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1.88 Å crystal structure of the C domain of hCyP33: A novel domain of peptidyl-prolyl *cis–trans* isomerase

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Abstract

Cyclophilins (CyPs) are a widespreading protein family in living organisms and possess the activity of peptidyl-prolyl *cis–trans* isomerase (PPIase), which is inhibited by cyclosporin A (CsA). The human nuclear cyclophilin (hCyP33) is the first protein which was found to contain two RNA binding domains at the amino-terminus and a PPIase domain at the carboxyl-terminus. We isolated the hCyP33 gene from the human hematopoietic stem/progenitor cells and expressed it in *Escherichia coli*, and determined the crystal structure of the C domain of hCyP33 at 1.88 Å resolution. The core structure is a β-barrel covered by two α-helices. Superposition of the structure of the C domain of hCyP33 with the structure of CypA suggests that the C domain contains PPIase active site which binds to CsA. Furthermore, C domain seems to be able to bind with the Gag-encoded capsid (CA) of HIV-1 and may affect the viral replication of HIV-1. A key residue of the active site is changed from Ala-103-CypA to Ser-239-hCyP33, which may affect the PPIase domain/substrates interactions.

Keywords: Human nuclear cyclophilin; Cyclophilin A; Peptidyl-prolyl *cis–trans* isomerase; Cyclosporin A

Among peptide bonds in proteins, rotation of prolyl imide bond is the slowest and is thought to be the rate-limiting step in the folding process of proteins. An enzyme which catalyses this slow rotation is called peptidyl prolyl *cis–trans* isomerase (PPIase) [1,2]. There are three PPIases families: cyclophilin (CyP), FK506-binding protein (FKBP), and parvulin (Pvn) [1,2]. Cyclophilins (CyPs) bind to the immunosuppressive drug cyclosporine A (CsA) and are widespread in prokaryotes and eukaryotes at different subcellular locations [3,4]. CyPs play important roles in living organisms and possess the PPIase activity, which accelerate protein folding [1,2,5,6], and CyP–CsA complexes act as inhibitors for signal transduction pathways in the immunological reactions [7,8]. CypA is one of the well-studied cyclophilins which possess PPIase activity and is inhibited by CsA [1–4]. CypA performs an essential role in HIV-1 replication and is affected by CsA. The formation of CA–CypA complex can be competitively inhibited by CsA's binding to the active site of CypA [9–12]. Although some of the functions of CypA have been largely known, the mechanisms of these functions are still elusive.

Human nuclear cyclophilin (hCyP33) (GenBank Accession No.: NM_006112) contains two RNA-binding domains at the amino-terminus and a PPIase domain at the carboxyl-terminus (C domain). The C domain of hCyP33 is homologous to CypA in amino acid sequence [13]. We isolated the hCyP33 gene from the hematopoietic stem/progenitor cells and expressed it in *Escherichia coli*. We also purified and crystallized hCyP33 protein. Molecular weight of the protein in

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the crystal is 18.2 kDa measured by MALDI-TOF-MS. The protein in the crystal is the C domain of hCyP33, which has been proved by its crystal structure.

After measuring the molecular weight by MALDI-TOF-MS, we determined the crystal structure of the C domain at 1.88 Å resolution by molecular replacement. We have deposited the structure of the C domain in PDB bank as 1ZMF. Comparing with CypA, we predict that C domain has PPIase activity and CsA-binding site, and C domain could also interact with CA of HIV-1. Further studies on the C domain may explain the interactions of CyPs–CA in detail and give clues to targets finding in drug design.

Materials and methods

Cloning, expression, and purification. Cloning of the gene encoding hCyP33 was carried out by the Shanghai Institute of Hematology. The hCyP33 gene was cloned into pET22b (+) (Novagen) between the restriction sites *Nde*I and *Xho*I, but it was expressed as an insoluble protein. We, then, cloned this gene into PQE30 (Qiagen) between the restriction sites *Bam*HI and *Hin*dIII, and got soluble expression of it. To the protein expressed by the vector PQE30-hCyP33, added a His_6-tag to the N-terminus. This construction was confirmed by DNA sequencing. hCyP33 was expressed in *E. coli* strain M15 (Qiagen) after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h at 30 °C. The cells were harvested by centrifugation at 5000 rpm for 10 min and were lysed by ultrasonic cell disrupter. The soluble fraction was loaded on a 5 ml Chelating Sepharose Fast Flow resin column preloaded with $NiSO_4$ and pre-equilibrated in 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 2 mM β-mercaptoethanol. After three washes with increasing concentrations of imidazole the protein was eluted with 150 mM imidazole. Proteins eluted from the resin were further purified by ion-exchange chromatography on Resource Q, hydrophobic interaction chromatography on Resource ISO, and size exclusion chromatography on Superdex 200. The molecular weight of the purified hCyP33 protein is about 34 kDa measured by SDS–PAGE.

Crystallization and data collection. The crystals were obtained by the hanging-drop, vapor diffusion method. Screening experiments were performed with commercial Screen kits from Hampton Research, microcrystals were obtained in 0.1 M Hepes (pH 7.5) and 25% w/v PEG 3350. The sample of hCyP33 was presented at a concentration of about 15 mg/ml in a storage buffer consisting of 10 mM Tris–HCl (pH 8.0), 10 mM NaCl, and 2 mM β-mercaptoethanol. The well solution contains 0.1 M Hepes (pH 7.5) and 20% (w/v) PEG 3350. We got crystals by mixing protein solution and well solution in 1:1 ratio at 20 °C within 2 weeks. Molecular weight of the protein in the crystal is 18.2 kDa measured by MALDI-TOF-MS.

Diffraction data were collected at Mar345 with seal-tube X-ray source in the National Key Laboratory of Biomacromolecules, Institute of Biophysics, CAS. Diffraction data were scaled and integrated with DENZO and SCALEPACK [14]. The results showed that the crystal belongs to space group $P4_22_12$, with one molecule in the crystallographic asymmetric unit. The diffraction data statistics are summarized in Table 1.

Structure determination and refinement. The structure was solved by molecular replacement with MolRep [15] in the CCP4 suite [16]. The search model was the structure of CypA (PDB entry 1AWV), which displays 67% sequence similarity to the C domain of hCyP33. The initial model was manually rebuilt with the program O [17] and refined by ARP/wARP [18] and refmac5 [19]. After several cycles of refinement, final model was obtained. A summary of the refinement statistics is given in Table 1.

Table 1
Crystal data, data collection, and refinement statistics

Space group	$P4_22_12$
Unit cell parameters (Å)	
a	66.61
b	66.61
c	76.78
Molecules (a.u.)	1
Resolution range (Å)	47.10–1.88
Unique reflections	14,600
Average redundancy	13.6
Completeness	99.9
R_{merge} (%)	0.082 [0.39]
$I/\sigma(I)$	37.1 [4.5]
Non-hydrogen atoms	1391
Amino-acid residues	162
Water molecules	142
R_{work} (%)	16.9%
R_{free} (%)	22.5%
Average *B*-values (Å^2) for main chain/ side chain/water	15.4/18.6/27.9

Results and discussion

Overall structure of the C domain of hCyP33

The PPIase domain of hCyP33 has 165 residues. The model displays the structure of hCyP33 from Asn-139 to Tyr-300 and is shown in Fig. 1. In this model, two amino acid residues at the N-terminus and one at the C-terminus are not included for their poor electron density. The core structure is a β-barrel similar to those of CyPs. The eight β-strands are arranged in an anti-parallel fashion around the barrel surface and two α-helices sit on the top and the bottom of the barrel, respectively. The β-turns and loops in the strands are probably responsible for the flexibility of the barrel. This β-barrel motif

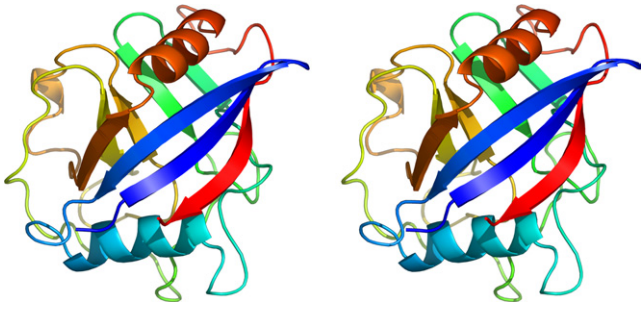

Fig. 1. The stereo view of the structure of the C domain of hCyP33. Red: N-terminus of the PPIase domain of hCyP33; blue: C-terminus of the PPIase domain of hCyP33. The core structure is displayed as a β-barrel formed by eight β-strands and the β-barrel is covered by two α-helices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

is highly conserved in CyPs and has a conserved hydrophobic core [20]. The conformation of the PPIase domain of hCyP33 is very similar to CypA (the r.m.s.d. of whole structure is 0.58 Å). The C domain of hCyP33 is highly conserved in key residues among the CyPs family. In the structure of the C domain of hCyP33, the loop of Gly-149-Asn-150-Lys-151-Pro-152 (compared with CypA, the r.m.s.d. is 1.96 Å) and the loop of Tyr-215-Gly-216-Lys-217-Lys-218 (compared with CypA, the r.m.s.d. is 1.5 Å) have remarkable difference with CypA (Fig. 2).

The PPIase active sites of the C domain of hCyP33

The PPIase domain of hCyP33 possesses the activity of peptidyl-prolyl *cis–trans* isomerase (PPIase) [13]. The PPIase active sites of the C domain of hCyP33 can be predicted by comparing with CypA in amino acid sequence and structure. Residues Arg-191, Phe-196, Met-197, Gln-199, Ala-237, Asn-238, Phe-249, Leu-258, and His-262 of the C domain are involved in hydrogen bonds formation or van der Wall interactions with its substrates. The side chains of Phe-196, Met-197, Phe-249, Leu-258, and His-262 in the C domain make up the hydrophobic pocket (Fig. 3), to which the proline residue of the substrates bind [21]. The residues Arg191 and Asn238 form hydrogen bonds with proline residue in the substrates. The underlying mechanism is unclear, and the structure of the C domain–substrate complex will give some insights into it.

The PPIase activity of the C domain of hCyP33 can be inhibited by CsA [13], and the CypA–CsA complex acts as inhibitor in the immunological reactions by binding to protein phosphatase calcineulin [7,8]. The superposition of the C domain and CypA–CsA complex suggests that the CsA-binding sites of the C domain are in fact its PPIase activity site. Those residues contributing to the CsA binding are similar to CypA [22–24], including Arg-191, Phe-196, Met-197, Gln-199, Gly-208, Ala-237, Asn-238, Ser-239, Gln-247, Phe-249, Trp-257, Leu-258, and His-262. The mutation of Trp-121 of CypA leads to dramatically decreased sensitivity to CsA [25]. So we predict that the Trp-257 of the C domain of hCyP33 plays a similar role in recognizing CsA.

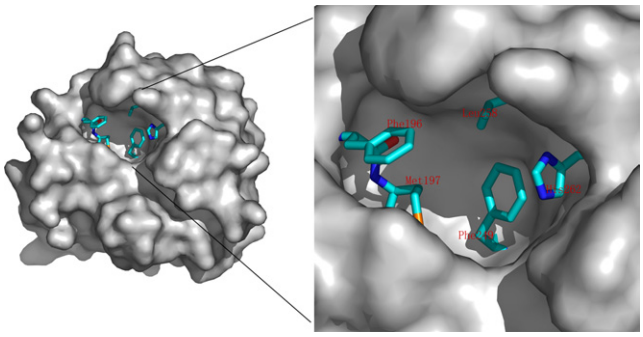

Fig. 3. The key residues in the hydrophobic pocket. Phe-196, Met-197, Phe-249, Leu-258, and His-262 form the hydrophobic pocket which is responsible for the activity of PPIase.

Analysis of the key residues and the binding site

Arg-55, Asn-102, and Ala-103 of CypA are the key residues for the interactions with the substrates and these interactions are affected by the neighboring residues [26]. Alignment of the amino acid sequence of the C domain of hCyP33 with CypA indicates that two of the three key residues (Arg and Asn) are the same, but Ala-103 in CypA is changed to Ser-239 in hCyP33 (Fig. 4). Comparing with the structure of CypA, Arg-191, and Asn-238 of the C domain sit in the same sites and adopt similar conformations. We predict that these three residues of the C domain play an important role as in CypA in the interactions with the substrates, and these interactions may also be affected by the neighboring residues. The movements of the neighboring

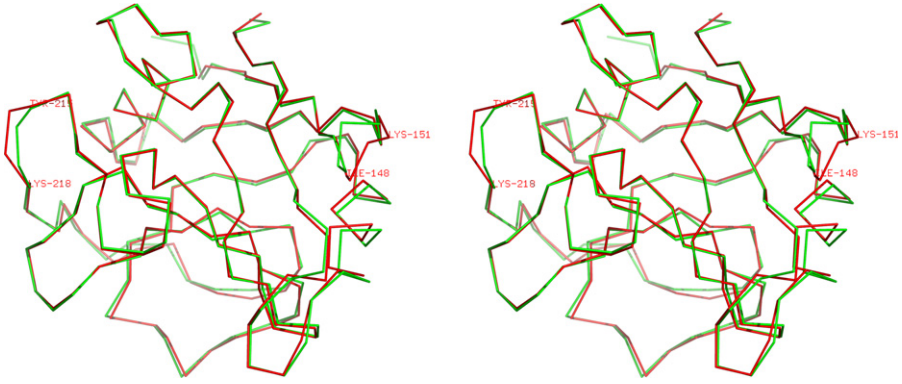

Fig. 2. The stereo view of two distinctive loops of the C domain of hCyP33. Red: the C domain of hCyP33; green: CypA (PDB:2RMA). Compared with CypA, the loop of G149-P152 and Y215-K218 in the structure of the C domain is different with high r.m.s.d. value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

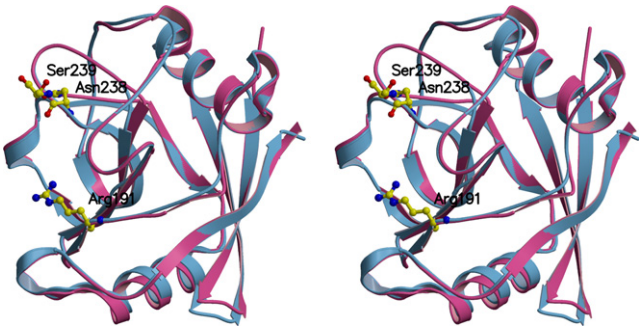

Fig. 4. Superposition of the C domain of hCyP33 with CypA (stereo view). Cyan: the C domain of hCyP33; purple: the CypA (PDB:2RMA, CsA not shown). Arg-191 and Asn-238 are the key residues for the hydrogen bond formation with the substrates. The Ala-103 in CypA is changed to Ser-239 in hCyP33. Asn-238 and Ser-239 are located in a loop region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

backbone will affect the enzymatic reaction. So the displacement of Ala with Ser may alter the PPIase/substrates interactions and influence the nature of the key residue Asn-238. The conformational fluctuation of this region where the three key residues lie may change the C domain/substrates interactions and therefore affect the PPIase activity and the sensitivity to CsA.

CypA binds specifically to proline-rich domain of the capsid (CA) of HIV-1 and is packaged into the viron. CypA has an essential function in HIV-1 replication and is inhibited by CsA's binding to the active site of it [10,12,27]. Comparison of the structure of the C domain of hCyP33 with the structure of CypA–CA complex indicates that the C domain adopts a very similar conformation with CypA. We propose that the C domain could also interact with CA and may recognize the main binding sequence (His-87-Ala-Gly-Pro-Ile-Ala-92) of CA [28]. The superposition of the structure of the C domain with the structure of CypA–CA complex also suggests that the C domain of hCyP33 may affects the viral replication of HIV-1, and the C domain–CA complex may have the similar function in HIV-1 replication and can be inhibited by CsA. Although some studies have revealed that CypA plays an important role in the HIV-1 infection cycle, the precise function of CypA is unclear. Further studies on the structure of the C domain–CA complex may give insights into the mechanism of the interactions between CyPs and CA and provide clues to targets searching for drug design.

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