Crystal Structure of Calcineurin-Cyclophilin-Cyclosporin Shows Common but Distinct Recognition of Immunophilin-Drug Complexes

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Introduction

Calcineurin (CN) is a unique serine/threonine protein phosphatase (PP3 or PP2B) because it is regulated by the second messenger Ca^{2+} and calmodulin. It is involved in many biological processes, including immune responses [1-5], the second messenger cAMP pathway [3, 6], Na/K ion transport in nephron [7], cell cycle regression in lower eukaryotes [8], cardiac hypertrophy [9], and memory formation [10, 11]. Calcineurin has been shown to be a common receptor for two immunophilin-immunosuppressant complexes: cyclophilin A-cyclosporin A (CyPA-CsA) and FKBP-FK506 [12, 13]. The binding of CyPA-CsA or FKBP-FK506 inhibits the calcium-dependent dephosphorylation of the transcription factor nuclear factor of activated T cell (NFAT), thus blocking T cell receptor-mediated cytokine transcription and T cell activation [14, 15]. However, we do not know how two structurally distinct immunophilin-immunosuppressant complexes recognize the common target. We report here on the crystal structure of CyPA-CsA-CN at a resolution of 2.8 Å and provide a structural comparison with FKBP-FK506-CN [16, 17].

Methods

The catalytic subunit (CNA, 61 kDa) and the regulatory subunit (CNB, 19 kDa) of the α isoform of human CN were co-expressed in *E coli* and purified by using a slightly modified version of a previously described procedure [18]. The tetragonal crystals of CyPA-CsA-CN were grown by the hanging drop method against a well buffer containing 0.1 M Na cacodylate (pH 6.5), 0.2 M $MgCl_2$, 13% PEG8000, and 2.5% ethanol. The protein drop was made up of protein and well buffer in a 2:1 ratio. The CyPA-CsA-CN crystals have the space group $P4_12_12$ with cell dimensions of a = b = 108.7 Å and c = 316.6 Å. The diffraction data for the CyPA-CsA-CN crystal were collected on the 14-C beamline of the Bio Consortium for Advanced Radiation Sources (BioCARS) at the APS at 100K and processed with HKL software.

The CyPA-CsA-CN structure was solved by the molecular replacement program AMoRe, which used individual subunits of CyPA [19], CNA, and CNB from the structure of FKBP-FK506-CN [16, 17]. Two solutions from the rotation and translation search yielded an R-factor of 0.44 and a correlation coefficient of 0.45 for 14,453 reflections at resolutions between 8 and 4 Å. The atomic model was rebuilt by program O [20] and refined by CNS [21] (Table 1).

Table 1. Statistics nf diffraction data and structure refinement of CyPA-CsA-CN.

Data collected	
Space group	$P4_12_12$
Unit cell (Å)	a = b = 108.7, c = 316.6
Total no. of measurements	183,803
No. of unique reflections	43,551
R-merge	0.136 (0.326)[a]
Resolution (Å)	2.8
Completeness (%)	90.9 (62.9)[a]
<I/sigma>	7.1 (1.5)[a]
Structure refinement	
R-factor	0.260
R-free	0.322
No. of reflections	42,377
Resolution (Å)	50 to 2.8
rms deviation	
Bond length (Å)	0.013
Angle (°)	1.67
Average B-factor (Å^2)	64.3 (all atoms)
CNA	60.6
CNB	84.6
CyPA	54.6
CsA	34.8
Zn-Fe	48.6
Ca	85.4

[a]The numbers in parentheses are the statistics for the shell at a resolution of 2.8-2.9 Å.

Results

Architecture of the CyPA-CsA-CN Structure

The CyPA-CsA-CN structure includes full-length CyPA (residues 1-165), CsA (residues 1-11), truncated CNA (residues 1-372), and CNB (residues 10-169). The truncated CNA subunit contains a catalytic domain and the CNB binding helix (BBH) (Fig. 1). The catalytic domain consists of a central β-sheet flanked by α-helices and has folding that is similar to that of other serine/threonine protein phosphatases, such as PP1 and PP2A [22]. The active site metal ions were assumed to be Fe^{3+} and Zn^{2+} without any further verification being conducted because of the resolution limit. The BBH of CNA embeds in CNB and, together with CNB, forms a composite surface for binding of CyPA-CsA as well as FKBP-FK506. CNB comprises two domains, each of which has two calcium-binding sites with a folding pattern similar to that of calmodulin. Cyclophilin A in the CyPA-CsA-CN complex is an eight-stranded β-barrel with the same folding as that of the unligated CyPA (Fig. 1).

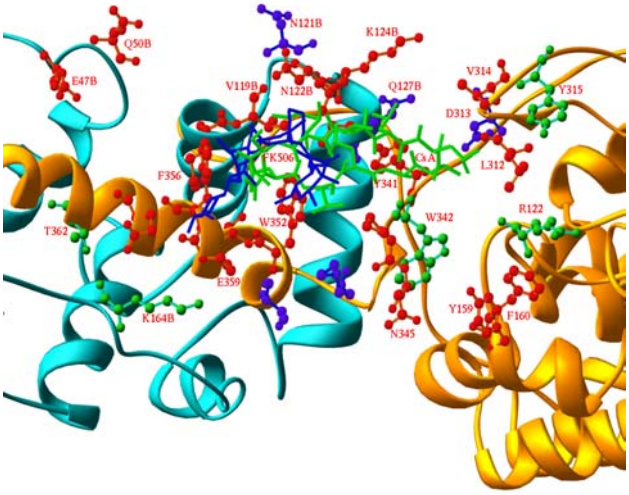
Common and Distinct Recognition Elements of CN for CyPA-CsA and FKBP-FK506

The conformations of CyPA, CsA, CNA, and CNB in the CyPA-CsA-CN complex are similar to the corresponding subunits in the CyPA-CsA binary complex [19, 23], unbound CN [17], and the FKBP-FK506-CN complex [16, 17]. A comparison of CyPA-CsA-CN with FKBP-FK506-CN revealed that both CyPA-CsA and FKBP-FK506 bind to the same region of CN and share major common recognition elements (Fig. 2). Trp352 of CNA is a key residue commonly recognized by both immunosuppressive drugs, since its Ne2 forms a hydrogen bond with the backbone carbonyl oxygen of Val5 of CsA in CyPA-CsA-CN or with O7 and O8 of FK506 in FKBP-FK506-CN. In addition, Trp352 and Phe356 of CNA and Met118 and Val119 of CNB make multiple hydrophobic contacts with residues 2-6 of CsA or a group of atoms of FK506 (Fig. 2). At the protein level, Oe1 of Glu47, Ne2 of Gln50, and Nd2 of Asn122 of CNB form hydrogen bonds with O of Gly80, Oe2 of Glu81, and O of Ala103 of CyPA, respectively. These three residues also form hydrogen bonds with Gln53, Glu54, and Lys47 of FKBP. For the common van der Waals' interactions, residues Tyr159, Phe160, Leu312, Val314, Asn345, and Pro355 of CNA contact with Pro50, Arg148, Ser147, Trp121, Asn149, and Thr73 of CyPA, or with Asp32, Lys34, Lys35, Gly89, and Pro88 of FKBP [16, 17].

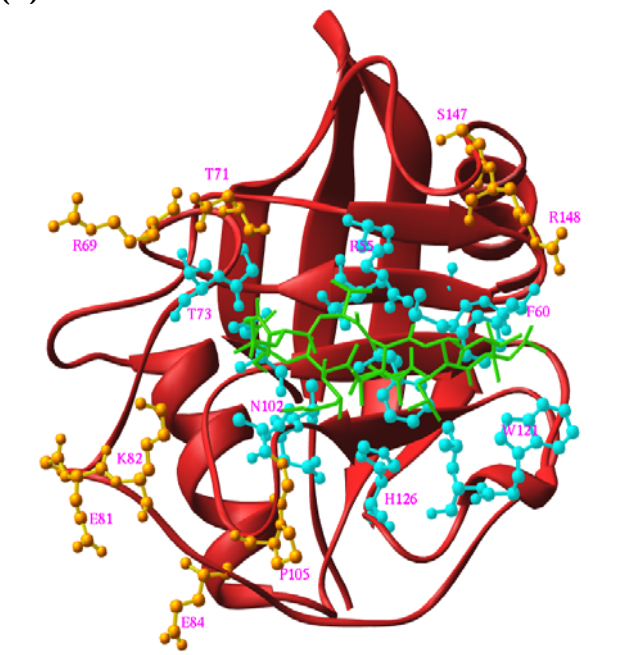
FIG. 1. Ribbon presentation of CyPA-CsA-CN. CNA and CsA are green; CNB is cyan; CyPA, Zn^{2+}, and Fe^{3+} are red; and calcium is blue. The pink balls are CN residues involved in the binding of CsA. The cyan balls indicate CyPA binding. Red balls represent Zn^{2+} and Fe^{3+}, and blue balls represent calcium.

On the other hand, a significant number of CN residues interacting with CyPA-CsA differ from those interacting with FKBP-FK506. First, the pattern of the hydrogen bonds is different between CyPA-CsA-CN and FKBP-FK506-CN. Thus, five hydrogen bonds were observed to be unique to CyPA-CsA-CN: Arg122 (CNA)-Arg148 (CyPA), Tyr341 (CNA)-Ala7 (CsA), Glu359 (CNA)-Arg69 (CyPA), Gln50 (CNB)-Lys82 (CyPA), and Lys164 (CNB)-Asn71 (CyPA). In comparison, four hydrogen bonds are unique to FKBP-FK506-CN: Tyr159 (CNA)-Asp32 (FKBP), Leu312 (CNA)-Lys35 (FKBP), Asn121 (CNB)-Lys44 (FKBP), and Gln127 (CNB)-Arg42 (FKBP) [16, 17]. Particularly interesting is the hydrogen bond between Arg148 of CyPA and the active site residue Arg122 of CNA, which may force reorientation of the side chain of Arg122, thus directly affecting the catalytic activity of CN. In contrast, the nearest residue from FKBP, Lys34, situates more than 5 Å away from Arg122, too far to exert the same effect as Arg148 of CyPA. In addition, a group of CN residues involved in van der Waals' interactions with CyPA-CsA are distinct from those interacting with FKBP-FK506. Thus, Arg122, Tyr315, Trp342, and Thr362 of CNA and Lys164 of CNB are unique in forming van der Waals' interactions with CyPA-CsA, while Asp313, Met347, and Thr351 of CNA and Asn121 and Gln127 of CNB are unique in contacting FKBP-FK506.

In summary, among the 25 CN residues involved in hydrogen bond or hydrophobic interactions, 20 are common to CyPA-CsA and FKBP-FK506, while 5 are unique to CyPA-CsA (Arg122, Tyr315, Trp342, Thr362 of CAN and Lys164 of CNB) or FKBP-FK506 (Asp313, Met347, and Thr351 of CAN and Asn121 and Gln127 of



(A)



(B)

FIG. 2. (A) CN Interface. CNA is shown as a gold ribbon, and CNB is shown as a cyan ribbon. A total of 23 CN residues are involved in interaction with CyPA-CsA: Arg122, Tyr159, Phe160, Leu312, Val314, Tyr315, Tyr341, Trp342, Pro344, Asn345, Trp352, Ser353, Pro355, Phe356, and Glu359 of CNA and Glu47, Gln50, Met118, Val119, Asn122, Leu123, Lys124, and Lys164 of CNB. Red balls represent residues for binding of both CyPA-CsA and FKBP-FK506. Green and blue sticks are CsA and FK506. Green balls are residues interacting with CyPA-CsA, and blue balls are residues interacting with FKBP-FK506. (B) CyPA interface. Gold balls are residues interacting with CN, and cyan balls are residues interacting with CsA (green sticks).

CNB). Although the majority of the CN residues are common with regard to binding the two structurally distinct immunophilin-drug complexes, the patterns of recognition are dramatically different. Of the nine hydrogen bonds between CyPA-CsA and CN, only four are common to FKBP-FK506. Especially notable is the fact that Tyr341 forms a hydrogen bond with CsA but a van der Waals interaction with Arg42 in FKBP. This recognition diversity of the composite surface may indicate its capacity for binding a variety of protein substrates.

CNA-CNB Composite Surface May Be a Recognition Site That Defines the Narrow Substrate Specificity of CN

Calcineurin has relatively narrow substrate specificity in comparison to other protein phosphotases. The molecular basis of the substrate specificity of CN has remained largely unknown. We have shown that the structurally distinct complexes of CyPA-CsA and FKBP-FK506 share their binding sites on the composite surface formed by CNA and CNB. On the basis of the observation that CyPA-CsA simultaneously interacts with the CNA-CNB composite surface and the active site residue, we speculate that the CNA-CNB composite surface may serve as a general substrate recognition site, defining the substrate specificity of CN. The nearly orthogonal extension of the composite surface from one end of the catalytic domain forms an L-shaped clamp, restricting the binding of phosphoprotein substrates with a matchable shape and size. Inherent in this model is the existence of a separate substrate binding domain from the active site of CN, as observed in the case of NFAT [24]. This model is also consistent with the fact that BBH of CN represents a unique C-terminal extension of the phosphatase catalytic domain of CN among protein serine/threonine phosphotases. The molecular recognition of two structurally distinct immunophilin-drug complexes by the same composite surface of CN is reminiscent of the complex between the human growth hormone and the homodimer of its receptor, in which two identical receptor subunits employed two largely distinct sets of residues to bind two different surfaces of the growth hormone [25].

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