hematopoietic progenitor cells from the toxic effects of two classes of chemotherapeutic drugs *in vitro*. Such multiple chemoprotection has the potential to decrease or eliminate much of the host toxicity associated with combination chemotherapy. Furthermore, it is noticed that the transduction efficiency in this experiment is relatively low. This limitation may be due to the apparent low titer of retrovirus containing the large size of inserted *mdr1* cDNA (~4.1 kb), as well as the interference between two separate promoters in dual gene vectors. By choosing different configurations of retroviral vector and optimized transduction protocol, the improved transduction efficiency can be expected. Indeed, low transduction efficiency is the main obstacle in current gene therapy for human hematopoietic progenitor cells. The highest efficiency reported so far in progenitor cells was only 5% after reinfusion of transduced autologous bone marrow. Although a variety of reasons contribute to this issue, retroviral construct carrying drug resistance gene, especially dual drug resistance genes, might permit enrichment of the transduced hematopoietic cells population by *in vivo* selection. This strategy may have significant implications for future cancer gene therapy protocols.

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Activities and properties of calcineurin catalytic domain

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Abstract Calcineurin (CN) is the only protein phosphatase known to be under the control of calcium (Ca^{2+}) and calmodulin (CaM). The enzyme consists of two subunits, the catalytic A subunit of 61 ku (CNA) and a regulatory B subunit of 19 ku (CNB). In this study, we used PCR amplication to construct a truncation consisting of only the CNA catalytic domain. The truncation was induced by IPTG and expressed in *E. coli*. PNPP was used as a substrate to study the phosphatase activity of the CNA catalytic domain. The findings show that its activity is 20 times greater than CNA in the presence of CNB and CaM. The optimum reaction temperature for the CNA catalytic domain protein is 40℃, and the optimum reaction pH value is 8.0. Mn^{2+} is still an effective activator for the CNA catalytic domain, but its activity is not controlled by Ca^{2+}. In the presence of 6 mmol/L Mg^{2+}, adding either Ca^{2+} or EGTA did not change the activity of the CNA catalytic domain.

Keywords: calcineurin, protein phosphatase, catalytic domain, activity, enzyme property.

Calcineurin (also called protein phophatase 2B, PP2B), a major calmodulin-binding protein in the brain and the only serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin, plays a critical role in the coupling of Ca^{2+} signals to cellular responses[1, 2].

Calcineurin (PP2B) shares high sequence homology with other members of the Ser/Thr PPase family (PPP family), PP1, PP2A and λPPase[3]. The major homology region is in their catalytic domains. In the PPP family, analysis of the primary sequence of all the protein phosphatases revealed considerable conservation with some 42 invariant residues dispersed throughout the primary sequences . Furthermore, the members of the PPP family had very similar tertiary structures. CN and PP1 have the same tertiary structure in their catalytic domain[4]. X-ray crystal structure analysis indicates that CN, PP1, and λPPase have similar catalytic mechanisms[5—8]. They are all metalloenzymes with two divalent metal ions located at the center of the catalytic site. The substrate dephosphorylation binds through a metal-bound water molecule acting as a nucleophile to attach the phosphorus atom of a phosphate group in an S_N2 mechanism[9,10]. In the PPP family, considerable structural and functional diversity of individual protein phosphatases are probably created as a result of a combination of associated regulatory domains and subunits. Compared with PP1 and PP2A, CNA contains 170 extra residues at its C-terminus[3]. This sequence has been shown to contain the CNB-binding domain, a CaM-binding domain, and an autoinhibitory domain (AID), all of which are involved in regulating calcineurin phosphatase activity.

In order to find the structural composition of CN and the relationship between different domains and phosphatase activity, early in 1989 Dr. C.B. Klee's group did extensive research work on the CN using the limited proteolysis method. They first used trypsin to study the limited degradation of CNA. Then, they used limited proteolysis of CN with clostripain sequentially to define four functional domains in CNA: the catalytic domain, CNB-binding domain, CaM-binding domain and autoinhibitory domain. The autoinhibitory and CaM-binding domains are readily removed from the protease-resistant core which contains the CNB-binding domain and the catalytic domain. The CNB-binding domain and the catalytic domain are not easily separated by proteolysis. Therefore, the properties of the catalytic domain have not been carefully studied[11].

The cDNA of CN was first available in the early 1990s, which provided the foundation to develop the structural and functional research on CN at the molecular level. Our laboratory has performed studies on the CNA catalytic domain after developing a good foundation to efficiently express high activity of mammalian CN in *E. coli*. We constructed the CNA catalytic domain truncation which was expressed into the same bacteria strain induced by IPTG. A large amount of CNA catalytic domain protein was then purified to study the difference between its activities and properties and those of CNA.

1 Materials and methods

(i) Materials. The cDNA for the αδ isoform of CNA, isolated from rat brain cDNA libraries, was the generous gift from Drs. B. Perrine and T. R. Soderling of Vollum Insitute, Portland, Oregon. Calmodulin was isolated from bovine brain[12] and CaM-sepharose was prepared by coupling to CNBr-activated Sepharose (Rharmacia-LKB Biotech). Polyclonal antibodies against the expressed CNA catalytic domain were raised in mice using the methods described in ref. [13]. DEAE-52 cellulose was obtained from Whatman. Restriction enzymes and ligase were obtained from New England Biolabs. Taq polymerase was obtained from the PE company. Prestained protein standard was obtained from New England Biolabs. DNA sequence analysis was performed by dideoxynucleotide sequencing.

(ii) Construction of vectors and expression. The coding sequences of the CNA catalytic domain were isolated by PCR amplification. *Nde* I and *Hin*dIII restriction sites were engineered at the 5′ and 3′ ends, respectively, using the primers 5′-AGG AGA TAT ACA TAT GTC CGA GCC CAA-3′ and 5′-CGC GAA GCT TCA CAT GAA ATT TGG GAG CC-3′. PCR fragment of the CNA catalytic domain was subcloned into the pET-21a expression vector (Novagen) using *Nde* I and *Hin*dIII restriction sites. Expression of CNA catalytic domain was performed in *E. coli* HMS174(DE_3) host cells grown at 37℃ in Terrific media (1.2% tryptone, 2.4% yeast extract, 1% NaCl, 0.6% glycerol, 50

μg/mL ampicillin) in 1 liter cultures. Cells were grown at 37℃ until the optical density at 600 nm reached a value of 0.7. Expression was then induced by addition of 0.5 mmol/L isopropyl β-thio galactoside (IPTG), followed by an additional 4-h growth at 37℃. CNA was expressed according to the method in refs. [14, 15].

(iii) Assay of phosphatase activity using *p*-nitrophenyl phosphate (PNPP). The activities of CNA and CNA catalytic domain were assayed using PNPP as the substrate. The assays using Mn^{2+} as an activator were performed in 50 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L $MnCl_2$, 0.5 mmol/L dithiothreitol (DTT), 0.2 mg/mL bovine serum albumin, 20 mmol/L PNPP, with different Mn^{2+} concentrations from 0.1—2 mmol/L. When 6 mmol/L $MgCl_2$ was used as an activator, these variations were studied: with Ca^{2+}, without Ca^{2+} and with EGTA. Reactions were performed in a volume of 0.2 mL at 30℃ for 20 min (except for the experiments to determine the optimum reaction temperature for which the enzyme was assayed for l0 min at different temperatures) and terminated by the addition of 1.8 mL 0.5 mol/L Na_2CO_3, 20 mmol/L EGTA. The absorbance at 410 nm was measured using a control lacking enzyme. One unit of activity was defined as that catalyzing the hydrolysis of 1 mmole of PNPP per minute. CaM and CNB, when being added, had concentrations of 1.5 and 0.1 μmol/L, respectively (Kinetic analyses were performed in the presence of Mn^{2+} at PNPP concentrations of 1.25, 2.5, 5, 10 and 20 mmol/L). The data were analyzed using Excel.

(iv) Protein determination and Western blotting determination. Protein determinations were performed by the Bradford method[16]. SDS-PAGE used 12% separation gel to detect purified CNA, crude lysate of the CNA catalytic domain and purified CNA catalytic domain. Western blotting was performed according to the method described in ref. [17].

2 Results

(i) Expression and purification of CNA catalytic domain. As described above in the Materials and methods, the coding sequence of CNA catalytic domain was isolated by PCR amplification. The PCR fragment was subcloned into the pET-21a expression vector. The full-length DNA sequence was confirmed by dideoxynucleotide sequencing. Expression of the CNA catalytic domain was performed in *E. coli* HMS174 (DE_3) host cells. The cells from 1 liter cultures were harvested by centrifugation at 4 000 r/min at 4℃ for 20 min and resuspended in 1/10 volume of buffer (100 mL) containing 50 mmol/L Tris-HCl, 1 mmol/L EDTA and 1 mmol/L DTT, pH 7.4. The cells were disrupted by supersonic sound waves. The lysate was then centrifuged at 20 000 r/min for 1 h at 4℃. The supernatant contained CNA catalytic domain protein. The supernatant was adjusted to pH 7.7, then poured into a DEAE-52 ion exchange column, washed with the equilibrating buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L DTT, pH 7.4). The last peak contained the CNA catalytic domain protein. CNA was expressed and purified according to the method in ref. [16]. SDS-PAGE used 12% separation gel to detect purified CNA, crude lysate of the CNA catalytic domain and purified CNA catalytic domain. The output from the SDS-PAGE shows that the purified CNA and CNA catalytic domain are of electrophoresis purity (fig. 1). Western blotting was used to detect the CNA and CNA catalytic domain through the polyclonal antibody against CNA. The results proved that CNA catalytic domain is the truncation of CNA.

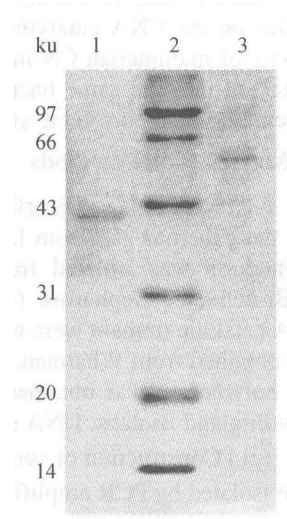

Fig. 1. Purification of CNA and CNA catalytic domain expressed in *E. coli* samples subjected to SDS-PAGE. Lane 1, Purified CNA catalytic domain; lane 2, protein standards; lane 3, purified CNA.

(ii) Comparison of CNA and CNA catalytic domain activities. The phosphatase assay was done using Mn^{2+} as the activator. The phosphatase activities of CNA and CNA catalytic domain were analyzed in the following four cases: in the presence of CaM and CNB, in the presence of only CaM, in the presence of only CNB, and in the absence of both CaM and CNB. The CNA activity increased sequentially, but the CNA catalytic domain activity remained unchanged (fig. 2). Moreover, in the presence of both CaM and CNB,

the activity of the CNA catalytic domain was 20 times greater than that of CNA.

(iii) Regulation of metal ions to phosphatase activity.

(1) Regulation of Ca^{2+} and Mg^{2+} to phosphatase activity. 6 mmol/L Mg^{2+} was used as an activator at pH 8.5. The phosphatase activity was determined in four cases: 1 mmol/L EGTA, in the absence of EGTA and Ca^{2+}, 1 mmol/L Ca^{2+} and 2 mmol/L Ca^{2+}. The phosphatase activity remained unchanged (fig. 3), suggesting that the CNA catalytic domain was not regulated by Ca^{2+}. However Mg^{2+} is still an effective activator of the CNA catalytic domain at pH 8.5.

(2) Regulation of phosphatase activity by Mn^{2+}. The phosphatase activity was detected at different

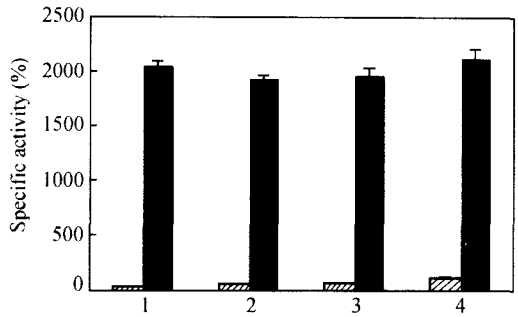

Fig. 2. Comparison of CNA and CNA catalytic domain activities. 1, In the absence of both CaM and CNB; 2, in the presence of only CaM; 3, in the presence of only CNB; 4, in the presence of both CaM and CNB. ▨ CNA; ■ CNA catalytic domain. In the presence of both CaM and CNB, the phosphatase activity for CNA was defined as 100%. The above results were average value of three-time experiments.

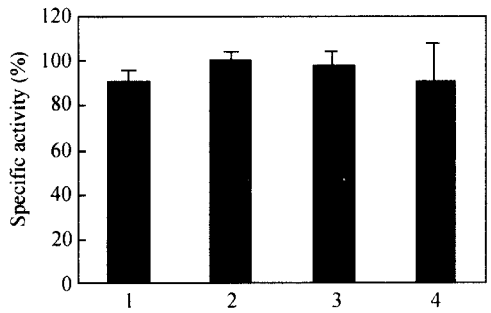

Fig. 3. Regulation to phosphatase activity by Ca^{2+} and Mg^{2+}. 1, 1 mmol/L EGTA; 2, in the absence of EGTA and Ca^{2+}; 3, 1 mmol/L Ca^{2+}; 4, 2 mmol/L Ca^{2+}. In the absence of EGTA and Ca^{2+}, the phosphatase activity for CNA catalytic domain was defined as 100%. The above results were average value of six-time experiments.

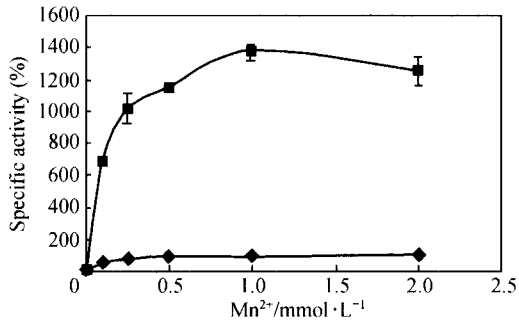

Fig. 4. Regulation of phosphatase activity by Mn^{2+}, ◆, CNA; ■, CNA catalytic domain. When Mn^{2+} concentrations is 1 mmol/L, the phosphatase activity for CNA was defined as 100%. The above results were average values of three-time experiments.

Mn^{2+} concentrations: 0.1, 0.25, 0.5, 1 and 2 mmol/L. The CNA and CNA catalytic domain activities all increased with the increasing of Mn^{2+} concentrations (fig. 4). The data indicate that the CNA catalytic domain activity is regulated by Mn^{2+}.

(iv) Optimum reaction temperature curve and optimum reaction pH curve. The phosphatase activity was detected at different temperatures (fig. 5(a)) and different pH (fig. 5(b)). As the

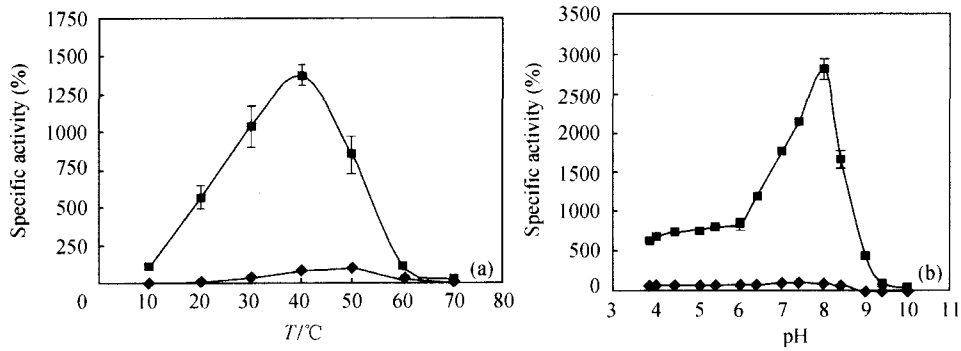

Fig. 5. Optimum reaction temperature curve (a) and optimum reaction pH curve (b) of CNA and catalytic domain. When the reaction temperature is 50℃ and the reaction pH is 7.0, the phosphatase activity for CNA was defined as 100%. The above results were average values of three-time experiments. ◆, CNA; ■, CNA catalytic domain.

temperature and pH increased, the CNA catalytic domain activity first increased, then decreased. The optimum CNA reaction temperature was 50℃, while the optimum CNA catalytic domain reaction temperature was 40℃. The optimum CNA reaction pH was pH 7.0, while the optimum CNA catalytic domain pH was 8.0.

3 Discussion

Molecular biology was used to construct, express and purify CNA catalytic domain. Study of the phosphatase activities and properties showed that the CNA catalytic domain phosphatase activity is very high. In the presence of CaM and CNB, its activity is 20 times greater than that of natural CNA. Before our study of the CNA catalytic domain, we have studied many CNA derivatives with truncated functional domains. The results suggest that when the auto inhibitory is deleted from CNA, its activity is 5 times greater than the original CNA. When the autoinhibitory and the CaM binding domain were deleted, the CNA activity increased more than 10 times[18]. However, this study showed that when the autoinhibitory, CaM-binding domain and CNB binding domain were all deleted, the activity of the remaining catalytic domain was 20 times greater than the original CNA. Therefore, the CNA catalytic domain is the core region for the CN catalysis function, and the role of the other CNA functional domains is to regulate the activity of the CNA catalytic domain. Also this regulation by the other domains is additive.

In the Ser/Thr phosphatase family (PPP family), the catalytic domains are highly conserved and the tertiary structures and catalytic mechanisms are also similar. Studies of the CNA catalytic domain activity indicate that the actual catalytic region in CN (PP2B) is its catalytic domain. The data also suggest that the catalytic domains and the catalytic mechanisms in the PPP family are highly homologous.

The Ca^{2+} dependence of the CN phosphatase activity is controlled by two structurally similar but functionally different Ca^{2+} binding proteins, CaM and CNB[2]. CNB and CaM are Ca^{2+} binding proteins with four "EF-hand" Ca^{2+} binding sites. CNB and CaM share some degree of similarity (35% identity). Despite their structural similarity, the two proteins are not functionally interchangeable. CNB cannot replace CaM in the activation of CNA, and CaM cannot substitute for CNB in the reconstitution of CN[18]. CNB only binds to the CNB-binding domain of CNA, while CaM only binds to the CaM-binding domain of CNA. In the presence of Ca^{2+}, CNB and CaM interact cooperatively to regulate the CNA phosphase activity[19]. Thus, we analyzed the phosphatase activity of CNA and the CNA catalytic domain in the following four cases: in the absence of CaM and CNB, in the presence of CaM, in the presence of CNB and in the presence of both CaM and CNB. The CNA activity increased sequentially, but the CNA catalytic domain activity remained unchanged. The data indicated that the phosphatase activity of the CNA catalytic domain was not regulated by CaM and CNB, which is to be expected since the CNB and CaM-binding domains had been deleted, so it could not be affected by the two proteins.

To prove that the CNA catalytic domain was not regulated by Ca^{2+}, the phosphates activity of the CNA catalytic domain was measured in the presence of Ca^{2+} or EGTA. The data showed that at pH 8.5 with 6 mmol/L $MgCl_2$, the phosphatase activity of the CNA catalytic domain was not altered by the presence or absence of Ca^{2+} or EGTA.

The Mn^{2+} activation curve of the CNA catalytic domain showed that as with CNA, Mn^{2+} is still an effective activator of the CNA catalytic domain. Though the phosphatase activity of the CNA catalytic domain was already 10 times greater than that of CNA, the activity continued to increase with the increasing of Mn^{2+} concentration. The investigation (sec. 2(iii)(1)) not only shows that the CNA catalytic domain is not regulated by Ca^{2+}, but also shows that the CNA catalytic domain activity is activated by Mg^{2+} in alkaline pH. All the experimental data further indicate that the CNA catalytic domain retains the catalytic properties of natural CN.

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Recombinant constructions and infectivity analysis of tobacco mosaic virus and attenuated tomato mosaic virus N14 genomes

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Abstract The recombinant clones of pTN and pNT have been constructed by exchanging the coding regions of the movement proteins (MP), coat proteins (CP) and 3′ noncoding regions between the cDNAs of the tobacco mosaic virus (Chinese Isolate, TMV-Cv) and the attenuated tomato mosaic virus N14 genomes, and used as templates for *in vitro* runoff transcription. Their transcripts have been used for tobacco infection assays. The infection results show that the transcripts of pTN and pNT are infectious. Local lesions were observed in the leaves of *Nicotiana tabacum cv. Samsun NN* inoculated with pTN transcript, but were fewer than those in the same kind of plant induced by pTMV-Cv transcript. Systemic symptoms were also observed in *N. tabacum cv. Huangmiaoyu* induced by pTN transcript, but were slighter than those on the same kind of tobacco induced by pTMV-Cv transcript. Local lesions were shown in *N. tabacum cv. Samsun NN* inoculated with pNT transcript, but were more than those in the same kind of plant induced by pN14 transcript while no systemic symptom was displayed in *N. tabacum cv. Huangmiaoyu*. These results suggest that the recombinant viruses of TN and NT are able to