Specific Interaction of the Cyclophilin-Cyclosporin Complex with the B Subunit of Calcineurin*

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Calcineurin (CaN), a Ca²⁺/calmodulin-dependent serine/threonine phosphatase, has been shown to be inhibited by the complex of the immunosuppressant cyclosporin A (CsA) and its receptor, cyclophilin (CyP), but not by either alone. In the current study, the topological relationship between cyclophilin and the subunits of CaN has been explored using chemical cross-linking agents. In the presence of cyclosporin, ¹²⁵I-CyP, shown to bind to CsA, is extensively cross-linked to the B subunit of CaN but not the catalytic A subunit. However, the A subunit is required for binding of the CyP-CsA complex, since cross-linking to recombinant B subunit alone does not occur. The kinetics of association indicate a saturable reaction with a K₅₀ less than 70 nM. Cross-linking to the B subunit occurs with cross-linkers that span from 0 to 16 Å and employ different cross-linking chemistry, indicating direct contact between the B subunit and CyP. Similar cross-linking to the B subunit has been observed with the complex of ¹²⁵I-labeled FK506 binding protein (FKBP) and FK506 but not with the FKBP-rapamycin complex. CyP-CsA cross-linking to CaN is Ca²⁺/calmodulin-dependent with intact CaN, but Ca²⁺/calmodulin-independent after digestion to remove the calmodulin binding and autoinhibitory domain.

Cyclosporin A (CsA), FK506, and rapamycin are microbial products with potent immunosuppressive activity that exert selective inhibition of T lymphocyte activation and lymphokine production (1–3). In addition to their value as immunosuppressants, these compounds are serving as valuable reagents to dissect the signal transduction pathway in T cells. In general, T cell activation pathways associated with a rise in intracellular Ca²⁺ are sensitive to CsA and FK506 (4). The products with potent immunosuppressive activity that exert in vivo activity with intact CaN, but Ca²⁺/calmodulin-independent after digestion to remove the calmodulin binding and autoinhibitory domain.

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1 The abbreviations used are: CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506-binding protein; CaN, calcineurin; CaNa, calcineurin A subunit; CaNB, calcineurin B subunit; FGS, ethylene glycol-bis(succinimidyl succinate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PAGE, polyacrylamide gel electrophoresis; DSS, disuccinimidyl suberate; DST, disuccinimidyl tartarate.

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EXPERIMENTAL PROCEDURES

Materials—Human recombinant CyP was prepared as described previously (18). Human recombinant FKBP was a gift from Dr. M. W. Harding, Vertex Inc. Human recombinant CaN B subunit was a gift from Drs. R. Hao and C. M. Klee. CaN was a gift from Sandoz Pharmaceuticals Corp. ¹²⁵I-Bolton-Hunter reagent was purchased from Du Pont-New England Nuclear. ¹²⁵I-Calmodulin was purchased from Amersham Corp. The 8-ornithino-CaNa matrix was prepared as described previously (19). Ethylene glycolbis(succinimidyl succinate) (EGS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and other cross-linkers were purchased from Pierce Chemical Co. CaN, calmodulin, and all other reagents were of the highest purity available from Sigma.

Preparation of ¹²⁵I-immunophilins—CyP was labeled by ¹²⁵I-Bolton-Hunter reagent as described previously (20). Briefly, human recombinant CyP (1 μg) in 10 μl of 0.1 M sodium phosphate buffer (pH 8.5) was added to 1 mCi of ¹²⁵I-Bolton-Hunter reagent and incubated for 15 min at 4 °C. The remaining active reagent was quenched by the addition of 0.5 ml of 0.2 M glycine in phosphate buffer (pH 8.5). ¹²⁵I-CyP was separated from the radioactive products by loading the reaction mixture onto an 8-ornithino-CaN affinity column. Any ¹²⁵I-CyP that was unable to bind CaN was removed, and the active form was eluted from the column by 20 mM
sodium phosphate buffer (pH 3.5) containing 100 mM NaCl and 5 mM β-mercaptoethanol and immediately neutralized to pH 7.0. The final specific activity was 1100 Ci/mmol.

Recombinant FKBP-12 was labeled by the same procedure and separated from free iodine and other low molecular weight by-products by a Sephadex G-25 column. The specific activity of 125I-FKBP was about 100 Ci/mmol.

Preparation of Calf Brain Cytosol—Quick-frozen calf brain was homogenized with a glass Potter-Elvehjem homogenizer in 4 volumes of buffer A (50 mM HEPES (pH 7.4), 100 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000 g for 1 h. The supernatant (6 ml) was depleted of CyP by passage through a 1.5-ml 8-ornithino-Csa column and CsA binding activity of the cytosol determined by the LH-20 assay (5) before and after the passage. Protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

Cross-linking of Radiolabeled CyP to CaN—Purified CaN (100 nM) or tissue cytosol (2 mg protein/ml) was incubated with 0.3 nM of 125I-CyP or 125I-FKBP in the presence or absence of 10 μM CsA or FK506 or tissue cytosol (2 mg protein/ml) was incubated with 0.3 nM of 125I-CyP for 1 h in a final volume of 30 μl of buffer A. In the case of CaN binding, CaCl2 (1 mM), MgCl2 (1 mM), MnSO4 (1 mM), calmodulin (0.1 mM), and 0.1% bovine serum albumin were included in the binding solution. EGTA (2 mM) was added as indicated. The cross-linking reaction was initiated by the addition of EGS (0.5 mM final concentration) to the binding solution and incubation for 40 min at 4 °C (21). Alternatively, the cross-linking was accomplished with 20 mM EDC and incubation at 25 °C for 1 h as described previously (22). The cross-linking reaction was quenched by the addition of 20 μl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol and 60 mM Tris. Samples were analyzed by SDS-PAGE as described under "Experimental Procedures." Radioactive bands were cut out using the autoradiogram as a template and counted in a γ counter.

Limited Proteolysis of CaN with Trypsin—CaN was digested with trypsin as described previously (17) with minor modifications. Briefly, CaN (2 μM) was incubated at 30 °C for 2 min with 35 nM trypsin in 25 μl of 50 mM HEPES buffer (pH 7.0) containing 0.1 mM CaCl2 and 0.1% β-mercaptoethanol; 1 μl of trypsin inhibitor (4 μM) was added to stop the reaction. Aliquots (1.5 μl) were removed to individual samples were cross-linked with EGS (0.5 mM) for 40 min at 4 °C followed by SDS-PAGE as described under "Experimental Procedures." A, native and CyP-depleted calf brain cytosol; B, calf brain extract; C, CaN.

RESULTS

Cross-linking of 125I-CyP—To determine the nature of proteins with which CyP associates, 125I-CyP was incubated with normal or CyP-depleted calf brain cytosol in the absence or presence of CsA and covalently cross-linked using the homobifunctional N-hydroxysuccinimide ester, EGS. The appearance of a major 34-kDa radioactive band in both normal, and to a much greater degree in CyP-free cytosol, indicated that 125I-CyP was closely associated to a 16-kDa protein, possibly the CaN B subunit. This band was observed only in the presence of CsA (Fig. 1A). In addition, radiolabeled CyP was cross-linked to two minor bands of higher molecular weight in CyP-free cytosol. The lower band (~100 kDa) was the expected size of a tetrameric complex of 125I-CyP, and the B and A subunits of CaN were formed. 125I-CyP cross-linking to these proteins was completely inhibited by the addition of excess cold CyP (Fig. 1B). The identity of the primary protein associated with the CyP-CsA complex was confirmed by using pure CaN (Fig. 1C). Again the 125I-CyP-CsA was primarily cross-linked to CaN B subunit instead of the expected association with the catalytic A subunit. This interaction was also saturable as shown by the addition of excess cold CyP. The addition of FK506 which binds to endogenous FKBP in the extract greatly diminished cross-linking of 125I-CyP and the further addition of excess pure FKBP eliminated cross-linking. This result suggests competition for the same site. Furthermore, our results confirm the previous finding (12) that the FKBP-rapamycin complex does not compete with CyP-CsA binding to CaN, a result consistent with its failure to inhibit the activity of CaN. Cross-linking to CaN B requires the presence of the A subunit, since 125I-CyP-CsA was not cross-linked to purified recombinant B subunit in the absence of A subunit (Fig. 2).

To estimate the association kinetics, increasing concentrations of cold CyP were added in the presence of a fixed concentration of CaN (100 nM), and cross-linking to the B subunit was quantitated as described under "Experimental Procedures." As little as 70 nM final concentration of CyP caused about half-maximal inhibition, and since the concentration of CaN was only 100 nM, the apparent Kd is less than this value (Fig. 3), a result consistent with some of the kinetic data for inhibition of protein phosphatase activity (14). A
Concentrations of cold CyP in the presence or absence of CsA (10 pM) as indicated. Other experimental conditions were the same as described in Fig. 1. Radioactivity in the 34-kDa bands was quantitated by a γ counter.

Fig. 3. Inhibition of [125I]-CyP cross-linking to CaN. [125I]-CyP (0.3 nM) was incubated with pure CaN (0.1 μM) and increasing concentrations of different CsA analogs as indicated. Other experimental conditions were the same as described in the legend to Fig. 1. The radioactivity of the 34-kDa bands was quantified.

Fig. 4. Concentration dependence of CyP cross-linking to CaN by CsA and its analogs. [125I]-CyP (0.3 nM) was incubated with pure CaN (0.1 μM) and increasing concentrations of different CsA analogs as indicated. Other experimental conditions were the same as described in the legend to Fig. 1. The radioactivity of the 34-kDa bands was quantified.

titation of the dependence of the cross-linking on CsA indicated an EC₅₀ of about 30 nM (Fig. 4), a result similar to the EC₅₀ of CsA with CyP in the peptidyl-prolyl cis-trans-isomerase assay (24). Compared with CsA, MeBmt-CsA has similar efficacy but is about 10-fold less potent. Dihydro-CsA and 1-furfuryl-CsA are also capable of promoting similar association to the B subunit but MeAla⁶-CsA has little effect even at 10 μM concentration (Fig. 4), despite its ability to occupy the CsA binding site and inhibit peptidyl-prolyl cis-trans-isomerase activity (24).

To determine if the FKBP-FK506 complex also is in contact with the B subunit of CaN and affects CyP-CsA cross-linking, [125I]-FKBP was prepared and cross-linked to CaN. A 28-kDa protein band (16 kDa for CaN B plus 12 kDa for FKBP) was observed with both calf brain cytosol and pure CaN in the presence of FK506 (Fig. 5A). Formation of this 28-kDa band was inhibited by cold FKBP and the CyP-CsA complex, but not by CsA or CyP alone (Fig. 5B). Furthermore, the FKBP-rapamycin complex was not cross-linked to the B subunit.

Cross-linkers of Different Spacing and Chemistry—In addition to EGS, a reagent with a 16-Å spacer, cross-linking was performed with the following other homobifunctional N-hydroxysuccinimide ester cross-linkers: disuccinimidyl suberate (DSS), 11.4-Å spacer; disuccinimidyl tartarate (DST), 6.4-Å spacer (Fig. 6A); 3,3’-dithiobis(sulfosuccinimidylpropionate), 12-Å spacer; and homobifunctional imidoester cross-linker, dimethyl suberimidate, 11-Å spacer (data not shown). In all cases predominant cross-linking of CyP occurred to the B subunit. Even with the “zero-length” cross-linker, EDC, [125I]-CyP was cross-linked to the B subunit in both pure CaN and calf brain cytosol in the presence of CsA (Fig. 6B), confirming a direct contact between bound CyP and the B subunit.

Ca²⁺/Calmodulin Effect on CyP-CsA Binding to Intact and Limited Digested CaN—Ca²⁺ and calmodulin are essential to the phosphatase activity of intact CaN as well as its inhibition by the CyP-CsA complex. We find that CyP cross-linking to purified CaN was dramatically enhanced by calmodulin and blocked by EGTA (Fig. 7). CyP was not directly cross-linked to [125I]-calmodulin itself in the absence or presence of CsA (data not shown), even in the presence of CaN. Furthermore, limited digestion of CaN with trypsin to remove the auto-inhibitory domain permitted cross-linking of [125I]-CyP to the B subunit with or without calmodulin (Fig. 7). EGTA was not able to block CyP-CsA binding to the truncated form of CaN. The previously reported upward mobility shift of CaNB (12) was also observed in the cross-linked complex with CyP-CsA when EGTA was present. The two higher molecular weight bands cross-linked to [125I]-CyP seen with intact CaN were also shifted to proportionately lower molecular weight positions, a
result consistent with the size of the truncated A subunit. The same results were observed with CaN after limited digestion by clostripain (data not shown).

**DISCUSSION**

Previous studies by Liu et al. (14) had suggested that the A subunit of CaN plays an important role in the binding of immunophilin-ligand complexes. However, recent reports indicate that the presence of the B subunit is required for CyP-CsA to affect CaN (15, 16). In the current studies we used an independent chemical approach to define in greater detail the spatial relationships between the CaN complex (CaNA, CaNB, CaNβ, and calmodulin) and CyP-CsA or FKBP-FK506 complexes. Our finding that the B subunit is the primary site of interaction is not the result of using a single cross-linking reagent that might have had unique access only to the B subunit for covalent reaction. Reagents with different cross-linking chemistry that react with amino and carboxyl groups and even a "zero" distance cross-linker all show a predominant binding to the B subunit of CaN. These findings support an intimate juxtaposition of CyP with the B subunit but do not rule out an interaction with the A subunit. In fact, the dependence of cross-linking on the presence of both subunits (binding does not occur with recombinant B alone) as well as CaN+calmodulin gives assurance that a coordinate complex must be formed to permit cross-linkage.

Since the B subunit of CaN and calmodulin are of similar molecular weight and evidence of cross-linking was highly dependent on calmodulin, it was important to demonstrate that the primary band at ~34 kDa was not a complex with the carboxyl groups and even a "zero" distance cross-linker all show a predominant binding to the B subunit of CaN. These findings support an intimate juxtaposition of CyP with the B subunit but do not rule out an interaction with the A subunit. In fact, the dependence of cross-linking on the presence of both subunits (binding does not occur with recombinant B alone) as well as CaN+calmodulin gives assurance that a coordinate complex must be formed to permit cross-linkage.

To date the relationship between the FKBP-FK506 and CyP-CsA binding sites on CaN has not been explored. Our current data demonstrate that endogenous FKBP in brain cytosol is sufficient to generate competition for cross-linkage in the presence of excess FK506 (Fig. 1B) and that this effect can be enhanced by adding FKBP. Similarly, CyP-CsA can inhibit the binding of FKBP-FK506. These results suggest that FKBP-FK506 and CyP-CsA complexes may compete for the same site.

Cross-linking reagents cause the formation of many protein complexes. Two less prominent radioactive higher molecular weight bands were observed; one of these is consistent with the formation of secondary cross-linkages between CaN B and CaN A, a result confirmed by the truncation of its size by limited tryptic digestion of the A subunit. The larger of the two bands is of a size consistent with cross-linking of the CaN-CyP-CsA complex to calmodulin.

Evidence for an approximation of CyP-CsA to the CaN B subunit is compelling, but one must question why direct cross-linking to the A subunit is not observed, since it has been suggested that the catalytic subunit is the target (14). One explanation would be the lack of accessible cross-linking sites on the surface of this subunit as it is found in the hexameric complex of CyP-CsA-CaNA-CaNB-CaNβ-calmodulin. However, the failure of reagents with different chemistries and distance spans to reveal binding argues against this. It remains possible that the requirement for the presence of the A subunit indicates a locus of interaction between the two subunits, perhaps in the vicinity of the catalytic site. Alternatively, formation of the A-B dimer may induce conformation changes at the B subunit that generate the CyP-CsA binding site.

It has been assumed that endogenous CaN-like molecules may exist and associate with CyP (25-26); these could modulate the effects on CaN. Preliminary evidence for a 77-kDa protein that associates with CyP has also been reported in the absence of CsA (27). The present cross-linking method provides a very sensitive approach to detect the existence of these intrinsic molecules as shown in Fig. 4. However, with the current techniques we were unable to demonstrate CaN-like activity, as measured by enhancement of CyP cross-linking to CaN, in either normal or CyP-depleted extracts from brain (Fig. 1), thymus, Jurkat cells, and other tissues (data not shown).

These findings with pure CaN and brain cytosol afford confirmation of an association of CyP with CaN when CsA is present. What is not known is whether in the absence of CsA if CyP interacts specifically with CaN or whether other proteins may be alternative targets. In this regard, we find that in extracts of the Jurkat T cell line, which contains much less CaN than brain extracts, cross-linking to the B subunit could not be detected. In the Jurkat extracts, however, 125I-CyP cross-links with a yet to be identified 100-kDa protein species in the presence of CsA. This protein may represent an alternative or supplementary target for the action of CsA.

A detailed understanding of the interactions we have detected with the B subunit must await x-ray crystallographic and NMR studies, but these findings provide an initial insight into the topology of the complexes.

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**REFERENCES**


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CyP-CsA Cross-linking to Calcineurin B Subunit