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Function and Structure of N-Terminal and C-Terminal Domains of Calcineurin B Subunit

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Calcineurin (CN), a Ca^{2+}/calmodulin-dependent protein phosphatase, plays a critical role in T-cell activation by regulating the activity of NF-AT. CN is a heterodimer consisting of a catalytic subunit (CNA) and a Ca^{2+}-binding regulatory subunit (CNB). CNB is composed of two global domains: the C-terminal domain (DC) and the N-terminal domain (DN), each containing two Ca^{2+} binding sites. In this study, using purified DN and DC derived from constructed expression systems, we revealed that intact CNB and DC can stimulate the phosphatase activity of CNA, about 2.2 and 1.6 times the phosphatase activity of CNA alone, respectively; DN itself has little effect on the phosphatase activity of CNA. Fluorescence spectroscopy of an ANS-hydrophobic fluorescence probe shows that binding of Ca^{2+} to CNB, DC or DN leads to exposure of the hydrophobic surface of the proteins and that the hydrophobicity of CNB is the greatest, that of DC is less, and that of DN is the least. The hydrophobic surface of CNB may be an important structural basis for stimulating CN phosphatase activity.

Key words: Calcineurin / Domain / Gene expression / Mutation / Phosphatase activity / Protein purification.

Introduction

Calcineurin (CN), a calcium- and calmodulin-dependent protein serine/threonine phosphatase, plays a critical role in various cellular processes (Crabtree *et al.*, 1994). It is a key enzyme of T-cell action. CN is a target of the immunosuppressive drugs cyclosporine A and FK506. Recently, some reports stated that cardiac hypertrophy and skeletal muscle hypertrophy are inducted by calcineurin (Molkentin *et al.*, 1998; Sussman *et al.*, 1998; Semsarin *et al.*, 1999).

CN is a heterodimer consisting of two tightly associated subunits: a M_r 61 000 catalytic subunit (calcineurin A, CNA) and a M_r 19 000 regulatory subunit (calcineurin B, CNB). Biochemical and crystal structural studies have identified that CNA is the catalytic core of the holoenzyme composed of a catalytic domain, a CNB binding domain, a calmodulin (CaM) binding domain and an autoinhibitory domain. CNB, with 35% sequence identity with CaM, is a member of the family of Ca^{2+} binding proteins with four EF hand Ca^{2+} binding sites. Two global Ca^{2+} binding domains of CNB, with each domain containing two Ca^{2+}-binding EF hand motifs, are flanked by a long C-terminal β-strand. Domain 1 of CNB (residues 1 – 84) connects to domain 2 (residues 85 – 155) *via* an α-helix that is kinked at Gly85. The two domains are arranged linearly on a long CNB-binding α-helix of CNA (Klee *et al.*, 1979; Griffith *et al.*, 1995; Kissinger *et al.*, 1995; Watanabe *et al.*, 1995). The linear arrangement of Ca^{2+} binding domains on the α-helix is in contrast with the way that CaM binds to α-helix of the target, where two CaM domains are arranged on opposite sides of an α-helix (Ikura *et al.*, 1992). Although CNB is a regulatory subunit of CN, it plays an important role in stimulating the phosphatase activity of CNA. Without CNB, CNA has weak phosphatase activity even in the presence of CaM. The reconstituted CN heterodimer of CNA and CNB has increased catalytic efficiency (increased K_{cat}/K_m) compared with isolated CNA (Perrino *et al.*, 1992). CNB is able to decrease the K_m of CN remarkably (Perrino *et al.*, 1995). However, some intriguing question are: What is the structural basis by which CNB stimulates CN phosphatase activity?, and: What roles do the two domains of CNB play in stimulating CN phosphatase activity?

In this study we try to answer these questions at the domain level. We constructed a system to express the N-terminal domain (residues 1 – 84) and the C-terminal domain (residues 85 – 169) of CNB using PCR and purified the two proteins. Then we studied their ability to stimulate CNA phosphatase activity. The hydrophobic probe ANS was used to study the changes of the hydrophobic surface of CNB and its two domains caused by Ca^{2+} binding. Our results show that there are striking differences between the effect of DC and that of DN on the activation of CNA phosphatase activity. Ca^{2+} is able to induce CNB and its two domains to expose the hydrophobic surface. The hydrophobic surface of CNB and its two domains may be an important structural basis for stimulating CN phosphatase activity.

Results

Construction, Expression and Purification of DC and DN

The DNA fragments of DC and DN were amplified using the PCR method, cloned into the expression vector

pet21a, then transformed into *E. coli* BL21plysS (DE3). Positive clones were selected. Sequences of DN and DC were as expected.

Homogeneous DC and DN were obtained. The yields of purified DC and DN were 30 mg/l and 50 mg/l culture, respectively. SDS-PAGE gel analysis showed that the sizes of DN and DC were, as expected, approximately M_r 10 000 (Figure 1).

Effect of DC and DN on the Activation of CNA Phosphatase Activity

There were striking differences between the effects of DC and DN on the activation of CNA phosphatase activity (Figure 2). The phosphatase activity of isolated CNA was 50.5 units/mg. When bound to CNB, DC, and DN, CNA phosphatase activity increased to 122.8 units/mg, 77.2 units/mg, and 52.4 units/mg, respectively. When both DC and DN bound to CNA, its phosphatase activity increased to 81 units/mg. In the presence of CaM, the phosphatase activity of CNA was 101.4 units/mg. In the presence of CaM, CNA phosphatase activity increased to 226.7, 160.4, and 103.2 units/mg when bound to CNB, DC, and DN, respectively. In the presence of DC, DN and CaM, its phosphatase activity increased to 160 units/mg. Thus, CNB and DC stimulate the phosphatase activity, to about 2.2 and 1.6 times the phosphate activity of isolated CNA, respectively, in the presence of CaM, while DN has little effect on the CNA phosphatase activity. The CNA phosphatase activation effects of the two domains of intact CNB may differ.

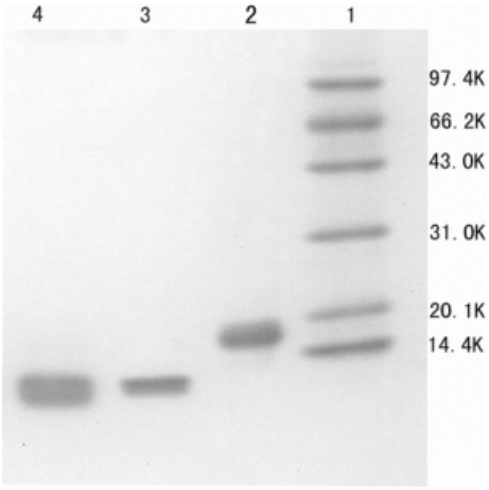

Fig. 1 Purification of CNB and Its Two Domains.
The three proteins were expressed in *E. coli* and purified as described in Materials and Methods. The proteins were run on a 12% acrylamide gel and stained with Coomassie Brilliant Blue. Lane 1: protein marker, lane 2: purified CNB, lane 3: purified DN, lane 4: purified DC.

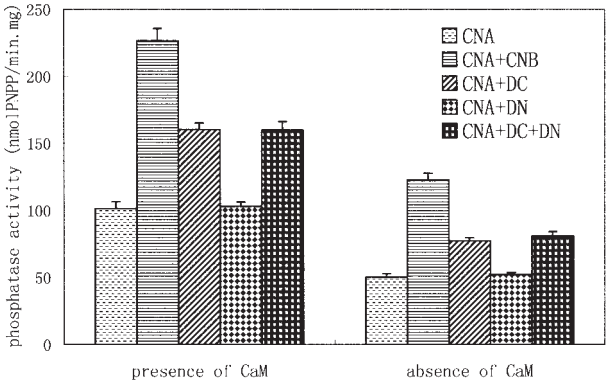

Fig. 2 Effect of CNB and the Two Domains of CNB on CNA Phosphatase Activity.
Phosphatase activities were assayed with pNPP in 50 mM Tris-HCl pH 7.4 containing 0.5 mM $MnCl_2$, 3 mM $CaCl_2$, 0.5 mM DTT, 0.2 mg/ml BSA and 20 mM pNPP at 30 °C. The CaM concentration was 0.3 μM. Data points represent the mean ± SD of the phosphatase activity in three separate experiments.

Interaction of Proteins with the Hydrophobic Fluorescence Probe ANS

The exposure of hydrophobic surfaces of the proteins CNB, DC and DN in the presence of Ca^{2+} was studied by measuring the fluorescence spectrum change of the hydrophobic probe ANS (Figure 3A, B, C). After Ca^{2+} bound to the proteins, ANS fluorescence intensity in CNB increased to 7×10^5 cps from 2.2×10^5 cps, while the maximum emission wavelength shifted to 472 nm from 506 nm. The ANS fluorescence intensity in DC increased to 3.5×10^5 cps from 1.8×10^5 cps, while the maximum emission wavelength shifted to 485 nm from 513 nm. The ANS fluorescence intensity in DN increased to 2.4×10^5 cps from 1.5×10^5 cps, while the maximum emission wavelength shifted to 498 nm from 520 nm. Ca^{2+} binding to the proteins leads to increased ANS fluorescence intensity in the proteins with the blue shifting of maximum emission wavelength, indicating large differences in the hydrophobic characteristics of the proteins (Figure 3D). ANS fluorescence intensity with CNB was the most intensive with the shortest maximum emission wavelength $\lambda_{max}=472$ nm; while that of DC was less intensive with a longer maximum emission wavelength $\lambda_{max}=487$ nm and that of DN was the least intensive with the longest maximum emission wavelength $\lambda_{max}=498$ nm. The results show that CNB has the most hydrophobic surface, with DC having less, and DN having the least.

The magnitude of the hydrophobic surface of the proteins is in agreement with its effects on CNA phosphatase activity. The surface hydrophobicity differences in the proteins may be the structural basis accountable for the differences in stimulation of CNA.

Discussion

CNB, a regulatory subunit of CN, is composed of two global domains. The current results showed that there were striking differences between the effects of DC and of DN on the activation of CNA phosphatase activity. DN

itself has little effect on CNA phosphatase activity, while DC stimulates CNA phosphatase activity to about 1.6-fold in the presence of CaM, approximately 50% of wild-type CNB. Therefore, although DN itself cannot stimulate CNA phosphatase activity, it may play an important role in intact CNB in activating CNA phosphatase activity. The mixture of DN and DC can only stimulate CNA phosphatase activity approximately to the level stimulated by DC, but not to the level stimulated by intact CNB, which indicates that the function of the DN requires the complete structure of the CNB molecule.

CNB is a member of the family of EF-hand calcium-binding proteins. Ca^{2+} is absolutely necessary in reconstituting the CN heterodimer of CNA and CNB, and in CNB stimulating CN phosphatase activity (Milian *et al.*, 1994). The change of conformation of CNB and its two domains induced by binding Ca^{2+} is important to further understand the interaction between CNA and CNB. The effect of Ca^{2+} on surface hydrophobicity of the proteins was studied with the hydrophobic fluorescence probe ANS as described in this paper. The results show that after binding Ca^{2+}, intensity illustrated by the ANS fluorescence increases remarkably, as the emission peak is blue-shifted. These results indicated that after Ca^{2+} bound to the proteins, surface hydrophobicity of the proteins increased greatly. These findings further our understanding of why Ca^{2+} is necessary in recombination between CNA and CNB. We speculate that before Ca^{2+} binds to CNB, the surface hydrophobicity of CNB is too weak to recombine with CNA to form a heterodimer; after the binding, the hydrophobic areas of CNB are exposed, the surface hydrophobicity of CNB is increased to the extent that CNB can recombine with CNA to form a heterodimer.

The differences between CNB and its two domains in stimulating CNA phosphatase activity are closely related to surface hydrophobicity of the above three proteins. CNB has the greatest surface hydrophobicity, and is the most competent in stimulating CNA phosphatase activity, while DC ranked second and DN ranked third. Watan-

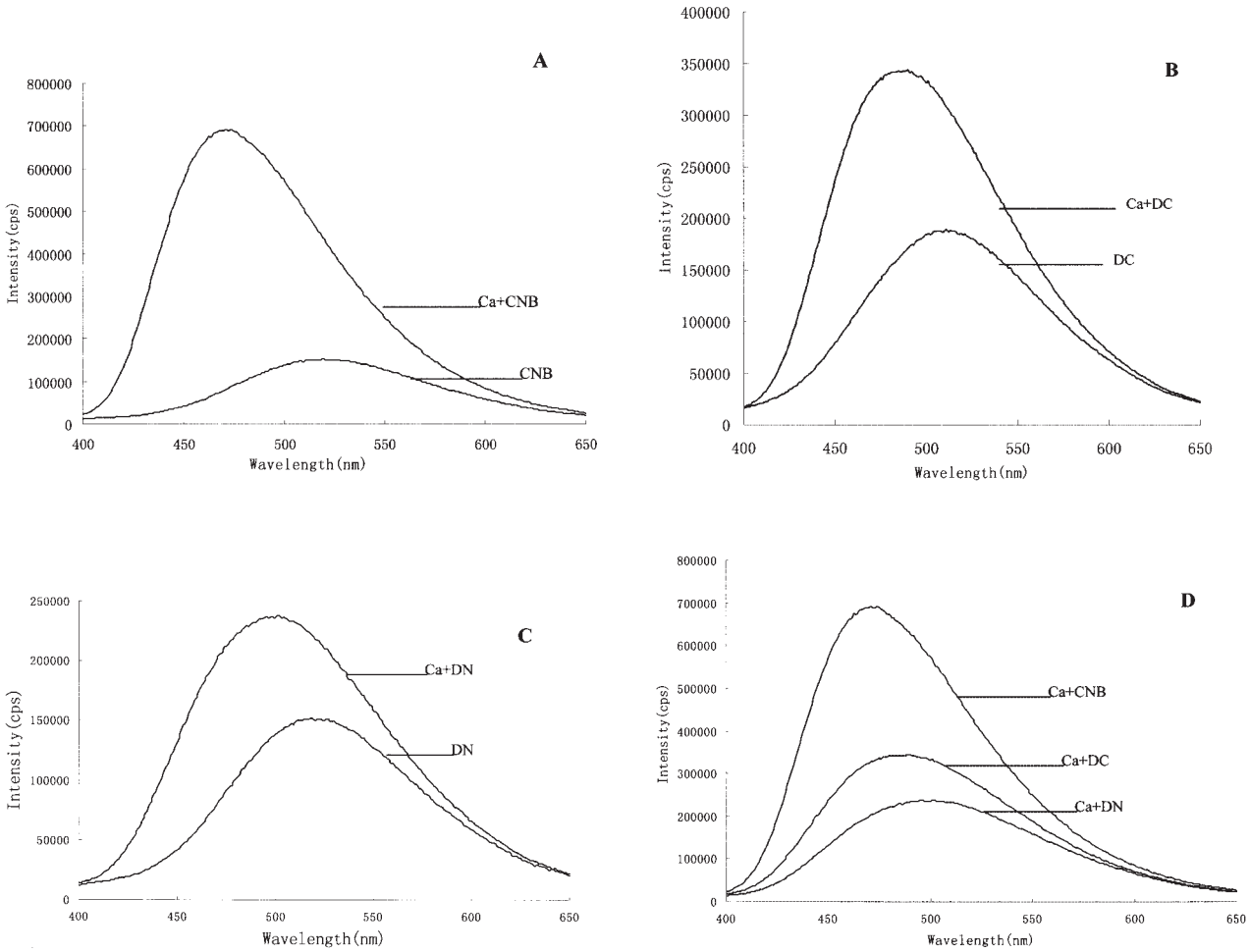

Fig. 3 Interaction of ANS with CNB and Its Two Domains.
Fluorescence of 50 mM ANS in 0.1 mg/ml CNB and its two domains was recorded in 20 mM Tris-HCl pH 7.4 buffer. The Ca^{2+} concentration was 1 mM. (A) ANS fluorescence in CNB solution in the presence and absence of Ca^{2+}. (B) ANS fluorescence in DC solution in the presence and absence of Ca^{2+}. (C) ANS fluorescence in DN solution in the presence and absence of Ca^{2+}. (D) ANS fluorescence of CNB and its two domains in the presence of Ca^{2+}.

abe *et al.* changed the non-polar amino acid residues Val115/Leu116 into polar Glu (1996). However, these changes only increased the CNA phosphatase activity to 25% of wild-type CNB. The binding of the mutants with CNA is of lower affinity than wild-type CNB. We speculate that replacing hydrophobic amino acids by hydrophilic amino acids may change the surface hydrophobicity of the mutants when Ca^{2+} binds with the mutants; consequently the mutants have decreased affinity with CNA and decreased ability to stimulate CNA phosphatase activity. Therefore we suggest that the surface hydrophobicity of CNB might be very important for CNB to stimulate CNA phosphatase activity. Previous studies (Feng *et al.*, 1999) stated that the Ca^{2+} binding sites in the N-terminal domain of CNB might act in dynamic modulation of enzyme function, whereas the Ca^{2+} binding sites in the C-terminal domain are structural. However, our results showed that after Ca^{2+} binding, DC could stimulate CNA phosphatase activity to about 1.6-fold, or half, of that of CNB. DC possesses intensive ability to modulate enzyme function. Although there are two main hydrophobic clusters in the C-terminal of CNB (Watanabe *et al.*, 1996), and the surface hydrophobicity of DC is stronger than that of DN, the surface hydrophobicity of CNB composed of DN and DC is strikingly stronger than that of DC. These results indicated that when forming the hydrophobic surface, the two CNB domains might have some synergism. Intensive surface hydrophobicity of CNB might be a reason that it has ability to activate CN phosphatase activity. Therefore, we suggest that the CNB hydrophobic surface produced by Ca^{2+} might be an important structural basis for stimulating CN phosphatase activity.

Materials and Methods

Materials

Restriction endonucleases (*Nde*I, *Bam*HI) and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA), TaqDNA polymerase from Roche (Mannheim, Germany), primers from Cybersyn, (Beijing, China), Tryptone and yeast extract from Oxford (Basingstoke, Hampshire, England), Agarose from GIBCO (Carlsbad, USA), Phenyl-sepharose, DEAE-Cellulose and Sephadex G75 from Pharmacia Inc. (Uppsala, Sweden), acrylamide, ampicillin, IPTG (isopropyl-1-thio-β-D-galactoside), PMSF (phenylmethylsulfonyl fluoride), DTT (dithiothreitol), and ANS (1-anilinonaphthalene-8-sulfonate) from Sigma Chemical Co. (St. Louis, USA). CNA, CNB and CaM were purified and prepared in our laboratory following the method of Wei *et al.* (1993, 1997a). All other reagents were of standard laboratory grade or the highest quality available from commercial suppliers.

Plasmid Construction

The DNA fragment of DN was amplified using the PCR method. The template was the cDNA for rat CNB. The primers were 5'-CCGCCATATGGGAAATGAGGCGAGTT-3' and 5'-CGCGGGATCCTCATTTGACACTGAAC-3'. The DNA fragment of DC was also amplified using the PCR method with a cDNA template for rat CNB. The primers were: 5'-CCGCCAATGGGCGATAAGGGACAG-3' and 5'-CGCGGGATCCTCACACATCTACCACCA-3'.

The DNA fragments of DN and DC were cloned into a pet21a expression plasmid after digestion with *Nde*I and *Bam*HI and transformed into competent *E. coli* BL21plysS(DE3). Positive recombinant clones were selected with sequences DN and DC. DNA sequence was confirmed by DNA sequencing.

Protein Expression and Purification

DC was expressed as described by Wei and Lee (1997a) with a few modifications. *E. coli* BL21plysS(DE3) containing the expression vector pet21a of DC was grown at 37 °C while shaking in a TM culture media (1.2% Tryptone, 2.4% yeast extract, 1% NaCl, 0.6% glycerol, 50 mg/l ampicillin) for 9 h. Cells were harvested by centrifugation at 2000 *g* for 20 min, disrupted by ultrasound and centrifuged at 12 000 *g* for 20 min. After adding PMSF and DTT, the supernatant was loaded onto a DEAE-Cellulose column equilibrated with 20 mM Tris-HCl pH 7.4 buffer. The column was washed with 20 mM Tris-HCl pH 7.4 buffer. The crude solution of DC was eluted using 0.2 M NaCl, 20 mM Tris-HCl pH 7.4 buffer. After adding PMSF, DTT, $CaCl_2$ (final concentration 3 mM), and NaCl (final concentration 1 M), the crude solution of DC was loaded onto a phenyl-sepharose column equilibrated with 20 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$, 1 M NaCl buffer. The column was washed with 20 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$, 1 M NaCl buffer and 20 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$ free NaCl buffer. The DC was eluted with 20 mM Tris-HCl pH 7.4 1 mM EGTA buffer, concentrated, and then loaded on a G75 gel-filtration column to obtain homogeneous protein.

E. coli BL21plysS (DE3) containing the expression vector pet21a for DN was grown at 37 °C while shaking in TM culture media until the absorbance at 600 nm reached about 0.7, at which point IPTG was added to a final concentration of 50 μM. The cells were grown for an additional 5 h, harvested by centrifugation at 2000 *g* for 20 min, disrupted by ultrasound, then boiled for 20 min, and centrifuged at 12 000 *g* for 20 min. After adding PMSF and DTT, the supernatant was loaded onto a DEAE-Cellulose column equilibrated with 20 mM Tris-HCl pH 7.4 buffer. The column was washed with 20 mM Tris-HCl pH 7.4 buffer. The DN was eluted using gradient elution (0 – 0.5 M) NaCl, 20 mM Tris-HCl pH 7.4 buffer. The concentrated DN was loaded onto a G75 gel filtration column to obtain homogeneous protein. All protein concentration measurements were performed using the procedure of Bradford (1976).

Assay of Phosphatase Activity

The activity assay with *p*-nitrophenyl phosphate (PNPP) as substrate (Wei *et al.,* 1997b) was performed in 50 mM Tris-HCl pH 7.4, 0.5 mM $MnCl_2$, 3 mM $CaCl_2$, 0.5 mM DTT, 0.2 mg/ml bovine serum albumin (BSA) and 20 mM PNPP. Measurements using CNB to stimulate CNA phosphatase activity used a CNA to CNB ratio of 1:1 on a molar basis. Measurements using DC and DN to stimulate CNA phosphatase activity used a ratio of DC or DN to CNA of 2:1 on a molar basis. The reactions were performed in a 0.2 ml volume at 30 °C for 20 min and terminated by the addition of 1.8 ml 0.5 mM Na_2CO_3, 20 mM EDTA solution. The absorbance was read at 410 nm. The units of activity were defined as nanomoles of PNPP hydrolyzed per minute. The CaM concentration was 0.3 μM when used.

Measurement of ANS Fluorescence Spectroscopy

The ANS fluorescence spectrum was recorded on a FLUOR MAX-2 fluorometer. The fluorescence spectrum of 50 μM ANS in 0.1 mg/ml of the three protein solutions was measured at room temperature in a 1 cm path length cuvette with an excitation wavelength of 345 nm and a slit of 10 nm. Emissions scans were

taken from 400 nm to 600 nm. When measuring the effect of Ca^{2+} on the proteins, Ca^{2+} was added to the sample to a final concentration of 1 mM.

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