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Enzymatic and thermodynamic analysis of calcineurin inhibition by RCAN1

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ABSTRACT

Calcineurin (CN) is the target of the immunophilin–immunosuppressant complex, cyclophilin/cyclosporin A (CyP/CsA). RCAN1 has recently been shown to be an endogenous regulator of CN activity. We determined the enzymatic and thermodynamic aspects of CN inhibition by RCAN1. The IC50 values of isoforms RCAN1-1L and RCAN1-4 for CN were 2.7 μM and 2.6 μM, respectively. Two deletions in the CN catalytic subunit, one a deletion of Val314 in the Loop7 domain (ΔV314) and the other in the autoinhibitory domain (CNAabc), increased the sensitivity of CN to inhibition by RCAN1-1L. The IC50s of RCAN1-1L and RCAN1-4 for CN in homogenates of mouse brain were 141 nM and 100 nM, respectively. Using isothermal titration calorimetry (ITC), we found that the RCAN1-1L/CN or CyP/CsA/CN interactions were exothermic with a dissociation constant of 0.46 μM or 0.17 μM, respectively. Our ITC results show that the interactions between CN and its two inhibitors were both characterized by a favorable binding enthalpy change. We also confirmed that overexpression of RCAN1-1L could inhibit the transcriptional activation of an NFAT-dependent promoter in response to PMA and ionomycin by inhibiting CN activity in HEK293T cells. Our data should contribute to our understanding of the regulation of CN activity by endogenous inhibitors.

1. Introduction

Calcineurin (CN), a member of the serine/threonine phosphatase family of enzymes, is highly abundant in the brain. It consists of a catalytic subunit, CNA (61 kDa), and a regulatory subunit, CNB (19 kDa). CNA comprises four regions: a catalytic domain (residues 20–340), a CNB-binding domain (BBH, residues 349–372), a calmodulin-binding segment (CBD, residues 390–414), and a C-terminal autoinhibitory domain (AID, residues 469–486) [1]. CN is widely distributed among eukaryotes with a structure that is conserved from yeast all the way to man. However, it has a narrower range of substrates than other phosphatases; its substrates include the transcription factor NFAT, which is involved in T-cell activation, and cytoskeletal proteins such as tau, which is phosphorylated at multiple serine/threonine sites in early Alzheimer's disease [2].

CN is the only phosphatase regulated by the second messenger Ca^{2+} together with calmodulin (CaM). Activation of CN upon an increase in intracellular Ca^{2+} leads to dephosphorylation of its substrates, including NFAT. Dephosphorylated NFAT translocates to the nucleus, where, in cooperation with other transcription factors such as AP-1, it induces the expression of target genes, such as cytokine genes in human T cells [3]. Because CN has important roles in signal transduction, many studies have focused on the regulation of its phosphatase activity.

The immunosuppressant cyclosporin A (CsA) is the most specific and well-known inhibitor of CN. CsA inhibits CN activity after forming a complex with the cytoplasmic immunophilin, cyclophilin (CyP) [4]. This immunophilin–immunosuppressant complex binds CN and inhibits its function by sterically hindering access of substrates to its catalytic site. Interestingly, *in vitro* CN inhibition by CyP–CsA complexes only occurs when a physiological substrate is used to assay the enzyme, e.g., phospho-RII peptide whose sequence corresponds to the phosphorylation site of the regulatory subunit of cAMP-dependent protein kinase. The inhibitory effect of CsA on CN has been elucidated in studies of the immune system. In T lymphocytes, CsA specifically inhibits CN activity and turns off the cascade of T cell activation [5].

RCAN1, an endogenous regulator of CN, has been shown recently to modulate CN activity under both physiological and pathological

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conditions. *RCAN1* was first identified as a gene in the Down's syndrome critical region on human chromosome 21. It contains seven exons, and differential promoter usage and first exon choice can generate several transcripts. The different mRNAs comprise one of the four possible initial exons (E1 through E4) plus the three exons (E5 through E7) common to all forms of *RCAN1* mRNA. All mRNA isoforms contain exons 5–7, while the three isoforms most studied also contain either 29 amino acids (RCAN1-1S), 55 amino acids (RCAN1-1L) encoded by exon 1, or 29 amino acids (RCAN1-4) encoded by exon 4 [6]. RCAN1 proteins can bind to CN and inhibit its activity. The primary CN binding portion of RCAN1 is encoded by exon 7, which is common to all RCAN1 isoforms [7–10]. RCAN1 is implicated in calcium-mediated oxidative stress [11], cardiac hypertrophy [12], VEGF-mediated signaling during angiogenesis [13] and Alzheimer's disease [14,15]. Hence, RCAN1 plays a role in many physiological and pathological CN-dependent processes [16].

Usually, the CN phosphatase activity can be determined using the chromogenic substrate *para*-nitrophenyl phosphate (pNPP) or the phosphor-RII peptide as the specific substrate. In the previous study, the full-length RCAN1 proteins have been used to determine the kinetic parameters toward pNPP substrate [8]. Compared to the conveniently measurable pNPP assay, the RII peptide assay is more sensitive and specific. The sequence of phospho-RII peptide represents the phosphorylation site of the regulatory subunit of cAMP-dependent protein kinase, a well characterized and more physiological phosphopeptide substrate [17]. But most researchers used synthetic RCAN1-derived peptides to determine its inhibitory effect on CN activity with phospho-RII as the substrate [18]. In our present study, we purified the full-length RCAN1 proteins and examined their effects on CN activity with phosphor-RII as the substrate. We also compared of the extent of inhibition of native CN by RCAN1 proteins and CsA in homogenates of mouse brain. In addition, we used isothermal titration calorimetry (ITC) to determine the thermodynamic parameters of RCAN1-1L binding to a recombinant single chain calcineurin (called BA). ITC is the most quantitative means available for measuring the thermodynamic properties of protein–protein interactions. Our results could be helpful in understanding the regulation of CN activity by endogenous inhibitors.

2. Materials and methods

2.1. Materials

RII peptide, a CN substrate, was obtained from BioMoL Research Laboratories, Inc. (PA, USA). CsA and okadaic acid were from Sigma Chemical Co. (Missouri, USA). All other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

2.2. Construction of vectors

RCAN1-1L and *RCAN1-4* were amplified by PCR and subcloned into pET21a vector using the *XhoI* and *EcoRI* restriction sites. The recombinant plasmids were transformed into *Escherichia coli* DH5α for screening, and transformed into *E. coli* BL21 (DE3) for expression. EGFP/RCAN1-1L and pcDNA3.1/RCAN1-1L were generated using primers GCCTCGAGATGG AGGACGGCGTGGC (*XhoI*)/GCGAATTCTCAGCT GAGGTGGATC (*EcoRI*) and TAGAATTC-GATATGGAGGACGGCGTG (*EcoRI*)/TGCCTCGAGTCAGCTGA GGTG-GATGG (*XhoI*). The expression vector pTrcHisC/CyP was kindly supplied by Prof. Yin Gao of Capital Normal University of China.

2.3. Expression and purification of proteins

RCAN1-1L and RCAN1-4 were expressed in *E. coli* BL21 (DE3). Cultures were induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 16–18 h at 18 °C. They were then centrifuged at 5000 rpm for 20 min at 4 °C, and the pellets were stored at −20 °C. Lysates obtained by sonication were centrifuged at 16,000 rpm for 30 min at 4 °C. The supernatants were collected and applied to a Ni–nitrilotriacetic acid–agarose column equilibrated in the binding buffer (50 mM Tris–HCl, 300 mM NaCl and 0.1% phenylmethylsulfonyl fluoride, pH 8.0). The proteins were eluted with a gradient of 10–100 mM imidazole in elution buffer (50 mM Tris–HCl and 0.1% phenylmethylsulfonyl fluoride, pH 8.0). Protein purity was assessed by SDS-PAGE, and protein concentrations were measured by the procedure of Bradford.

For subsequent thermodynamic experiments, a recombinant single chain CN (called BA) was used. It is a fusion of the A and B subunits using a pair of linker primers including six glycine residues. The recombinant enzyme has high specific activity, and biochemical properties and kinetic parameters similar to those of the native enzyme from bovine brain [19].

We also used two mutant forms of CNA: one, called ΔV314, has a deletion of Val314 in the Loop7 region, and the other called CNAabc has a deletion of the autoinhibitory domain. CNA, CNB, CaM, BA, ΔV314 and CNAabc were expressed and purified as previously described [20].

2.4. Assay of calcineurin activity

Purified CN was concentrated with an Amicon Ultra Filter Unit and diluted in 50 mM Tris–HCl, 0.5 mM dithiothreitol, 0.1 mg/ml BSA, and 50% glycerol. Phosphatase activity was measured mainly as described [21].

Male Kunming mice (weight 16 ± 2 g, 4 weeks old) were supplied by the Experimental Animals Center of Peking University. Animals were group housed under following laboratory conditions: temperature 20 ± 1 °C, humidity 40–60%, 12:12 – L/D cycle, lights on at 08:00 h. Mice had free access to food and water. Mice were killed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The experimental procedures were approved by the Animal Ethics Committee of Beijing Normal University and were carried out in strict accordance with the NIH Guide for Care and Use of Laboratory Animals. Mice were killed and the brains were immediately removed and homogenized by passing through a syringe at 4 °C into 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM dithiothreitol, 0.2% NP-40, 1.0 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 μg/ml pepstatin. Air bubbles should be avoided at this stage. The tissue homogenate was then centrifuged at 16,000 rpm, and the supernatant was used in phosphatase assays [22]. CN activity in mouse tissues was determined with a Calcineurin Cellular PLUS-AK-816 Assay Kit (BioMol) according to the manufacturer's instructions. The kit is provided with the BIOMOL GREEN™ QuantiZyme™ Assay system, and is a complete colorimetric assay kit for measuring CN phosphatase activity in tissue/cell extracts. CN activity was measured as the rate of dephosphorylation of a synthetic phosphopeptide substrate (RII peptide). The amount of PO_4 released was determined colorimetrically with the BIOMOL GREEN reagent. Phosphatase activities are presented as picomoles phosphate released/mg protein/min.

HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5 μM L-glutamine, and penicillin streptomycin. Cells were grown at 37 °C in humidified air containing 5% CO_2. They were washed twice with 1 ml of phosphate-buffered saline on ice, and lysed in 40 μl of hypotonic buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA,