Effect of different immunosuppressive drugs on calcineurin and its mutants

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Abstract Several mutants in Loop7 region and near Loop7 region of calcineurin A (CN A) subunit have been constructed and purified using site-directed mutagenesis. Their phosphatase activity and the corresponding solution conformation were examined. Their phosphatase activities between wild-type CN and mutants were compared to identify the interaction of different immunosuppressive drugs with CN. The results showed that the phosphatase activities of the mutants at Loop7 were much higher than the one of wild-type CN. Furthermore, circular dichroism spectra of the mutants revealed that their solution conformations gave rise in changes in native structure of the protein. Cyclophilin-CyclosporinA (CyP-CsA) significantly inhibited the phosphatase activity of wild-type CN, and had no effects on the phosphatase activity of mutants in Loop7 region, which indicates that the site-directed mutagenesis at Loop7 region made a significant change in the interaction between CyP-CsA and CN. Examination of the activities of these mutants resulted in the presence of immunosuppressive component from traditional Chinese drugs. The component of Chinese drug, ZIP1, could directly inhibit both CN and CN mutants without drug binding protein. These results suggest that the Loop7 region is an important structural area involved in the inhibition by CyP-CsA. It is valuable to further study the inhibition by ZIP1.

Keywords: calcineurin, site-directed mutagenesis, immunosuppressive drugs, circular dichroism spectrum.

The immunosuppressants CsA and FK506 have highly similar biological properties. These drugs exert their major therapeutic effects by inhibiting T-cell activation. A wealth of biochemical data[1—4] has been demonstrated that these immunophilin-immunosuppresant complexes suppress T cell activation by binding to CN and inhibiting its phosphatase activity. CN inhibition by FKBP12-FK506 or by CyP-CsA correlates with their ability to inhibit IL-2 production in T cells, a critical step in T-cell proliferation.

CN, also known as protein phosphatase 2B, is a serine/threonine protein phosphatase whose activity is regulated by Ca^{2+}/Calmodulin. CN is a heterodimer consisting of a 61-ku catalytic subunit, CN A, and a 19-ku regulatory subunit, CN B[5—7]. CNA is the catalytic and regulatory core which include 4 domains: catalytic domain, CN B binding domain, calmodulin binding domain and autoinhibitory domain. Loop7 region belongs to catalytic domain of A subunit (the sequence numbers are 307—319) and its tertiary structure is near the active site of the enzyme. CN plays an

important role in the Ca^{2+} signaling pathway, especially as a critical signaling enzyme in the processes of T lymphocytes activation. CN has been implicated in the nuclear transport of cytoplasmic transcription factors, primarily NF-AT (the nuclear factor for activated T cells) in the processes of interleukin 2 activation[3—4]. NF-AT consists of an AP1 nuclear component, composed of Jun and Fos, and a pre-existing cytoplasmic component (NF-ATp). The nuclear translocation of NF-ATp requires for the activation of T lymphocytes[8,9]. Increases in intracellular calcium induce the NF-ATp dephosphorylation by the activated CN, the nuclear appearance of NF-ATp DNA binding activity, and the translocation of NF-ATp to the nucleus, where NF-ATp interacts with the AP1 elements Fos and Jun and binds to its cognate DNA-binding site, i.e. IL-2 enhancer, thereby starting IL-2 gene transcription (CN binds phosphated or dephosphated NF-AT[10]). IL-2 gene expression is an essential factor for most of T cells proliferation and differentiation. NF-AT is one of several transcription factors which require for T cell specific expression, IL-2 gene activation and protein synthesis. It is clear that CN could regulate the phosphorylation level of NF-AT, thereby regulating the translocation of the cytosolic component of NF-AT to the nucleus[11]. The study on the crystal structure[12] of a tertiary complex containing CN, FKBP12 and FK506 addresses much of the speculation that has surrounded the critical event in immunosuppression. FKBP12-FK506 and CyP-CsA appear to block the nuclear translocation and have the effects of immunosuppression drugs by inhibiting CN phosphatase activity. In the structure, the FKBP12-FK506 binary complex does not contact the phosphatase active site on CNA. The loop 7 of CN A is in close proximity to the FKBP12-FK506 complex in the tertiary structure. We aimed at loop7 region and near Loop7 region using mutagenesis to identify the interaction of immunosuppressant CyP-CsA with CN. In the meantime, effect of immunosuppressive component from Chinese drug, ZIP1, on CN was studied.

1 Materials and methods

1.1 Materials

Restriction endonuclease (*Nde* I, *Hind* III), primer oligonucleotide, T4 polynucleotide kinase, annealing 10×buffer, synthesis 10×buffer, T4 DNA ligase, T4 DNA polymerase were obtained from New England Biolabs; CsA, CyP, ampicillin (Amp.), Adenosine 5'-triphosphate, MOPS, $RbCl_2$, phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma; HEPES, RNaseA were purchased from Boehringer Mannheim Gmbh; R II peptide (DLDVDIPGRFDRRVSVAAE) was obtained from BioMoL Lab; PO [$OC(C_6H_5)$ = NCH = CC_6H_5] and POPOP {[$OC(C_6H_5)$= CHN = $C]_2C_6H_4$} were obtained from E. Merck; Dowex AG1-X8 and AG50W-X8 were purchased from Bio-Rad. All other reagents were of standard laboratory grade and the highest quality available from commercial suppliers domestically. Calmodulin, CN B subunit and anti-CN polyclonalantibody were purified and prepared in our lab. The specific activity of the [r-^{32}P] TAP was >5 000 Ci/mmol.

1.2 Site-directed mutagenesis

The rat brain δ CN A cDNA (1.5 kb) was cloned into the pET21a vector and expressed in *E. coli* strain HMS 174(DE3). The rat δ protein sequence consists of 511 residues. Site-directed mutagenesis was performed with the Altered Sites *in vitro* mutagenesis system (Promega). Primers used for each specific mutation were as follows: V314 (deletion V at 314): 5' CCA AAT TAC TTA GAT TAC AAT AAT AAA GC 3'; V314Y315 (deletion V at 314 and Y at 315): 5' CCA AAT TAC TTA GAT AAT AAT AAA GCT GC 3'; YRG (D313Y, V314R, deletion Y at 315, N316G): 5' CC GCA CCA AAT TAC TTA TAT CGG AAT AAA GCT GCA GTG TTG AAG TACG 3'; Y311F: 5' CGG CAC CAA ATT TCT TAG ATG TGT AC 3'; P300 (deletion P at 300): 5' GCC AAA CAA CTG GCT TCT CTC TAA TTA CG 3'. Mutagenesis procedure involves annealing of the ampicillin repair oligonucleotide and the 5'-phosphorylated mutagenic oligonucleotide to the ssDNA template, followed by synthesis of the mutant strand with T4 DNA polymerase. The heteroduplex DNA is then transformed into the repair minus *E. coli* strain BMH 71-18 mut S. Mutants are selected by overnight growth in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109 ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants. The sequences of the mutanted cDNAs were confirmed by dideoxynucleotide termination DNA sequencing and cloned into the expression vector pET-21a and expressed in *E. coli* strain HMS174(DE3).

1.3 Expression and isolation of the mutants

The wild-type and mutant cDNAs in the pET21a vector were used to transform *E. coli* HMS174(DE3) cells. One liter cultures were grown as described by Wei et al.[13]. Isolation and purification of protein were performed by the procedure of Wei et al.[13] with the exception of using ultrasonication instead of a French press.

1.4 Electrophoresis and Western blotting

SDS-PAGE was performed using 12% acrylamide gels. Western blotting was performed using a mouse polycolonal antibody against CN. Bound antibody was detected using a biotin labelled second antibody and an avidin-labelled horseradish peroxidase third antibody. Protein determination was performed by the procedure of Bradford[14].

1.5 Assay of phosphatase activity

R II peptide was labelled with [r-^{32}P] ATP as described by Perrino et al.[15]. CN A and CN B were 1 : 1 mol/mol. Assays were performed in a volume of 20 μL at 30℃ for 10 min and terminated by the addition of 0.18 mL of 75 mmol/L phosphoric acid . For the assay, the reaction mixture contained 50 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L $MnCl_2$, 0.5 mmol/L dithiothreitol, 0.1 mg/mL bovine serum albumin, and 50 μmol/L phosphorylated R II peptide. The amount of ^{32}P released was determined by applying each sample to 0.5 mL of Dowex AG50W-X8, collecting the flow-through and washing with 1 mL water, and counting in a liquid scintillation counter. Units of

activity were defined as nanomoles of [^{32}P]Pi released per minute per milligram enzyme. Calmodulin and B-subunit added were 0.3 μmol/L and 1 ∶ 1 mol/mol, respectively. CsA and CyP added were 20 μmol/L and 1 μmol/L, respectively. The immunosuppressive component from traditional Chinese drug ZIP1 added was 50 μg/mL.

1. 6 Circular dichroism spectroscopy

The enzymes were prepared to a final concentration of 0.4 mg/mL. CD spectra were recorded with a Jasco Spectropolarimeter J-700 using 1 mm spectral path length.

2 Results

2. 1 Construction of CN A mutants

δ CN A mutants were constructed using the altered sites *in vitro* mutagenesis system. The mutated cDNAs were cloned into the expression vector pET-21a and transformed in *E. coli* strain HMS174(DE3). The sequencing of the mutated cDNAs was identified by DNA sequencing and resulted in the confirmation of expectant designing. The positive recombinant colonies were successfully demonstrated.

2. 2 Expression, isolation and characterization of mutants

The expression of the rat brain δ CN A mutants was finished, then the cells were disrupted using ultrasonication. An SDS-PAGE gel stained for the wild-type CN A and the mutants showed the bands at the positions expressed for their estimated molecular masses of 60 ku. All the mutants made in this study were found to be expressed similar to the wild-type enzyme, and all were purified to near-homogeneity (fig. 1). Yields of the purified mutants ranged from 10 to 20 mg per liter of culture. Expression of the mutants made in this study was monitored by Western blot analysis and these mutants migrated at the positions of 60 ku similar to the pattern of PAGE gel (fig. 2).

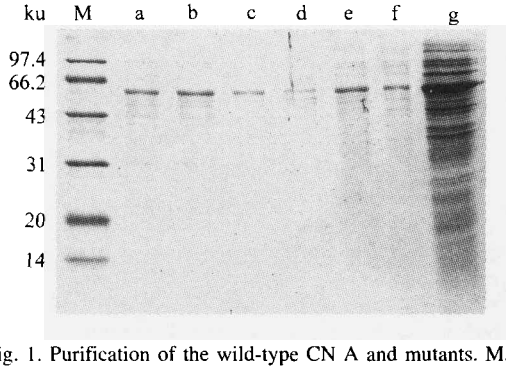

Fig. 1. Purification of the wild-type CN A and mutants. M, Protein marker; a, V314; b, V314Y315; c,Y311F; d, P300; e, wild-type δ- CN A; f, YRG; g, lyzed *E. coli* cell containing pET-21a plasmid.

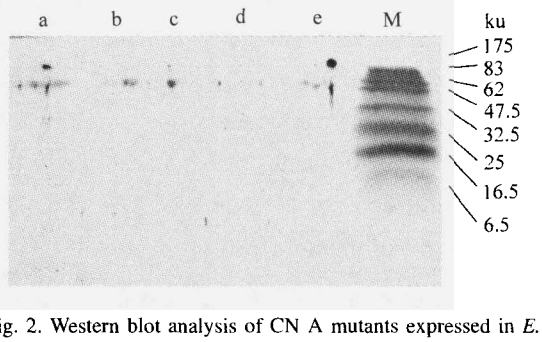

Fig. 2. Western blot analysis of CN A mutants expressed in *E. coli*. M, Marker; a, Y311F; b, P300; c,YRG; d, V314Y315; e, V314.

2.3 Effect of mutation on the phosphatase activities

When assayed with R II peptide as the substrate, a striking contrast between the wide-type

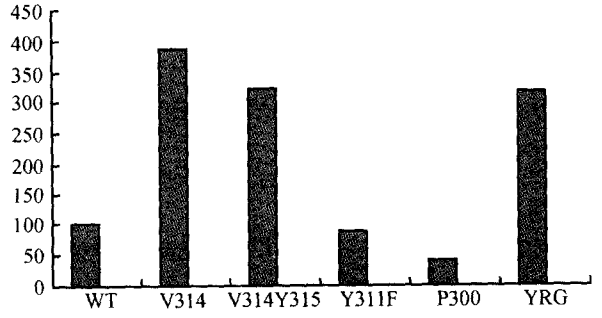

Fig. 3. Comparison of phosphatase activity between wild-type CN and mutants. The phosphatase activity for wild-type CN was defined 100 percent.

mutants, implying that the backbone conformation of mutants led to a significant change in the secondary structure of these proteins as the patterns of negative grooves showed measurable differences. The spectra of wild-type CN A and mutants measured in the far-UV CD and the near-UV CD region are overlaid in fig. 4.

2.5 Effect of different immunosuppressive drugs on the phosphatase activity of wild-type CN and mutants

CyP-CsA significantly inhibits the phosphatase activity of wild-type CN, and induces a slight increase or few change in the phosphatase activity of mutants. The direct-mutagenesis in Loop7 region makes a significant change in the interaction between CyP-CsA and CN. The immunosuppressive components from traditional Chinese drugs appear to inhibit significantly the phosphatase activity of wild-type CN and mutants except for mutant P300 shown in table 1.

CN A and the mutants presents in fig. 3. The phosphatase activities of mutants V314, V314Y315 and YRG were higher than that of the wild-type CN, and activities of mutants Y311F and P300 were lower than that of wild-type CN.

2.4 Determination of circular dichroism spectroscopy

The CD spectrum of wild-type CN A was very different from that of these

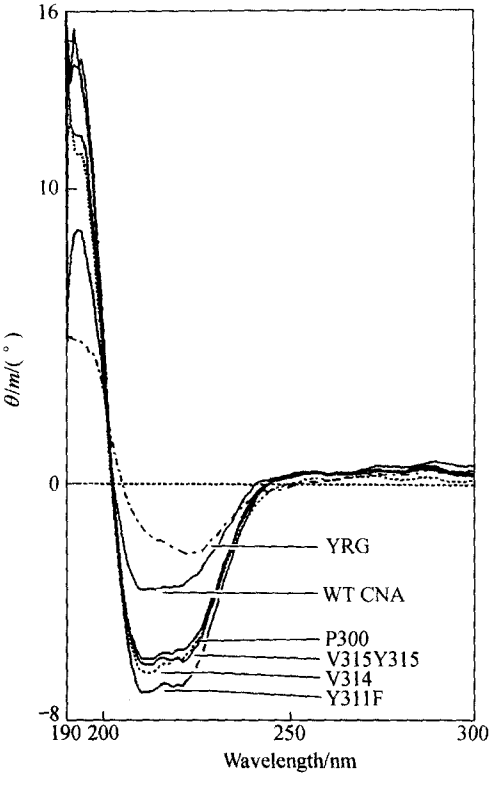

Fig. 4. Spectrophotometric analysis of wild-type CN A and mutants.

Table 1 Effect of different immunosuppressive drugs on specific phosphatase activities of CN or mutants

Variant	CN	CN+CsA-CyP	CN+ZIP1
WT	100	37	30
V314	385	406	108
V314Y315	320	345	151
Y311F	87	100	30
P300	42	46	42
YRG	323	392	69

Each experiment was performed three times. The phosphatase activity for wild-type CN was defined 100 percent.

3 Discussion

The tertiary structure of CN with FKBP12-FK506 complex showed that Loop7 region was near both active site of enzyme and FKBP12-FK506 complex. In this study of CN A, we showed that mutation at the Loop7 and near Loop7 has significant effects on CN phosphatase activity. The phosphatase activities of the mutants were assayed using R II peptide as the substrate in the presence of CN B, calmodulin and Mn^{2+}. The results showed that the activities of three of the mutants (V314, V314Y315, YRG) which are on one side of Loop7 near the active site of enzyme were more active than that of the wild-type enzyme, and the activities of two of the mutants (Y311F, P300) which are on the other side of Loop7 beyond the active site of enzyme were less active than that of the wild-type enzyme. Furthermore, CD spectra of the mutants revealed that their solution conformations gave rise to changes in native structure of the protein. It was a structural basis on interaction among the enzyme, substrate and immunosuppresants. The immunophilin-immunosuppresant CyP-CsA complex significantly inhibited the phosphatase activity of wild-type CN and had no effects on the phosphatase activity of mutants. The results suggested that the site-directed mutagenesis in Loop7 region made a significant change in the interaction between CyP-CsA and CN.

The crystal structures of the human CN-FKBP12-FK506 complex and the bovine brain CN-FKBP12-FK506 complex have revealed that FKBP12-FK506 contacted two distinct areas on CN[8,9]. The composite surface formed by FK506 and surrounding residues of FKBP12 contacts residues on the exposed side of the CN B-binding helix of CN A and surrounding residues from hydrophobic groove of CN B. The large site of interaction was called a primary recognition site. Additional interactions were called secondary recognition site between FKBP12 and the catalytic domain of CN A. In the structure, the FKBP12-FK506 binary complex does not contact the phosphatase active site on CN A and the corresponding FKBP12 residues were involved extensively in interaction with CN A that is more than 1 nm removed. The sites of binding of FKBP12-FK506 appear to be shared by other non-competitive inhibitors of CN, including CyP-CsA complex and an anchoring protein, AKAP79 (A-kinase anchoring protein79). CyP-CsA appears to bind at the same general location as FKBP12-FK506 and it may inhibit CN through a similar mechanism. Loop7 of CN A consists of 12 amino acid residues (307—318). At the secondary recognition sites, contact by FKBP12-FK506 and CN A contain part of residues at Loop7. CyP-CsA induced different levels of increases in the phosphatase activities of mutants when assayed with R II peptide as the substrate. These results suggested that Loop7 region, an important structural element involved in the inhibition by CyP-CsA, may participate in different conformational transitions of CN.

The classical immunosuppressants have a practical defect because of their high toxicity. The immunosuppressive components from traditional Chinese drugs were selected using the property of CN (unpublished data). ZIP1 has been purified, and has a more typical effect among the im-

munosuppressive drugs. We found that ZIP1 had a direct inhibition on CN without binding protein. ZIP1 also appears to inhibit both the wild-type CN and Loop7 region mutants constructed at present. The binding of ZIP1 and wild-type CN also appears to change the conformation of CN (data not shown). The results suggested that ZIP1 inhibited CN through a mechanism different from CyP-CsA. The ZIP1 molecule is much bigger than FK506 or CsA. ZIP1 may stereometrically prevent binding of peptide substrate at the CN A active site and results in inhibition. The mechanism of inhibition has not been elucidated.

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