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Inhibition of calcineurin by cyclosporin A–cyclophilin requires calcineurin B

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The interaction of the immunosuppressive complex cyclosporin A–cyclophilin (CsA-CyP) with the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin is investigated using a recombinant form of the A subunit of calcineurin (rCNA). Only in the presence of purified calcineurin B (CNB) does rCNA show the response of native calcineurin, i.e. 50% inhibition of rCNA phosphatase activity at 6 nM human cyclophilin B and 0.6 μM human cyclophilin A using [^{32}P]casein as substrate, yet stimulation of activity with *p*-nitrophenyl phosphate as substrate. This study demonstrates that the B subunit is necessary to confer sensitivity of calcineurin to CsA-CyP.

Calcineurin; Cyclosporin A; Cyclophilin; Immunosuppression

1. INTRODUCTION

The immunosuppressants cyclosporin A (CsA) and FK506 inhibit T-cell activation via a common pathway by preventing the transcriptional activation of the interleukin-2 gene [1–5]. These drugs bind to distinct intracellular receptors, CsA to cyclophilin (CyP) [6,7] and FK506 to FK506-binding protein [8,9]. Recently, the Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase calcineurin (CN) was implicated as the common proximal target of these drug receptor complexes [10]. Several studies have since supported this by demonstrating that both CsA-Cyp and FK506–FKBP complexes inhibit calcineurin in vitro [10–13], and that overexpression of calcineurin in the T-cell line Jurkat resulted in an increased resistance to these drugs [14,15].

Calcineurin is a heterodimer comprised of a 59 kDa catalytic A subunit (CNA) and a 19 kDa Ca^{2+}-binding B subunit (CNB). It is distinguished from other serine/threonine protein phosphatases by the presence of the B subunit, which is required for maximal activity [16,17], and additional carboxy-terminal domains within the A subunit that confer CaM dependence [18,19]. From previous work, neither CaM nor the CaM-binding and autoinhibitory domains are necessary for inhibition by CsA-CyP [11,12].

In this report the role of CNB in the interaction of CN with CsA-CyP is investigated through the use of a recombinant form of CNA. It is found that the B subunit is required to confer sensitivity to CsA–CyP complexes.

2. EXPERIMENTAL

2.1. *Enzyme preparation*

Native CN was prepared from bovine brain as described previously [20]. The cDNA gene for the α isoform of rat CNA [21,22] was obtained as a gift from Drs. B. Perrino and T. Soderling. Recombinant CNA (rCNA) was overexpressed in *E. coli* using a T7 system and purified by a method similar to that for native bovine brain CN [20] (Haddy, A., Swanson, S.K.-H. and Rusnak, F., in preparation). CNB was purified from native bovine brain CN by gel filtration chromatography in the presence of SDS [17] and was essentially devoid of phosphatase activity (<1% of the native enzyme). Recombinant human cyclophilin A (hCyPA) [23] and human cyclophilin B (hCyPB) [24] were purified from *E. coli* expression systems provided as gifts from Dr. C.T. Walsh.

2.2. *rCNA-CNB reconstitution*

rCNA and CNB were combined in a ratio of approx. 1:2.5 and preincubated for 5 min at 30°C in 50 mM MOPS, pH 7.0, and 0.5 mM $CaCl_2$ before addition to assay tubes. For comparison with the activity of unreconstituted rCNA (Fig. 1), rCNA was similarly incubated in the absence of CNB.

2.3. *[^{32}P]Casein assays*

[^{32}P]Casein was prepared as described [25] by phosphorylation of casein (Sigma) using cAMP-dependent protein kinase catalytic subunit (Sigma). Assays using [^{32}P]casein as substrate were performed in 50 mM MOPS, pH 7.0, 1.0 mg/ml BSA, 0.5 mM DTT and 1.0 mM $CaCl_2$, $MnCl_2$, CaM, CsA, hCyPA and hCyPB were added as indicated. After addition of CN, rCNA, and/or CNB, the assay tubes were preincubated for 10 min at 30°C. Reactions were initiated by [^{32}P]ca-

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Abbreviations: CaM, calmodulin; CN, calcineurin; CNA, calcineurin A; CNB, calcineurin B; CsA, cyclosporin A; CyP, cyclophilin; hCyPA, recombinant human cyclophilin A; FKBP, FK506 binding protein; hCyPB, recombinant human cyclophilin B; PP, protein phosphatase; rCNA, recombinant calcineurin A.

sein addition and allowed to proceed for 10 min at 30°C before quenching with trichloroacetic acid and BSA as described [25].

2.4. *pNPP assays*

Assays using *p*-nitrophenyl phosphate (pNPP) as substrate were performed in 25 mM MOPS, pH 7.0, 1.0 mM $CaCl_2$, 1.0 mM $MnCl_2$ and 0.40 μM CaM at room temperature (23°C). CsA and hCyPA or hCyPB were added as indicated. After addition of rCNA or rCNA-CNB the solution was preincubated for 15 min at 37°C before initiation of the reaction by the addition of pNPP to a final concentration of 10 mM. The absorbance change at 405 nm was monitored using a microtiter plate reader.

4.5. *Addition of CsA, CyP*

A 5 mM stock solution of CsA, which was provided as a gift from Sandoz, was prepared in 50% ethanol. A 50 μM solution used for addition to the assay tubes was prepared from the 5 mM stock by serial dilution with distilled water. hCyPA and hCyPB stock solutions were diluted to appropriate stock solutions in 50 mM MOPS, pH 7.0. CsA, hCyPA and hCyPB were added to the assay tubes after all other components except for calcineurin.

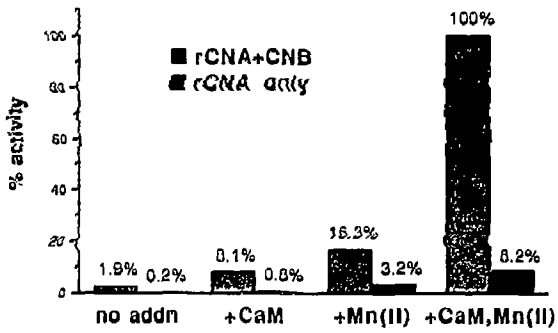

Fig. 1. Effect of CNB on [^{32}P]casein phosphatase activity of rCNA in the presence and absence of CaM and $MnCl_2$: solid bars, rCNA alone; shaded bars, rCNA reconstituted with CNB. Assays were performed in the presence of 81 nM rCNA alone or 20 nM rCNA plus 35 nM CNB and 0.67 μM [^{32}P]casein. When present the concentrations of CaM and $MnCl_2$ were 0.58 μM and 1.0 mM, respectively. % activity is defined as the activity relative to that of rCNA-CNB in the presence of CaM and $MnCl_2$, which showed a specific activity of 28 nmol·min^{-1}·mg rCNA^{-1}.

3. RESULTS

Recombinant calcineurin A (rCNA) was purified from an *E. coli* overexpression system and was therefore devoid of calcineurin B (CNB). The rCNA produced in this manner included a 10 residue fusion sequence with the bacteriophage T7 gene 10 protein [26]. The cDNA for this protein corresponded to a rat α isoform that lacked the polyproline sequence at the N-terminus and a 10 amino acid sequence at the C-terminus [21].

Phosphatase activity was assessed using [^{32}P]casein phosphorylated by cAMP-dependent protein kinase catalytic subunit, a commonly used calcineurin substrate [25,27] with which native CN shows the same inhibition by CsA-CyP as it does with [Ser(^{32}P)^{15}R]$_{11}$ peptide [11]. The phosphatase activity of rCNA was very low, only 1–3 nmol·min^{-1}·mg rCNA^{-1} in the presence of calmodulin (CaM) and $MnCl_2$, compared with 100–150 nmol min^{-1}·mg CN^{-1} for native bovine brain calcineurin (CN) under the same conditions. Addition of CNB purified from bovine brain [17] to rCNA in the presence of CaM and Mn^{2+} led to an increase in phosphatase activity to 25–35 nmol min^{-1}·mg rCNA^{-1}. The increase in specific activity of rCNA reconstituted with CNB (rCNA-CNB) to a level comparable to that of native CN (25%) indicates that a considerable portion of functional native-like rCNA-CNB molecules were formed.

rCNA alone, rCNA-CNB, and native CN showed similar responses to the effectors Mn^{2+} and CaM. In the absence of CNB, the activity of rCNA was stimulated 4-fold by CaM, 15-fold by $MnCl_2$, and 40-fold by both. Similarly, in the presence of CNB, rCNA activity was stimulated 4-fold by CaM, 8-fold by $MnCl_2$ and 50-fold by both (Fig. 1). Native CN is also stimulated by either CaM or $MnCl_2$ and manyfold more by both depending on the preparation [27,28]; the preparation used for these experiments had a low background activity that was stimulated about 25-fold by CaM, 21-fold by $MnCl_2$ and 185-fold by both. The similarity of native and recombinant calcineurin (with and without CNB) in their responses to CaM and $MnCl_2$ indicate that these properties are conferred by the catalytic subunit, as noted previously [16–19]. In addition, rCNA (without CNB) and native CN showed similar responses to the inorganic inhibitors PO_4^{3-} and MO_4^{2-} (data not shown). For comparison of the inhibition of rCNA and rCNA-CNB by CsA-CyP, both $MnCl_2$ and CaM were included in subsequent assays in order to achieve significant rates, as in previous studies [22].

The inhibition of rCNA activity CsA-CyP was tested in the absence and presence of CNB using [^{32}P]casein as substrate. Inhibition of native CN requires both CsA and CyP [10,11], although some inhibition ($\leq$ 25%) is observed with μM amounts of human cyclophilin B (hCyPB) alone. In the absence of CNB, rCNA was not inhibited by 1.3 μM human cyclophilin A (hCyPA) either alone or in combination with CsA, while 0.93 μM hCyPB caused only minor inhibition (25–30%) regardless of whether CsA was present (Table I). In contrast, rCNA reconstituted with CNB behaved like native CN in that it was inhibited by both CsA-hCyPB and CsA-hCyPA but not by CsA, hCyPA or hCyPB alone. Although rCNA-CNB was not completely inhibited by 1 μM CsA-hCyPA (37% activity remained), its activity could be further suppressed using higher concentrations of CsA-hCyPA (see Fig. 2A). These results indicate that calcineurin B is an essential component for conferring sensitivity to the drug–receptor complexes. It is also notable that the rCNA isoform used here lacks the polyproline region [18,21], indicating that this region is not important for binding of CsA-CyP.

To further demonstrate the requirement for CNB to reconstitute a native-like response to CsA-CyP, the phosphatase activity toward *p*-nitrophenyl phosphate

(pNPP) was tested in the presence of the complexes. With pNPP, the immunosuppressive complexes actually stimulated the activity of native CN by 3- to 4-fold [10,11]. Similarly, rCNA showed a 2-fold stimulation of activity by CsA-hCyPB or CsA-hCyPA only when reconstituted with CNB (Table I).

rCNA reconstituted with CNB showed nearly identical sensitivity as native CN to CsA-hCyPA and CsA-hCyPB using [^{32}P]casein as substrate. The concentration of CsA-CyP necessary for 50% inhibition (IC_{50}) of rCNA-CNB was found to be 6 nM for CsA-hCyPB and 0.6 μM for CsA-hCyPA (Fig. 2A). For tight-binding inhibitors, IC_{50} must be equal to or greater than the enzyme concentration [11]. Hence the comparison of IC_{50} for CsA-hCyPB (5 nM) with the assay concentration of calcineurin (26 nM) must reflect the presence of 25–30% functional rCNA-CNB, as suggested by comparison of its specific activity with native CN noted above. IC_{50}s for rCNA-CNB are close to the values for native CN of 10 nM for CsA-hCyPB and 1 μM for CsA-hCyPA under the same assay conditions (Fig. 2B).

4. DISCUSSION

This study shows that inhibition of calcineurin by CsA-CyP is dependent upon the presence of calcineurin B. This is demonstrated by the observation that rCNA activity in the absence of the B subunit, although lower in general, is not inhibited by the drug–receptor complex. This suggests that CNB somehow endows CN with the requisite molecular structure for interaction with the immunosuppressant drugs CsA and FK506. From the data presented here it is not clear whether CNB creates or stabilizes the binding site for CsA-Cyp on CNA or whether CNB itself interacts partially with CsA-CyP. Since FK506-FKBP has been found thus far to act by the same route as CsA-CyP, it would be expected to show a similar dependence on the B subunit for inhibition of the phosphatase activity of native CN.

Mn^{2+} has long been known to have a stimulatory effect on calcineurin. Indeed, it is shown here that Mn^{2+} stimulates rCNA activity in the presence or absence of CNB. Although it has been suggested that CN has a specific site for Mn^{2+} [29], immunoprecipitation experiments have been unable to demonstrate the presence of

Table I

Effect of calcineurin B on the sensitivity of recombinant calcineurin A (rCNA) to cyclosporin A–cyclophilin A and cyclosporin A–cyclophilin B

Conditions[a]	% activity	
	rCNA (%)	rCNA-CNB (%)
[^{32}P]casein as substrate[b]:		
no addition	100.0[d]	100.0[d]
+ CsA	90.7	96.8
+ hCyPA	96.2	96.9
+ CsA, hCyPA	88.3	37.2[e]
+ hCyPB	72.8	79.8
+ CsA, hCyPB	75.4	2.6
pNPP as substrate[c]:		
no addition	100.0	100.0
+ CsA, hCyPA	86.0	193.0
+ CsA, hCyPB	115.0	213.0

[a] When present, the concentrations of CsA, hCyPA and hCyPB were 1.0, 1.3, and 0.93 mM, respectively.

[b] Assays using [^{32}P]casein as substrate were performed in the presence of 1.0 mM $MnCl_2$ and 0.58 mM CaM using either 92 nM rCNA alone or 25 nM rCNA plus 60 nM CNB, and 0.85–1.6 mM [^{32}P]casein.

[c] Assays using pNPP were performed using either 460 nM rCNA alone, 63 nM rCNA plus 150 nM CNB

[d] 100% activity is defined as the activity in the absence of CsA, hCyPA or hCyPB for each column. Each % activity value is the average of 2 or 4 measurements.

[e] This % activity was obtained using a concentration of hCyPA close to the IC_{50} value (see text). Thus, the activity would decrease further at higher concentrations of CsA-hCyPA.

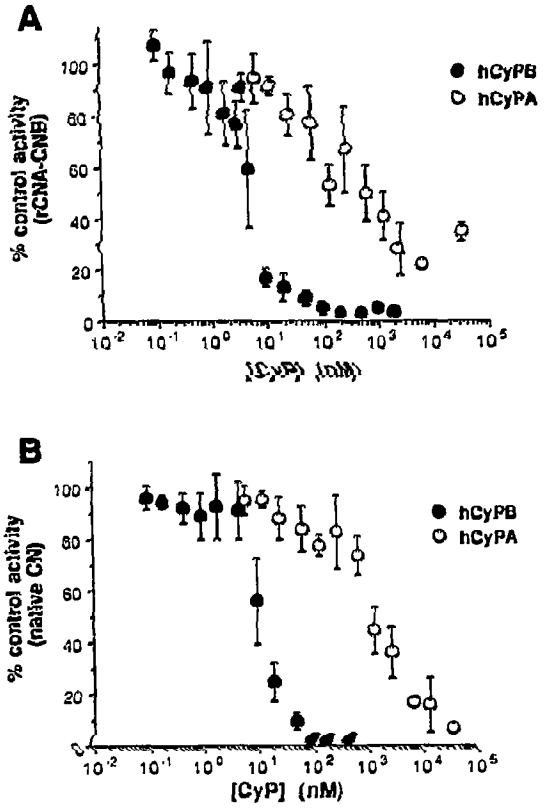

Fig. 2. Inhibition of calcineurin by CsA-hCyPA (open circles) and CsA-hCyPB (closed circles): A, reconstituted rCNA-CNB; B, native bovine brain CN. For rCNA-CNB, [^{32}P]casein assays were performed in the presence of 1.0 mM $MnCl_2$, 0.58 μM CaM, 0.75–2.0 μM [^{32}P]casein and 26 nM rCNA plus 59 nM CNB. For native CN, assays were performed in the presence of 1.0 mM $MnCl_2$, 0.40 μM CaM, 0.5–0.75 μM [^{32}P]casein and 5–10 nM CN. The final concentration of CsA was either 1 μM, equal to the concentration of hCyPA when the latter was greater than 1 μM, or 0.4 μM when the concentration of hCyPB was less than 100 nM. Each error bar represents the standard deviation of the value, for which 2, 4 or 6 measurements were averaged.

endogenous Mn [30]. Interestingly, CN does not display Mn^{2+} dependence prior to affinity purification on CaM-Sepharose [31]. In the experiments performed here, we have included $MnCl_2$ in the buffer system in order to raise the activity of rCNA above its low basal level. The inhibitory effect of the vesicular isoform of cyclophilin, CyPB, on native CN is essentially the same whether or not Mn^{2+} is present. Inhibition of native CN by the cytoplasmic isoform, CyPA, has been previously found to occur at a lower concentration (IC_{50} = 40 nM [11,12]) than observed here, with the difference possibly due to the use of $MnCl_2$ in these assays.

Although it is generally accepted that the functional unit of calcineurin comprises both CNA and CNB, little is known about the precise role of CNB in vivo other than that it conveys full activity to the catalytic subunit. The need for a regulatory B subunit has been unclear because calmodulin has the function of controlling calcineurin through cell signalling pathways. Furthermore, other serine/threonine protein phosphatases (PP1, PP2A and PP2C) do not require a CNB-like subunit for activity or in vivo regulation [28,32]. Recently a distinct isoform of CNB was discovered from rat testis [33], opening up the possibility that other tissue specific isoforms of CNB also exist. This suggests that distinct isoforms of either CNA or CNB may control the preference of the immunosuppressive drugs for action specifically on T-cells. Future studies aimed at investigating the interaction of calcineurin with immunophilins must consider the role CNB plays in the formation of the calcineurin-immunophilin complex.

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