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The salt bridge of calcineurin is important for transferring the effect of CNB binding to CNA

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Abstract Calcineurin (CN) is a heterodimer consisting of a catalytic subunit (CNA) and a regulatory subunit (CNB). The crystal structure shows that three residues or regions of CNA are mainly responsible for the interaction with CNB: the CNB binding helix (BBH), the N-terminus, and Glu53 that forms a salt bridge with Lys134 of CNB. In this report, we try to find the role that the salt bridge plays in the interaction between CNA and CNB. We found that mutation of Glu53 greatly reduced its responsiveness to CNB in the phosphatase assay and also that mutation of Lys134 of CNB affected its ability to activate the phosphatase activity of CNA. Structural analysis showed that disruption of the salt bridge affected the compact association of CNA and CNB. Thus, the salt bridge appears to help to stabilize CN and transfer the effects of CNB binding to CNA to activate its phosphatase activity.

Keywords: Calcineurin; Mutation; Salt bridge; N-terminus; Interaction

1. Introduction

Calcineurin (CN) is a major calmodulin (CaM)-binding protein in the brain and the only serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin [1–3]. It plays a critical role in coupling Ca^{2+} signals to cellular responses, and is involved in many signaling pathways, such as T-cell activation, the cell cycle, apoptosis and survival of certain cells [4–6].

CN is a heterodimer composed of a catalytic subunit A (CNA) and a regulatory subunit B (CNB) [7]. CNA contains four functional domains: a catalytic domain, a CNB binding domain (BBH), a CaM binding domain and an autoinhibitory domain (AID) [8–10]. CNB is an 'EF-hand' Ca^{2+} binding protein. The crystal structure of human CN-FKBP12-FK506 [11,12] shows that CNB has two globular domains flanked by a long C-terminal β-strand. The two domains are arranged linearly along the BBH and form a 33 Å-long hydrophobic groove with an amphipathic C-terminus. The top half of the BBH is embedded in the groove. Additional contacts occur between the N-terminus of CNA and the C-terminal lobe of CNB forming part of the binding cleft, and a salt bridge is formed between Glu53 of CNA and Lys134 of CNB.

It is well known that without CNB, CNA has little activity even in the presence of Ca^{2+}/CaM. However, the details of how CNB interacts with and regulates CNA are not well defined. The interaction between BBH and the hydrophobic groove of CNB is essential for the stability of CN and mutations that alter the hydrophobic character of the two regions often disrupt the association between CNA and CNB [13,14]. It is not known how the salt bridge and the N-terminus of CNA influence the interaction between CNA and CNB and the regulation by CNB of the phosphatase activity of CNA.

Here, we focus on the function of the salt bridge in the interaction between CNA and CNB. We separately mutated the Glu-53 of CNA to Glycine and Alanine, and the Lys134 of CNB to Glycine, to disrupt the salt bridge, yielding three salt bridge-disabled mutants: E53G, E53A, and K134G. Analysis of the phosphatase activity and structure of these mutants showed that the salt bridge is very important for CN function.

2. Materials and methods

2.1. Plasmid construction

All mutations were obtained by PCR. E53G and E53A were constructed with the following primer pairs and full-length rat CNA cDNA as template: E53G, sense 5′-CTC ATG AAG GGA GGC AGG CTG-3′, antisense 5′-CAG CCT GCC TCC CTT CAT GAG-3′. E53A, sense 5′-CTC ATG AAG GCA GGC AGG CTG-3′. antisense, 5′-CAG CCT GCC TGC CTT CAT GAG-3′. The CNB mutant K134G was constructed with full-length rat CNB cDNA as template and the primer pairs were: sense 5′ -CAG ATT GTA GAC GGA ACC ATA ATA AAC GC-3′, antisense 5′–GCG TTT ATT ATG GTT CCG TCT ACA ATC TG-3′. The CNA genes were cloned into the pET21a expression vector using the *Nde*I and *Hin*dIII restriction sites, and the corresponding primers were: sense 5′-AGG AGA TAT ACA TAT GTC CGA GCC CAA GGC-3′ and antisense 5′-CGC GAA GCT TTC ACT GAA TAT TGC T-3′. K134G was cloned into pET21a using the *Nde*I and *Bam*HI restriction sites, and the corresponding primers were: sense 5′-CCG CCA TAT GGG AAA TGA GGC GAG TT-3′ and antisense 5′-CGC GGG ATC CTC ACA CAT CTA CCA CCA-3′.

2.2. Expression and purification of proteins

The target proteins were expressed and purified as previously described [15,16]. Protein concentrations were measured by the procedure of Bradford [17] and protein purity was assessed by SDS–PAGE.

2.3. Assay of phosphatase activity

Phosphatase activity was assayed as described [18]. Briefly, 10 μl of 20 nM enzyme solution was mixed with 10 ml assay buffer (40 mM ^{32}P-

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Abbreviations: CN, calcineurin; CNA, calcineurin subunit A; CNB, calcineurin subunit B; BBH, CNB binding helix; CaM, calmodulin

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labeled RII peptide, 100 mM Tris–Cl, 0.2 mg/ml BSA, 1 mM DTT and 1 mM Mn^{2+}, and 0.2 mM $CaCl_2$, pH7.4; with or without 0.6 mM CaM) at 30 °C for 10 min. CNB or K134G was added in a molar ratio of 1:1 to CNA. The reaction was terminated with 0.18 ml of 83.3 mM H_3PO_4, and the released ^{32}P was separated from the RII peptide and quantified by liquid scintillation spectrometry.

2.4. CD spectroscopy

CD experiments were performed on a CD instrument (Jasco J-720, Japan) calibrated with d_{10}-camphorsulfonic acid. All spectra were recorded at 20 °C with constant N_2 flushing using a bandwidth of 1.0 nm in a cuvette of 1 mm path length. The enzyme concentration was 2.7 mM in 20 mM Tris–Cl, pH 7.4, 0.5 mM $MnCl_2$ and 3 mM $CaCl_2$. CNB/K134G was added in a molar ratio of 1:1 to CNA and their concentrations were set at 53 mM when their spectra were assayed separately. The measurement was repeated twice and the data were normalized to mean residue ellipticity (MRw). The percentage of secondary structures contents was estimated using the software in the CD instruments [19,20].

2.5. UV-absorbance spectroscopy

UV-absorbance spectroscopy was performed on a GBC UV-visible Cintra 10e spectrometer at 25 °C in a cuvette of 1 cm path length and slit-width of 1.5 nm. Absorbance spectra were recorded from 240 to 320 nm with protein free buffer as the blank control. The enzyme concentration was 8.2 mM in 20 mM Tris–Cl, pH 7.4, 0.5 mM $MnCl_2$ and 3 mM $CaCl_2$. CNB/K134G was added in a molar ratio of 1:1 to CNA and their concentrations were set at 26 mM when their spectra were assayed separately.

3. Results

3.1. Expression and purification of proteins

DNA sequence analysis confirmed the mutant sequences (date not shown). The proteins were purified as described in Section 2. SDS–PAGE analysis showed that mutants E53G and E53A had the same mobility as WT CNA as well as mutant K134G migrated with similar mobility to CNB. All proteins were electrophoretically pure (Fig. 1).

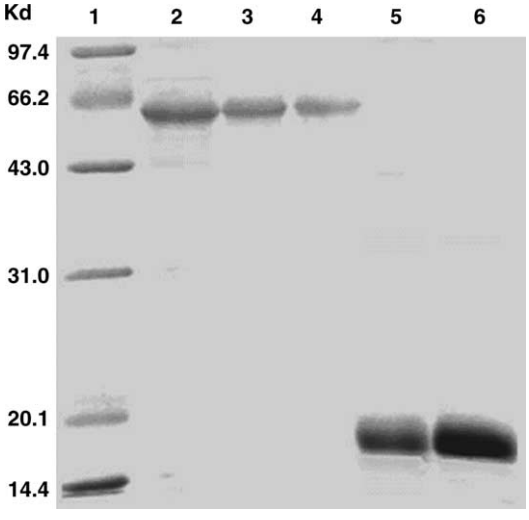

Fig. 1. SDS–PAGE analysis of purified proteins. Lane 1, protein markers; lane 2, CNA; lane 3, E53G; lane 4, E53A; lane 5, CNB; lane 6, K134G.

3.2. Phosphatase activity

3.2.1. The effect of CNB and K134G on the phosphatase activity of CNA. The effect of K134G on the phosphatase activity of CNA was weaker than that of CNB. The activity of CNA in the presence of K134G was only about 75% of that observed in the presence of CNB whether CaM was present or not (Fig. 2A).

3.2.2. The effect of CNB on CNA and its derivatives. It is out of our anticipation that mutation of the salt bridge of CNA greatly reduced the sensitivity of CNA to CNB. The presence of CNB increased the phosphatase activity of WT CNA about 24-fold, whereas with E53G and E53A it increased only 3.41- and 1.84-fold, respectively. When CaM and CNB

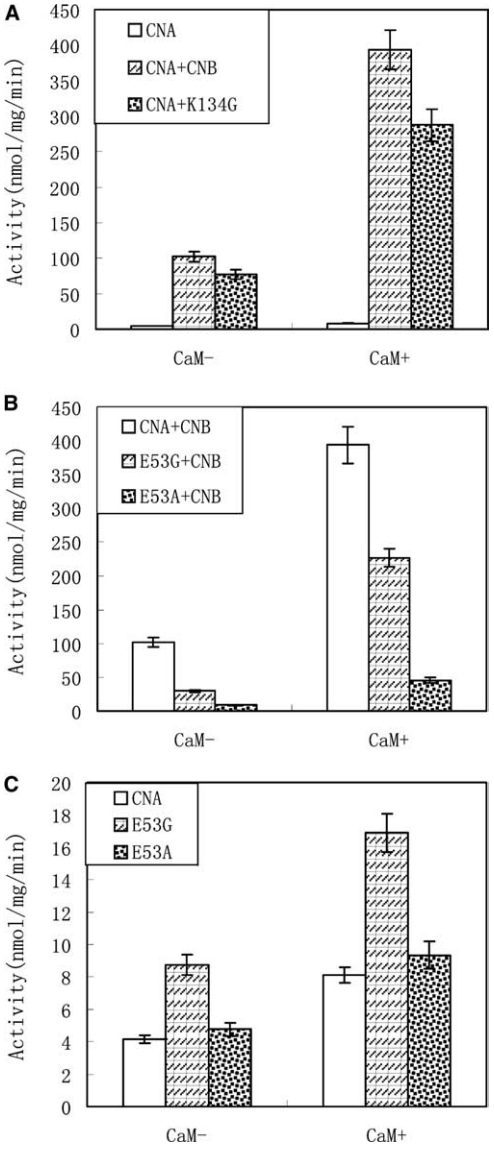

Fig. 2. Phosphatase activity was assayed as described in Section 2. (A) The effect of CNB and K134G on the phosphatase activity of CNA in the presence and absence of CaM. (B) The effect of CNB on the phosphatase activity of CNA and its mutant derivatives in the presence and absence of CaM. (C) The phosphatase activity of CNA and its mutants in the presence and absence of CaM. The data with error bars are expressed as means ± S.D. ($n = 3$).

were both present, the phosphatase activity of the three mutants was still much lower than WT CNA (Fig. 2B).

3.2.3. The phosphatase activity of CNA and its derivatives The basal phosphatase activities of CNA and its mutants did not differ greatly. The phosphatase activity of E53G and E53A alone were 2.1 and 1.1 times that of WT CNA, respectively (Fig. 2C). In addition, the effect of CaM on the phosphatase activity of the CNA derivatives was similar, indicating that the substitutions at Glu53 did not inhibit CaM binding to CNA.

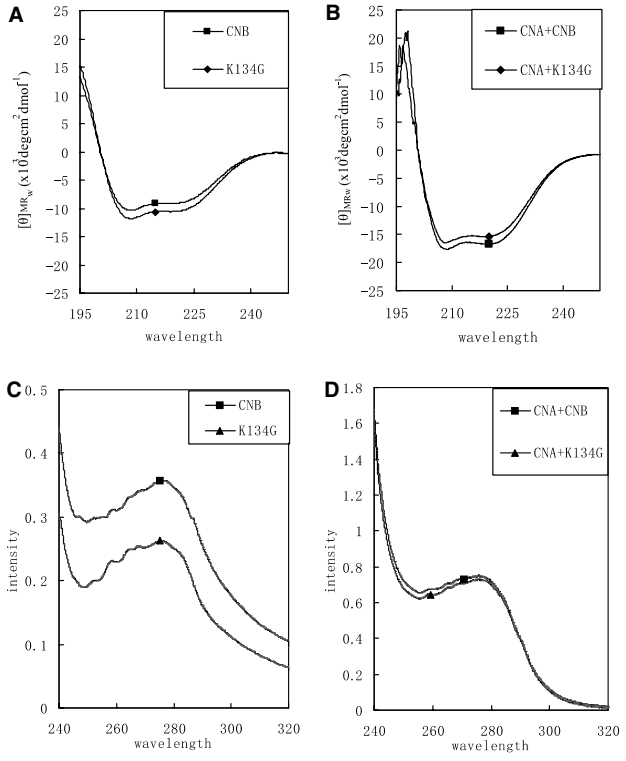

Fig. 3. CD and UV spectra assay of CNB and K134G. (A) CD spectra of CNB and K134G. (B) CD spectra of CNB and K134G in the presence of CNA. (C) UV spectra of CNB and K134G. (D) UV spectra of CNB and K134G in the presence of CNA.

3.3. CD and UV-absorbance spectra

3.3.1. The CD and UV-absorbance spectra of CNB and K134G. There are minor differences between both the CD spectra and UV spectra of CNB and K134G whether CNA present or not (Fig. 3). Table 1(A) indicates that the secondary structures of CNB and K134G are a little different. We presume that disruption of the salt bridge affects the correct conformation forming between CNA and CNB.

3.3.2. The CD and UV-absorbance spectra of CNA and its derivatives. The CD spectra of E53G and E53A had somewhat greater negative peaks than WT CNA (Fig. 4A), and their α-helix contents are much higher than WT CNA (Table 1(B)). The UV-absorbance spectra of WT CNA and E53A were similar, but E53G had a higher peak at 265 nm (Fig. 4C).

3.3.3. The CD and UV-absorbance spectra of CNA and its derivatives in the presence of CNB. In the presence of CNB, the CD spectra of E53G and E53A were almost the same, with a lower negative peak than WT CNA (Fig. 4B). When CNB added, the changes in the UV spectra of the various forms of CNA are quite similar when compared with the spectra in the absence of CNB (Fig. 4D). The fact that changes in secondary structures of CNA and its mutants are different upon addition of CNB (Table 1(B)) indicates that our mutation may affect the correct binding of CNB to CNA and the formation of correct conformation.

4. Discussion

The essential role of CNB in supporting CN phosphatase activity and the requirement of CNB for Ca^{2+} has been well established [21,22], but it is not clear how CNB interacts with and regulates CNA. The crystal structure of human CN-FKBP12-FK506 reveals that three regions of CNA are mainly responsible for the interaction with CNB: the BBH, the N-terminus and residue Glu53 that forms a salt bridge with Lys134 of CNB [11,12]. The corresponding region of CNB is the latch region, an element of the tertiary structure that forms when CNB binds CNA and is considered important in both docking of the immunosuppressant–immunophilin complex and phosphatase activation [13]. The hydrophobic features of BBH and CNB are important for complexing of CNA and CNB [13,23]. Little is known about the role of the salt bridge in the interaction of CNA and CNB.

Table 1
The percentage of secondary structures contents was estimated using the software in Jasco J-720 CD instruments according to the CD spectra showed in Figs. 3 and 4

	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
(A) The computed percentage of secondary structure contents of CNB/K134G, respectively and with CNA				
CNB	28.3	39.2	2.7	29.8
K134G	29.1	37.8	3.3	29.8
CNA + CNB	23.0	27.9	22.4	26.7
CNA + K134G	22.8	32.1	18.2	26.9

	α-Helix (%)		β-Sheet (%)		β-Turn (%)		Random coil (%)	
	CNB^-	CNB^+	CNB^-	CNB^+	CNB^-	CNB^+	CNB^-	CNB^+
(B) The computed percentage of secondary structure contents of CNA and its derivatives in the absence and presence of CNB								
CNA	21.3	23.0	34.5	27.9	19.6	22.4	24.6	26.8
E53G	33.0	20.9	21.5	41.1	12.9	13.2	32.6	24.8
E53A	30.3	20.6	33.1	38.5	10.1	16.2	26.6	24.7

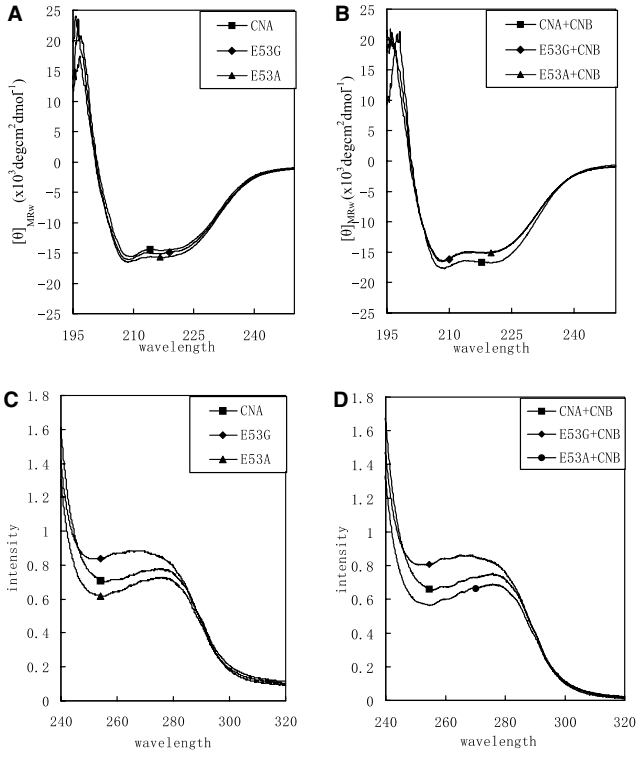

Fig. 4. CD spectra and UV spectra assay of CNA and its mutants. (A) CD spectra of WT CNA and its three mutants. (B) CD spectra of WT CNA and its three mutants in the presence of CNB. (C) UV spectra of WT CNA and its mutant derivatives. (D) UV spectra of WT CNA and its three mutants in the presence of CNB.

In order to clarify the role of salt bridge in the interaction between CNA and CNB, we mutated the Glu53 of CNA to Glycine (E53G) and Alanine (E53A), and the Lys134 of CNB to Glycine (K134G).

The mutations at Lys134 of CNB and Glu53 of CNA both block formation of the salt bridge, but their effects on the interaction of CNA and CNB were different. The effect of K134G on the phosphatase activity of WT CNA was weaker than that of CNB and the mutation at Glu53 of CNA greatly decreased its sensitivity to CNB. The presence of CNB increased the phosphatase activity of WT CNA about 24-fold, whereas with E53G and E53A it increased at most 3-fold. The differences between the CD spectra, as well as the computed secondary structures contents, and the UV-absorbance spectra indicate that our mutation affects the correct binding and the correct conformation forming between CNA and CNB. It is reported that a synthesized BBH peptide can bind to CNB in vitro [24]. Though much lower, CNB do can activate the phosphatase activity of E53G and E53A with RII as the substrate. In the preliminary experiment, CNB can activate E53G and E53A to a relative higher degree (data not shown). And so, our mutation at the salt bridge unlikely disrupts the interaction between CNA and CNB. Thus, the phosphatase activity assays and the structural assays both indicate that mutation of the salt bridge affects the closeness of the association between CNA and CNB and reduces the activation of CNA phosphatase activity by CNB.

A novel CN splice variant that contains the N-terminus, the CaM binding domain and the autoinhibitory domain of CNA has been cloned from human spinal cord cells. This splice variant revealed a stimulating effect on CN activity at low Ca^{2+} concentrations as well as protein-protein interaction with the catalytic CN holoenzyme [25]. This result brings a possibility that the N-terminus of CNA can regulate the phosphatase activity of CN, maybe. In our experiment, E53G and E53A both had somewhat higher phosphatase activity than WT CNA probably because glycine is more flexible than other amino acids. In the crystal structure, CNA residues 1–13 are invisible due to their high mobility. Mutation of glutamic acid to glycine may increase this flexibility greatly for the 54th residue of CNA is also glycine. This excess flexibility possibly affects the correct orientation of the N-terminus and even cause unwanted interaction with other domains of CNA. This maybe the reason that E53G shows obviously higher phosphatase activity than WT CNA and when glutamic acid is substituted by alanine, an amino acid not so flexible as glycine, its phosphatase activity is only 1.1 times that of WT CNA.

This speculation can also explain why K134G retained 75% of the normal ability to activate CNA, but that mutation at Glu53 made CNA almost completely insensitive to CNB. Our mutation at Glu53 of CNA may cause incorrect orientation of the N-terminus of CNA, apart from disrupting the salt bridge. This may affect the interaction between the N-terminus and the C-terminus of CNB, and thus the effect of CNB binding to CNA can not be transferred to CNA effectively. Furthermore, Glu53 of CNA is an important amino acid for CN to form correct conformation.

In conclusion, the phosphatase activity and structural assays both indicate that mutation of the salt bridge affects the closeness of the association between CNA and CNB and greatly reduces the activation of CNA phosphatase activity by CNB. We speculate that the salt bridge plays its role in transferring the effect of CNB binding to CNA through two ways: stabilize the interaction between two subunits and ensure the correct orientation of the N-terminus of CNA. That is maybe the reason that mutation at CNB and CNA to defect the salt bridge will cause distinctly different effect in CN function.

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