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Research Communication

The Polarity of the Amino Acid Residue 118 of Calcineurin B Is Closely Linked to Calcineurin Enzyme Activity

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Summary

Calcineurin(Cn), a multifunctional regulator expressed in several tissues and organs, consists of CnA (catalytic subunit) and CnB (regulatory subunit). The crystal structure shows that the hydrophobic groove formed by 118–123 residues of CnB is necessary for its interactions with two different immunosuppressant-immunophilin complexes and with CnA. In this report, we focus on Met118 of CnB to study the association between conformational states of CnB and the phosphatase activity of Cn. We found that hydrophobicity in the region around site118 of CnB is essential for the Cn activity. Polar mutants significantly weakened the enzymatic activity compared with the nonpolar ones. The data showed that some modest alterations in the vicinity of site118 impaired the integrality and compactness of hydrophobic microenvironment, and this might explain why CnB mutants defective in hydrophobicity failed in activating Cn. This requirement of hydrophobic microenvironment around site118 in CnB suggests that, besides the mutations in the catalytic subunit CnA, which impairs Cn phosphatase activity, and had been identified to be associated with diseases such as Alzheimer's disease (AD), the mutations in CnB might also affect Cn enzymatic activity *in vivo*, and this might be helpful for our further research on mechanisms of diseases associated with Cn.

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Keywords calcineurin; Met118; hydrophobic; enzyme activity.

INTRODUCTION

Calcineurin (Cn), a heterodimer composed of two subunits, the 61-kD catalytic subunit (calcineurin A, CnA) and the 19 kD regulatory subunit (calcineurin B, CnB), is a Ca^{2+}-and calmodulin (CaM)-dependent serine/threonine protein phosphatase. As a multifunctional regulator, Cn is widely distributed in mammalian tissues, especially in brain (*1–3*), and participates in many physiological and pathological processes, such as Ca^{2+}-dependent signal transduction pathways, T-cell activation and memory formation (*4–6*). Additionally, it got extensive attention as a target enzyme of immunosuppressant FK506 and cyclosporin A (CsA) (*7–9*).

According to the crystal structure of Cn-FKBP12-FK506 complexes, CnB contains two globular Ca^{2+}-binding domains and form a long hydrophobic groove into which the top half of the BBH (CnB binding helix, the CnB binding domain) of CnA is embedded. This interaction is essential for the stability of Cn (*10*). Data showed that, in the presence of EGTA, CnB could interact with CnA (*11*), and the hydrophobic groove of CnB was largely retained in both holo-CnB (Ca^{2+}-saturated CnB) and apo-CnB (Ca^{2+}-free CnB). Additionally, apo-CnB is able to regulate the phosphatase activity, albeit at a lesser extent than holo-CnB (*12*). These results imply the importance of hydrophobic groove of CnB in regulating Cn enzyme activity. The hydrophobic cleft participates in the docking of immunophilin-drug complexes, and FKBP12-FK506 complex docks into the region formed by Leu115, Met118, Val119 and Leu123 of CnB (*10, 13*). Another immunosuppressant CsA, though having different structure with FK506, is similarly embedded into the hydrophobic region of CnB as shown in the crystal structure of Cn–Cyp–CsA complex. Despite there are overall residues of CnB involved in binding either with Cn-FKBP12-FK506 or Cn-CyP-CsA, residues in the region from 118 to 123 of CnB are conserved in binding with these two immunosuppressant complexes. This region is hypothesized to function in the CnB-mediated activation of CnA catalysis (*14–16*). However, the details of how this region of CnB regulates CnA are not well defined.

In this report, we focus on the Met118 site of CnB to study the association between the conformational states of CnB and phosphatase activity of Cn. We mutated Met118 to different kinds of amino acid residues, including hydrophobic amino acids, neutral polar amino acids and charged polar amino acids.

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Analysis of the phosphatase activity and structure of these mutants indicated that retaining hydrophobicity in site118 of CnB is essential for Cn enzyme activity. Hydrophobic microenvironment around site118 protects the integrality and compactness of hydrophobic groove, which has been disrupted by polar mutants.

EXPERIMENTAL PROCEDURES

Materials

The strains BL21(DE3)plysS, DH5α, expression vector pET-21a were obtained from Invitrogen corporation. The human CnB cDNA clone was cloned in our laboratory. The resins and columns for purification were obtained from Amersham Biosciences. RII peptide was purchased from BioMol Research Labs Inc. PPO[OC(C6H5)=NCH=CC6H5] and POPOP {[OC(C6H5)=CHN=C]2C6H4} were obtained from E. Merck and [γ-^{32}P]-ATP from Beijing Furi Biology Engineering. Anti-CnA and anti-CnB antibodies (mouse IgG) were prepared by our laboratory. Peroxidase-conjugated affinipure goat anti-mouse IgG (H+L) was from Jackson ImmunoResearch Laboratories. Protein A-Agarose (sc-2001) was purchased from Santa Cruz Biotechnology. The nitrocellulose membranes were Millipore product. Super-Enhanced chemiluminescence detection kit was obtained from Applygen Technologies. All other reagents were of standard laboratory grade and of the highest quality available from commercial suppliers.

Plasmid Construction

Mutants in full-length human CnB cDNA were constructed using a novel site-directed mutagenesis PCR method (*17*). Mutants cloned into the expression vector pET-21a were then transformed into *Escherichia coli* strain BL21 (DE3) plysS host cells, and aimed at productive expression.

Protein Expression and Purification

The wild-type CnB and its mutants were expressed and purified by similar methods with a few modifications as described earlier (*18*). Briefly, the target gene-transfected *E. coli* host cells were cultured in LB media for 10 h at 37 °C, 200 rpm and inoculated to TM media for 10 h at 37 °C, 240 rpm. The cells were harvested by centrifugation at 4,000 rpm for 30 min at 4 °C. The pellets were resuspended in buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM 2-mercaptoethanol and 0.4 mM PMSF) and disrupted by ultrasonication. The homogenates were treated at 100 °C for 40 min and centrifuged at 12,000 rpm for 40 min. After adding final concentration of 3 mM $CaCl_2$ and 0.5 M NaCl, the supernatant was loaded onto the phenyl-sepharose column (Amersham Biosciences) equilibrated with buffer B (20 mM Tris-HCl pH 7.4, 0.5 mM $CaCl_2$ and 0.5 M NaCl). After extensive desalting wash, the target protein was eluted with buffer C (20 mM Tris-HCl, pH 7.4 and 1 mM EGTA) and loaded on the G25 gel-diltration column (Amersham Biosciences) to obtain homogeneous protein. Protein purity was assessed by SDS-PAGE, and protein concentration was measured by the Bradford method (*19*).

Phosphatase Activity Assay

Protein phosphatase activity was assayed by measuring the release of ^{32}Pi from ^{32}P-labeled RII peptide (DLDVPIPGRFDRRV SVAAE) (*20*). The assay buffer (40 μM ^{32}P-labeled RII, 0.6 μM CaM, 0.2 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.1 mg/ml BSA, 0.5 mM DTT and 50 mM Tris-HCl, pH 7.4) was mixed with the enzyme buffer (0.2 μM CnA and 0.2 μM CnB or its mutant) in a ratio of 1:1 with 20 μl total volume, and incubated at 30 °C for 10 min, then added 0.18 ml of 83.3 mM H_3PO_4 to terminate the reaction. The free ^{32}P separated from RII peptide was quantified by liquid scintillation spectrometry (TRI-CARB 2900^{TM}, Packard Bioscience).

Coimmunoprecipitation Assay

The coimmunoprecipitation (co-IP) assays were operated using anti-CnA antibody as the bait antibody to test the interaction of CnB or the site118 mutants with CnA. Two microgram of antibody was mixed with 200 μl CnA-CnB (or mutants) complex in 20 mM Tris-HCl, pH 7.4, including 3 mM $CaCl_2$ and 0.5 mM $MnCl_2$, and the mixtures were incubated overnight with a gentle rotation at 4 °C. The molar concentrations of CnA and CnB were both 5 μM. The protein–antibody complexes were harvested with protein A-agarose for 2 h at 4 °C and washed five times with PBS according to the manufacturer's instructions. Elution of the protein complexes was performed at 100 °C for 5 min in 2× SDS loading buffer and then detected by Western blotting. The anti-CnA and anti-CnB antibody (mouse IgG) were both prepared by our laboratory.

Circular Dichroism Spectroscopy

Far-UV circular dichroic spectra studies were performed on a Jasco-810 spectropolarimeter (slit width, 2 mm). All spectra were recorded at 20 °C with constant N_2 flushing using a bandwidth of 1.0 nm in a cuvette of 2-mm path length and were averaged from three scans with a scan rate of 100 nm/min. CnB or its mutant was mixed with CnA in a molar ratio of 1:1 in assay buffer I (20 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$, 0.5 mM $MnCl_2$), and the enzyme concentration was set at 5 μM. Enzyme concentration was set at 10 μM in assay buffer II (20 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$) to test spectra of CnB series alone.

Molecular Dynamics Simulation

The calculations were carried out using coordinates of CnB from the crystal structure of Cn-FKBP12-FK506 complex (protein data bank code 1TCO) determined by X-ray crystallography at 2.5 Å (*10*). The structure of wild-type CnB and its mutants were simulated by using the InsightII software package (Biosym Technologies, San Diego, CA). Hydrogens are added using the AMBER suite (*12, 21*).

The proteins were all solvated in a periodic box of 66 × 81 × 74 Å^3 with about 9,600 water molecules. To neutralize the system

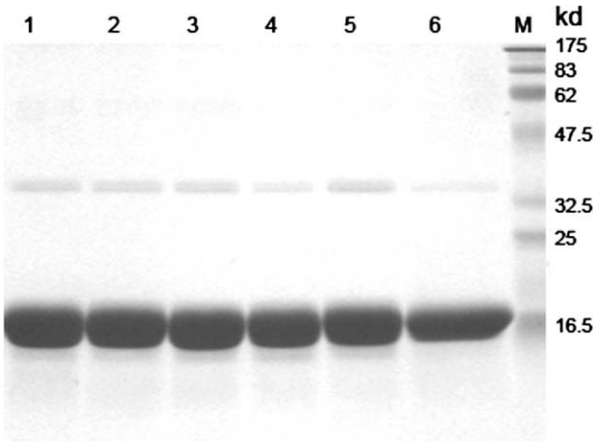

Figure 1. SDS-PAGE analysis of purified CnB and site118 mutants. The proteins were expressed in *E. coli* and purified as described in "Experimental Procedures" section. The proteins were run on a 12% acrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, wild-type CnB; Lane 2, M118W; Lane3, M118G; Lane 4, M118N; Lane 5, M118E; Lane 6, M118K; Lane M, protein markers (NewEngland biosciences).

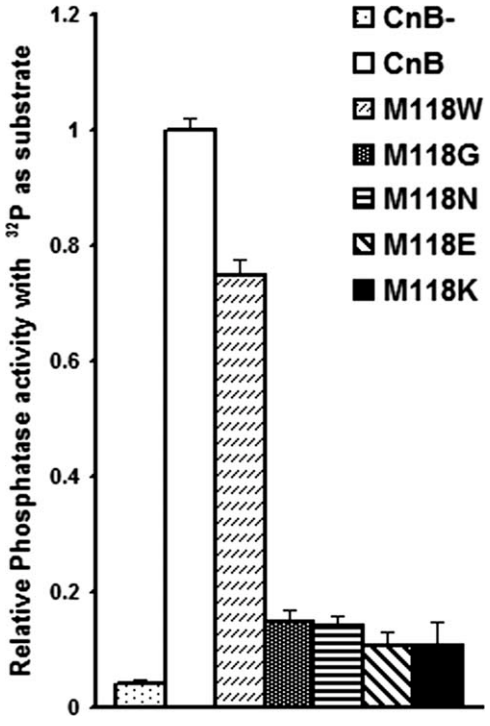

Figure 2. The effect of CnB and site118 mutants on the phosphatase activity of CnA. Dephosphorylation activities of CnA regulated by CnB or its site118 mutants were assayed using ^{32}P-labled RII peptide as substrate, CnB or its mutants was added in a molar ratio of 1:1 to CnA. (Details were described in the "Experimental Procedures" section.) The data with error bars were expressed as means ± SD (n = 3).

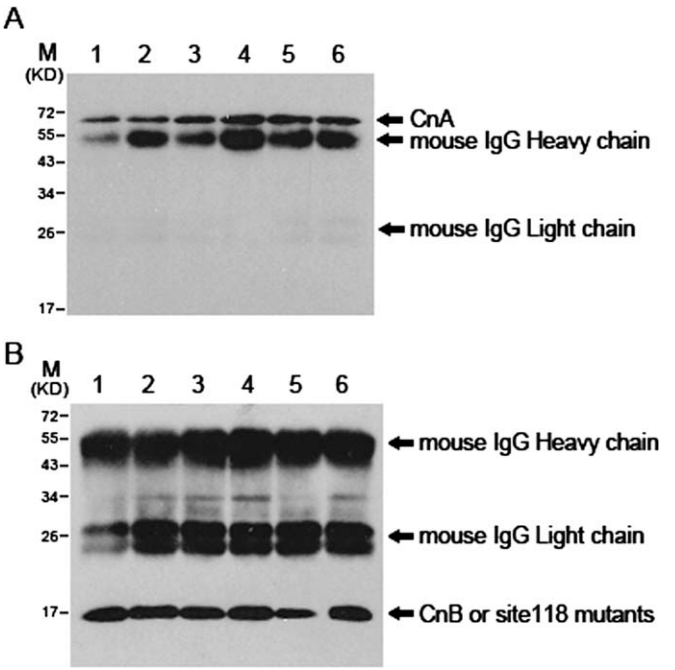

Figure 3. Coimmunoprecipitation analysis of the interactions between CnA and wild-type CnB or site118 mutants. (A) Detection of CnA (61 KD) seized by anti-CnA antibody in CnA-CnB (or mutants) complexes. The complexes were incubated with anti-CnA antibody overnight at 4 °C, and were harvested by protein A-agarose for 2 h and then washed five times according to the protocol. The primary antibody for detection of CnA by western blotting was the same as the bait antibody (dilution 1:1000). (B) Detection of CnB or the mutants (19 KD) that were coimmunoprecipitated by anti-CnA antibody by Western blotting. The dilution of CnB antibody was 1:400. Lane 1, wild-type CnB; Lane 2, M118W; Lane3, M118G; Lane 4, M118N; Lane 5, M118E; Lane 6, M118K; Lane M, prestained protein markers (Genstar Biotechnology).

of wild type of calcium-saturated CnB (Holo-WT), a total of 20 Na^+ ions and 17 Cl^- ions were added in each simulation to mimic the solution with an ionic strength of about 100 mM. The mutants added similar counterions to neutralize these systems and mimic the same ionic strength.

The molecular dynamic simulations were performed using the AMBER9 package of programs (*22, 23*), while the protein and water molecules were described by parameters from AMBER99 (*24*) and TIP3P force fields, respectively.

A cutoff radius of 9 Å was introduced for nonbonded interactions. The electrostatic interactions were calculated with the Particle Mesh Ewald method (*25*). The SHAKE algorithm (*26*) was used to constrain all bond lengths involving hydrogens. Optimization and relaxation of solvents and ions were performed at first by keeping the solute atoms constrained to their initial position with progressively decreasing force constants of 25, 20, 15, 10 and 0 Kcal/mol Å^2, respectively. Following the minimization and 60 ps equilibration, a molecular dynamics production run was performed for a duration of 8 ns.

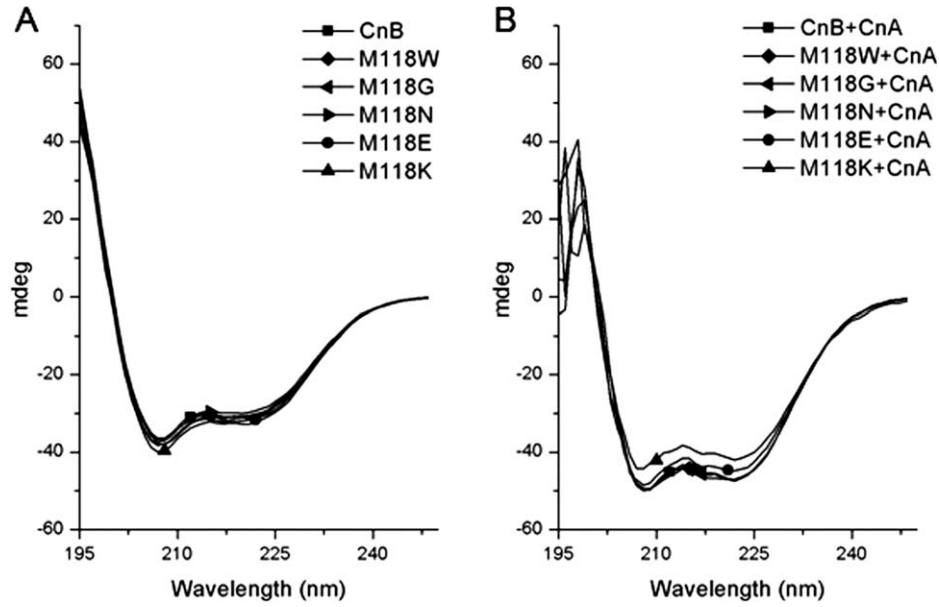

Figure 4. Circular Dichroic (CD) spectra assay of wild-type CnB and its mutants. (A) CD spectra of CnB and the mutants. The protein concentration was 10 μM diluted in 20 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$. (B) CD spectra of CnB and the mutants in the presence of CnA. CnB or its mutant was mixed with CnA in a molar ratio of 1:1 in 20 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$, 0.5 mM $MnCl_2$, and the enzyme concentration was set at 5 μM. All spectra were recorded at 20 °C with constant N_2 flushing and were averaged from three scans.

The three 10-ns simulations were carried out at a constant temperature (*27*) of 300 K and at a constant pressure of one atmosphere with a 2-fs time step. Atomic coordinates were saved every 1 ps for analysis during the production run.

RESULTS AND DISCUSSION

Expression and Purification of Wild-type CnB and M118 Mutants

The proteins were expressed and purified as described in "Experimental Procedures" section. SDS-PAGE gel analysis showed the proteins were electrophoretically pure and had the similar mobility (Fig. 1).

Polar Mutants in Site118 of CnB Impair Phosphatase Activity of Cn

We constructed a series of point mutations at residue 118 by mutating Met to various amino acids, and the relative activities of these mutants in comparison to wild-type CnB on Cn phosphatase activity were as follows: M118W(75%), M118G(15%), M118N(14%), M118E(10.75%) and M118K(10.7%) (Fig. 2).

The data showed that retaining hydrophobicity in site118 could retain enzyme activity of Cn, while mutating to polar residues such as neutral ones and charged ones, the enzyme activity of Cn was significantly reduced. It is worth noting that the neutral mutants of CnB kept more enzyme activity of Cn in comparison with the charged mutants.

These results suggest that hydrophobicity around residue 118 of CnB is essential for the enzymatic activity of Cn, while polar mutations in CnB attenuate phosphatase activity of Cn, and charged polar mutants are more defective in Cn enzyme activity in comparison with the uncharged ones.

Coimmunoprecipitation Analysis of the Interaction Between CnA and CnB or Its Mutants

To investigate whether the interaction between CnA and CnB would be disturbed by the mutation in residue 118, the co-IP assays were operated using anti-CnA antibody as the bait antibody. According to our result, both wild-type CnB and its site118 mutants could interact with CnA, and there seemed no obvious differences among the groups (Fig. 3). Mutations in site118 of CnB could influence the enzyme activity, but it had not much effect on the interaction between CnA and CnB. This result indicated that the binding of CnB to CnA was not the sufficient condition in the process of Cn activation.

Circular Dichroim Spectra Assay of Wild-type CnB and Its Mutants

We investigated the structure transformation of CnB and its mutants using CD spectra assay. As shown in Fig. 4, the differences between the CD spectra of CnB and its mutants were imperceptible (Fig. 4A), and the addition of CnA seemed not induce infusive changes of the spectra, the mutants were only a little different from the wild-type CnB (Fig. 4B). According to our previous work, we concluded that the structure change of CnB in the process of activating Cn was mild and the compactness of hydrophobic groove would be largely retained no matter

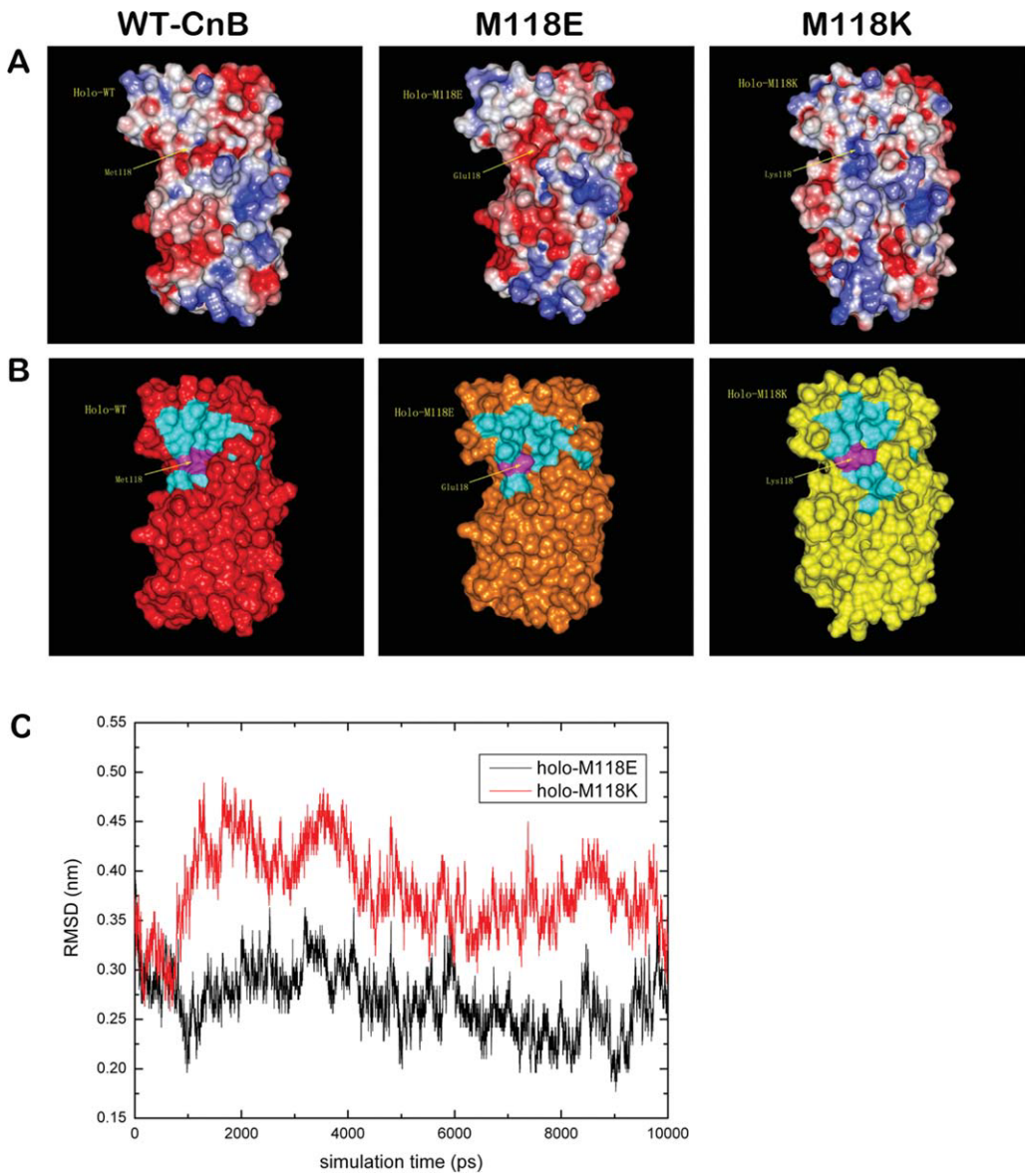

Figure 5. Molecular dynamics simulation scheme of wild-type CnB and mutants M118E M118K. (A) Distribution of the surface potential energy of wild-type CnB, M118E, M118K from left to right. Different value of the surface potential energy showed in different colors: positive (blue), negative (red), neutral (white). (B) Display the hydrophobic area formed by four residues of latch region (cyan), and specially mark the site 118(purple). (C) Global RMSDs are calculated between sequential frames in wild-type trajectory and the other two trajectories (M118E, M118K). The *X*-axis is the wild-type-CnB(WT-CnB) as baseline. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

it combined with CnA or not. The hydrophobic microenvironment transformation was efficient and modest. The regulation of CnB to activate CnA was a precise process.

Molecular Dynamics Simulations of Wild-type CnB and Its Mutants

To further explore the possible explanation for the failure of polar mutants in activating Cn, we chose M118E and M118K, which have the most obvious reduction in enzyme activity, to study the situation of hydrophobic microenvironment around site118 by molecular dynamics simulations.

The comparison of conformational variability during the simulation between WT and M118E or M118K were described by the root mean square deviations (RMSD) from corresponding frames at the same time t_i in both trajectories. The RMSD was calculated using the g_rms program available in Gromacs. Ratio of CT (non/polar) was calculated from the ratio of nonpolar solvent accessible surface areas in C-terminal hydrophobic groove to the polar areas. The C-terminal hydrophobic groove is

Table 1
The non/polar ratio of C-terminal (CT) or the four residues of WT CnB and mutants M118E M118K in Ca^{2+}-saturated status by molecular dynamics simulation

	WT-CnB	M118E	M118K
Ratio of CT (non/polar)	4.30 ± 0.63	4.06 ± 0.67	3.54 ± 0.74
Ratio of four residues (non/polar)	2.82 ± 0.34	2.63 ± 0.49	2.07 ± 0.54

defined as the residues whose distance to the residues (Val349–Val357) in BBH of CnA in crystal structure is less than 4 Å. The ratio of four residues (non/polar) was calculated from the ratio of nonpolar solvent accessible surface areas of four neighbor residues to their polar areas. These four residues are Leu115, Met118, Va1119 and Leu123 from CnB.

The results of molecular dynamics simulation showed: (1) The surface electrostatic potentials around Met118 of wild-type CnB keep an electric charge balance status, and it changed to negative or positive when Met118 was mutated into Glu(M118E), or Lys(M118K), respectively. We noticed that mutations in residue 118 influenced a much broader region than the site itself (Fig. 5A). (2) The wild-type CnB has a larger hydrophobic groove than the mutants (Table 1); this implies a more tight interaction with CnA. In comparison to the integral and compact structure of the hydrophobic region in WT CnB, similar region in CnB mutants were disrupted to some extent (Fig. 5B). Figure 5C shows the RMSD value of the three proteins using WT CnB as the baseline, the higher the RMSD value, the more the deviation between the structures, which in turn changed their stability and functional activity. We speculated that the modest change of hydrophobic region might explain why mutants failed in activating CnA.

In conclusion, the hydrophobic groove of CnB containing residues 115–123 is the main region for CnB to interact with CnA, the importance of this region is not only because it embeds the BBH of CnA and stabilizes the whole enzyme, but also it undertakes structural alterations when CnB interacts with CnA and regulates the enzyme activity. In our previous work, we used to mutate Val119 of CnB to polar amino acids, such as Arg, and the phosphatase activity results clearly showed that the mutant V119R decreased the enzyme activity of Cn compared with the wild-type CnB (data not shown). Taken together, these experiments suggest that the hydrophobic amino acid residues in the hydrophobic groove of CnB are optimized in wild-type CnB, and a slight change may affect the enzyme activity of Cn drastically. In this article, we focused on the key residue Met118 in the hydrophobic groove of CnB to study the role of residue 118 of CnB in regulating the phosphatase activity of Cn. This article indicated that retaining hydrophobicity in the surrounding region of residue 118 of CnB is essential to Cn enzyme activity. Polar mutants in residue 118 slightly change the shape of hydrophobic region, and as a result, apparent impairment of the enzyme activity of Cn is observed. In mammalian tissues, Cn acts as a Ser/Thr phosphatase and participates in many biological activities. The imbalance between kinases and phosphatases has long been proposed as the pathophysiological mechanisms of some diseases, as evidenced by the decreased Cn activity in the brain of human patients with Alzheimer's disease (AD) (*28, 29*). The requirement of hydrophobicity in the vicinity of residue 118 of CnB suggests that, in addition to the CnA mutations defective in catalytic activity, mutations in CnB might also affect enzyme activity *in vivo*. In summary, our current work on the regulation of Cn enzyme activity *via* the hydrophobic groove of CnB might be helpful for our further studies on mechanisms of diseases associated with deregulation of Cn signaling.

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