn with a central cis-proline that is essentially identical to the structure found in cis-CNA (Fig 3c). The parallel pairing of β14 strands from neighbor CNA subunits having to deviate from a symmetric two fold interaction can explain, at least in part, the presence of the alternative conformations. This supports the feasibility of the trans- to cis transition, not yet detected directly in vivo, when the appropriated interactions with other proteins are involved. The standard PxIxIT binding site in the trans-CNA subunit (sequence VMNIRQF starting with Val328), is occupied by the shifted sequence AAVLKYE (starting with Ala319) from the β14 strand of the neighbor cis-CNA (Figs 1b and 2b). However, the peptide NNKAAVLKYE, containing the sequence of β-14 strand of cis-domain is also explicitly indicated (AID in yellow). The acidic, C-terminal and hydrophobic substrate-binding grooves, defined for PPP phosphatases, are labeled as A, C and H, respectively. Loops 6 (light blue) and 7 (brown) are clearly visible between the acidic and C-terminal grooves. Surface representation of the catalytic domain of CNA subunit illustrating the trans- conformation (b) and the cis- conformation (c). In these views the two catalytic metal ions and a bound phosphate molecule are clearly visible. The C-terminal tail of cis-CNA, corresponding to the extended linker, has been omitted for clarity. (d) Cartoon of the cis-/trans-CNA transition with a schematic representation of the main structural differences between the trans-CNA (upper) and cis-CNA (bottom) conformations. Strands β13-β14 are colored in light blue and green for the trans- and cis-CNA conformations, respectively. Metal ions (red balls), loop6 (light blue) and loop7 (brown), with proline 309 (green pentagon), are depicted.

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Fig 5. Mutation of loop 6 residues affects NFATc activity. Luciferase reporter gene assays in HEK 293T cells transfected with 3xNFAT-luc plasmid, pBJ5-mCNB and Flag-CNA 2–389 wild type (wt) or related mutants. ΔNIR mutant lacks the CNA PXIXIT binding region VMNIR ranging from amino acids 328 to 332. All Flag-CNA constructs bear the Y341F mutation to achieve CsA resistance unless for the specified wt. Endogenous CN activity was abolished by treating the cells with 1 μM CsA 30 min before stimulation. Stimulation was achieved by treating the cells with Io/PMA/Ca2+ for 6 hours. FK506 was added as a control of CN inhibition of the CNA 2–389 Y341F. Absence of cell stimulation is shown with a minus symbol (-). Three experiments were performed in triplicates. CNA wt and mutants protein levels were assessed by densitometry of the electrophoretic bands detected by western blot analysis with anti-FLAG antibody. Data is given as mean percentage of NFAT activation normalized to CNA protein levels. Stimulation of the Y341F mutant was considered as 100% value.

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CNA, does not interfere with high affinity interactions between CN and peptides or proteins containing a PxIxIT motif such as the NFATc2-derived SPRIEIT peptide (amino acids 183–203, R3183–203) and assessed by fluorescence anisotropy. Anisotropic fluorescence emission values (mP) are represented as mean ± SEM of two independent experiments performed in triplicates. An unrelated peptide was included as negative control. Endogenous CNA (b) or Flag-CNA 2–389 (c) pull down assays using GST-RCAN3 (GSTR3) (b) or GST-NFATc2 (GSTNFATc2) (c) as bait and competed with increasing concentrations of cis-CNA-derived peptide NNKAAVLKYE or RCAN3-derived peptide (R3183–203). GST alone was used as negative control. Ponceau staining of the membrane shows equal GST fusion protein loading of each lane.

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(Fig 7a and 7b) having access to Pro309 if the cis-CNA conformation is adopted. Docking of the structure of the complex of CypA with the Ala-cis-Pro dipeptide (PDB code 2CYH) onto cis-CNA by superimposition of the Ala-cis-Pro fragments of both structures indicates a close interaction between CypA and the catalytic domain of CNA (Fig 7c and 7d), which can explain why CNB is dispensable for the FKBP12-CN binary complex but required for the ternary complex with FK506 [34]. A prolyl isomerization process provides a rationale for the intriguing interactions of CN and PPIs, with natural products CsA and FK506 capitalizing upon these inherent interactions [34]. A similar isomerization mechanism could also operate for the mammalian target of rapamycin kinase (mTOR), which forms a ternary complex with FKBP12 and the natural product rapamycin [36]. Phosphorylation-dependent prolyl isomerization has been proposed as a molecular timer to modulate the amplitude and duration of cellular processes [37]. Complex patterns of regulation could also result from prolyl isomerization and PPIs modulating Cn.

The high sequence and structural similarities between the catalytic domains of PPPs suggest that the alternative trans- cis- conformations associated with peptidyl isomerization of the proline from the conserved SAPNY sequence observed for CNA can be common to most PPPs. In particular, interactions at the RVxF binding site of PP1 could present a similar feature to the one proposed here for the PxIxIT binding site of CN. For PPPs an increased structural versatility can help to explain how they can achieve specificity towards a large number of substrates, besides other established mechanisms [1, 4, 24]. It also provides a new vista for the
development of therapeutic drugs with specificity directed towards one or the other of the two alternative conformers.

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Author Contributions

Conceived and designed the experiments: IF MPR. Performed the experiments: AG AAI MCM RPL ESC DA SMH. Analyzed the data: AG IF. Wrote the paper: IF MPR. Reviewed the manuscript: AG ÁAI RPL DA SMH MCM ESC MPR IF.

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