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Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes

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Summary

Although the immediate receptors (immunophilins) of the immunosuppressants cyclosporin A (CsA) and FK506 are distinct, their similar mechanisms of inhibition of cell signaling suggest that their associated immunophilin complexes interact with a common target. We report here that the complexes cyclophilin-CsA and FKBP-FK506 (but not cyclophilin, FKBP, FKBP-rapamycin, or FKBP-506BD) competitively bind to and inhibit the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin, although the binding and inhibition of calcineurin do not require calmodulin. These results suggest that calcineurin is involved in a common step associated with T cell receptor and IgE receptor signaling pathways and that cyclophilin and FKBP mediate the actions of CsA and FK506, respectively, by forming drug-dependent complexes with and altering the activity of calcineurin-calmodulin.

Introduction

Complexes of immunophilins (immunosuppressant-binding proteins) and their immunosuppressive ligands are proving to be valuable reagents for probing signal transduction pathways (Schreiber, 1991). Studies of T cell receptor (TCR)–mediated transcription and IgE receptor–mediated exocytosis with the immunosuppressants cyclosporin A (CsA) and FK506 suggest the existence of a common cytoplasmic step in these Ca^{2+}-dependent signaling pathways (Hultsch et al., 1991). Rapamycin, an immunosuppressant structurally related to FK506, appears to act upon a similar step that exists in distinct signaling pathways associated with growth factor receptors (Dumont et al., 1990a).

The biological properties of CsA and FK506 are remarkably similar (Johansson and Moller, 1990; Lin et al., 1991). Aside from differences in potency, the only distinction between these two compounds in studies of their actions in T cells and mast cells is their response to rapamycin (Dumont et al., 1990b; Bierer et al., 1990a; Hultsch et al., 1991) and 506BD (Bierer et al., 1990b; Hultsch et al., 1991). These latter agents block the actions of FK506, but not CsA, in T cells and mast cells. FK506, but not CsA, is able to block the actions of rapamycin in T cells (Dumont et al., 1990b; Bierer et al., 1990a). These results suggest that distinct immunophilins are responsible for mediating the actions of CsA and FK506, and a common immunophilin may be responsible for mediating the actions of both FK506 and rapamycin. Much evidence has accumulated that supports roles for cyclophilin–CsA, FKBP–FK506, and FKBP–rapamycin complexes in the inhibition of the aforementioned processes, although the relevant cyclophilin and FKBP family members have not been uniquely defined in most cases. The similarities in all other aspects of CsA and FK506 function suggest that their associated immunophilin complexes are acting upon a (possibly immediate) common biological target molecule that is not a target of the FKBP–rapamycin complex, free FKBP, or free cyclophilin.

We now report the competitive binding of a human cyclophilin A (or murine cyclophilin C)–CsA complex and a human FKBP12–FK506 complex to a common cellular target that is not bound by either cyclophilin A (or C), FKBP12, or FKBP12–rapamycin. The target is shown to be the Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase calcineurin (Klee and Krinks, 1978; Klee et al., 1979; Stewart et al., 1982), as well as a complex of calcineurin and calmodulin. Calcineurin meets the biochemical requirements of the common target of immunophilin–drug complexes and thus is a potential component of TCR and IgE receptor signaling pathways involved in transcription and exocytosis.

Results

A Common Set of Proteins Bind to Cyclophilin-CsA and FKBP-FK506, but Not Cyclophilin, FKBP, CsA, FK506, Rapamycin, or FKBP-Rapamycin

A chimeric gene encoding a glutathione S-transferase–FKBP12 fusion protein (GFK) was constructed by fusing the cDNA encoding FKBP12 to that encoding the carboxyl terminus of glutathione S-transferase (Smith and Johnson, 1988). After transformation of the resulting construct, pGFK12, into Escherichia coli XA90, induction with isopropyl-β-D-thiogalactopyranoside (IPTG) yielded the fusion protein GFK as the major constituent of soluble, cellular proteins. GFK was partially purified by ammonium sulfate fractionation (40%–80%), glutathione affinity chromatography, and DE-52 anion exchange chromatography as detailed in Experimental Procedures. GFK fractions after DE-52 chromatography were nearly homogeneous as judged by Coomassie blue staining. However, several other protein bands were visible with silver staining (Figure 1, lane 1).

The rotamase activity of GFK and its ability to be inhibited by FK506 and rapamycin were determined in the presence and absence of reduced glutathione. GFK has rotamase activity and affinities for FK506 and rapamycin similar to those of recombinant human FKBP12; furthermore, neither its rotamase activity nor affinities for the

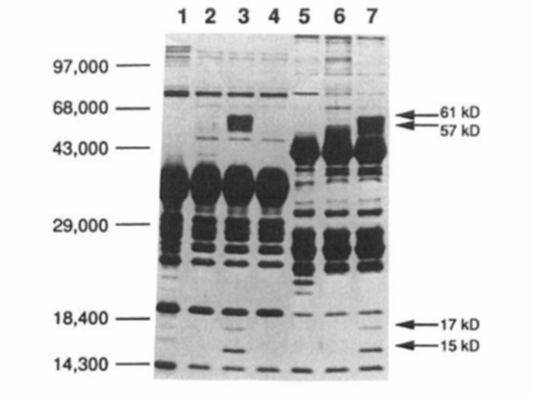

Figure 1. Detection of a Common Set of Proteins from Calf Brain Extract That Bind to GFK–FK506 and GCyP–CsA, But Not GFK, GCyP, or GFK–Rapamycin

The fusion proteins GFK and GCyP, with or without drugs, were incubated with calf brain extract. Glutathione–Sepharose was then added to adsorb the fusion proteins and associated target proteins. After three washes with the incubation buffer and 0.2% Triton X-100, the glutathione–Sepharose was resuspended in SDS sample loading buffer, heated in boiling water, and subjected to 12% SDS–polyacrylamide gel electrophoresis. The gel was silver stained. Lane 1, GFK control; lane 2, GFK plus extract; lane 3, GFK–FK506 plus extract; lane 4, GFK–rapamycin plus extract; lane 5, GCyP control; lane 6, GCyP plus extract; lane 7, GCyP–CsA plus extract.

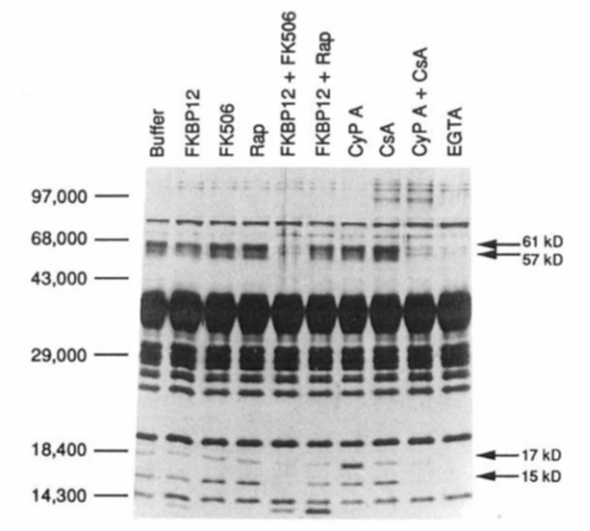

Figure 2. In Vitro Competition Experiments with Recombinant FKBP12, Cyclophilin A, Individual Drugs, Immunophilin–Drug Complexes, and EGTA

The four target proteins were first adsorbed onto glutathione–Sepharose using GFK–FK506 as shown in Figure 1, lane 3. The immobilized GFK–FK506–target protein complex was then incubated with the following: lane 1, buffer (as control); lane 2, recombinant human FKBP12; lane 3, FK506; lane 4, rapamycin; lane 5, FKBP12–FK506; lane 6, FKBP12–rapamycin; lane 7, recombinant human cyclophilin A; lane 8, CsA; lane 9, cyclophilin A–CsA; and lane 10, EGTA. The glutathione–Sepharose with adsorbed GFK–FK506 and bound target proteins was precipitated by centrifugation and washed twice before being subjected to 12% SDS–PAGE and silver staining. The 12 kd protein in lanes 2, 5, and 6 corresponds to FKBP12 from the incubation.

drugs are affected by the presence of glutathione (data not shown). Similar results were obtained with a glutathione S-transferase–cyclophilin C fusion protein, GCyP, described in the accompanying paper (Friedman and Weissman, 1991). Thus, it appears that immunophilin and glutathione S-transferase domains act independently in the fusion protein.

Since it is known that both CsA and FK506 inhibit Ca^{2+}-dependent signaling pathways (Lin et al., 1991), Ca^{2+} and Mg^{2+} were included in the incubation buffer (see Experimental Procedures). Under these conditions, proteins of M_r 61,000, 57,000, 17,000, and 15,000 from a calf thymus extract were found to be specifically adsorbed by the GFK–FK506 and GCyP–CsA complexes, but not by GFK or GCyP alone or the GFK–rapamycin complex (see Figure 1). Furthermore, the same set of proteins was detected in other tissues such as bovine brain and spleen, with brain being the most reliable source.

Competitive binding experiments were carried out with recombinant FKBP12 and cyclophilin A and their respective drug complexes. After the set of four target proteins was adsorbed onto the glutathione–Sepharose affinity matrix, elution was attempted with immunophilins, the drugs, or the immunophilin–drug complexes. As shown in Figure 2, the set of four proteins can be eluted from a GFK–FK506 affinity matrix with both recombinant FKBP12–FK506 and recombinant cyclophilin A–CsA complexes. In contrast, these proteins are not eluted by free immunophilins or unbound drugs. In addition, the FKBP12–rapamycin complex does not elute the target proteins (Figure 2), in agreement with previous observations (Figure 1, lane 4). The ability of both cyclophilins A and C, when complexed to CsA, to interact with the same set of proteins is noteworthy, as discussed below.

Divalent Metal Ion Dependence of Immunophilin Drug–Target Protein Complex Formation, and Purification of the Target Proteins by EGTA Elution

When Ca^{2+} and Mg^{2+} were omitted from the incubation buffer, no target proteins were retained by either GFK–FK506 or GCyP–CsA. To further test the importance of divalent metal ions for interaction between immunophilin–drug complexes and target proteins, the adsorption experiment was performed in the presence of the Ca^{2+} chelator EGTA. GFK–FK506 and GCyP–CsA complexes no longer retained the target proteins when EGTA was present. In addition, EGTA was found to be capable of eluting the four proteins from the GFK–FK506 complex immobilized on glutathione–Sepharose without a significant effect on the interactions between GFK and glutathione–Sepharose (Figure 2, lane 10).

GFK–FK506 bound to glutathione–Sepharose matrix was loaded with calf thymus extract and eluted with EGTA; analysis of the eluate is shown in Figure 3 (lanes 3 and 6). Two contaminant proteins coelute with the four target proteins, the 38 kd GFK and a less abundant M_r 26,000 protein that may be a glutathione S-transferase either from

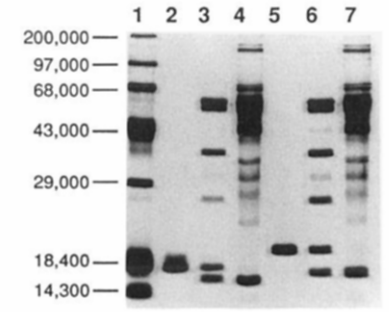

Figure 3. Ca^{2+}-Dependent Gel Mobility Shift of Calmodulin, the 17 kd and 15 kd EGTA-Eluted Target Proteins, and Calcineurin B

Standard calmodulin (from Sigma; 0.25 μg), EGTA eluate from calf thymus (2 μg), and standard calcineurin (from Sigma; 3 μg) were subjected to 12% SDS–PAGE in the presence of 5 mM $CaCl_2$ or 5 mM EGTA, followed by silver staining. Lane 1, molecular weight standards; lane 2, calmodulin plus Ca^{2+}; lane 3, EGTA eluate plus Ca^{2+}; lane 4, calcineurin plus Ca^{2+}; lane 5, calmodulin plus EGTA; lane 6, EGTA eluate plus EGTA; lane 7, calcineurin plus EGTA.

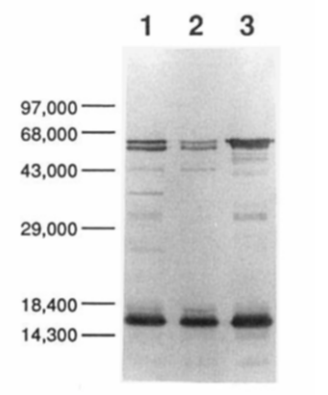

Figure 4. Western Blot Analysis of EGTA Eluate with Anti-Calcineurin Antibodies

EGTA-eluted target proteins (lane 1, 2 μg of total protein as used in Figure 3, lanes 3 and 6), calf brain calcineurin from Sigma (lane 2, 0.5 μg), and purified calf brain calcineurin (lane 3, 0.6 μg provided by Dr. C. B. Klee) were subjected to 12% SDS–PAGE and electroblotted onto nitrocellulose, which was then developed with rabbit anti-calcineurin IgG and goat anti-rabbit IgG conjugated with alkaline phosphatase.

the proteolytic cleavage of GFK or from the calf thymus extract. With this procedure, over 40 μg of proteins can be purified from 20 ml of calf thymus extract (120 mg of protein).

Identification of the M_r 17,000 Protein as Calmodulin, the M_r 61,000 and 57,000 Proteins as Calcineurin A, and the M_r 15,000 Protein as Calcineurin B

Since the binding of the target proteins to the immunophilin–drug complexes is metal ion (especially Ca^{2+}) dependent, we speculated that the M_r 17,000 protein was calmodulin. One of the distinctive properties of calmodulin is its Ca^{2+}-dependent gel mobility shift; i.e., calmodulin migrates faster in the presence of Ca^{2+} during SDS–polyacrylamide gel electrophoresis (Klee et al., 1979). Indeed, when the EGTA eluate was subjected to SDS–PAGE beside a calmodulin standard (Sigma), the M_r 17,000 protein bands exhibited the Ca^{2+}-dependent gel mobility shift characteristic of calmodulin (Figure 3, lanes 2 and 3 versus 5 and 6). That the M_r 17,000 protein is calmodulin was confirmed in subsequent experiments with authentic calmodulin.

We reasoned that the remaining three proteins could be part of a multisubunit complex of calmodulin-binding proteins, such as a Ca^{2+}- and calmodulin-dependent kinase or Ca^{2+}- and calmodulin-dependent phosphatase. Calcineurin (Klee et al., 1988) is composed of two subunits, calcineurin A, a 61 kd polypeptide, and calcineurin B, a 19 kd polypeptide. As the 19 kd calcineurin B migrates at about 15 kd on SDS–PAGE, we considered that the M_r 61,000 and 15,000 proteins are calcineurin A and B, respectively. It was also known that calcineurin A undergoes proteolysis to yield a 57 kd protein.

When the four target protein bands were blotted onto polyvinylidene difluoride membranes and subjected to N-terminal sequencing, they were all found to be N-terminally blocked (not shown). This is in agreement with the fact that both subunits of calcineurin, the 57 kd proteolytic fragment of calcineurin A, and calmodulin have covalent modifications of their N-termini (Klee et al., 1988; Aitken et al., 1984; Klee and Vanaman, 1982).

Calcineurin B is a calcium-binding protein with four Ca^{2+}-binding EF hands (Aitken et al., 1984); like calmodulin, it exhibits a gel mobility shift in the presence of calcium during SDS–PAGE. The M_r 15,000 EGTA-eluted protein did migrate faster in the presence of calcium (Figure 3, lanes 3 and 6). It was also found to comigrate with a calcineurin B standard (Sigma) in the presence or absence of calcium (Figure 3, lanes 4 and 7). The M_r 61,000 and M_r 57,000 EGTA-eluted proteins comigrate with a calcineurin A standard (Sigma); these proteins do not undergo a gel mobility shift in the presence of Ca^{2+}.

A Western blot of the EGTA eluate from calf thymus with polyclonal antibodies against bovine brain calcineurin helped to establish that the M_r 61,000, 57,000, and 15,000 proteins are calcineurin A, a proteolytic fragment of calcineurin A, and calcineurin B, respectively (Figure 4). A $^{45}Ca^{2+}$ ligand-blotting experiment with the EGTA eluate further supports the identity of the 15 kd protein as calcineurin B (Figure 5). The weaker response of calmodulin (M_r 17,000) was anticipated, as it is known that under the same blotting conditions calmodulin provides a weaker signal (C. B. Klee, personal communication). The identification of the retained proteins was next confirmed by experiments with authentic samples of calcineurin and calmodulin.

Calcineurin Binds to FKBP–FK506 and Cyclophilin–CsA in a Ca^{2+}-Dependent Fashion, and Its Phosphatase Activity toward a Phosphopeptide Substrate is Specifically Inhibited by the Two Immunophilin–Drug Complexes

The identification of calcineurin as a common target of immunophilin–drug complexes was confirmed by experi-

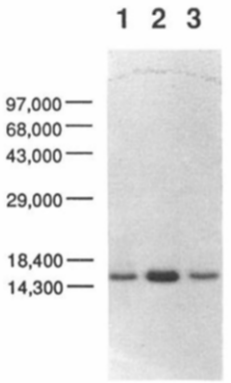

Figure 5. $^{45}Ca^{2+}$ Ligand Blotting of Calcineurin B

Lane 1, EGTA-eluted target proteins from calf thymus (2 μg of total proteins); lane 2, purified calf brain calcineurin (1 μg, provided by Dr. C. B. Klee); lane 3, calf brain calcineurin from Sigma (1 μg).

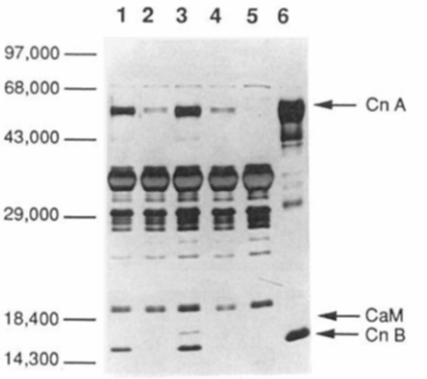

Figure 6. Glutathione–Sepharose Adsorption Experiments Using Bovine Brain Calcineurin and GFK–FK506

The experiments were carried out in the same way as described in the legend to Figure 1, except 2 μg of authentic calf brain calcineurin was used in place of the calf brain extract. The incubation buffer contained 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ (buffer A) or 50 mM Tris–HCl (pH 7.5), 100 mM NaCl (buffer B). For each adsorption experiment, the final concentrations of GFK and FK506 were 2 μM and 20 μM, respectively. Lane 1, calcineurin with buffer A; lane 2, calcineurin with buffer B and 20 mM EGTA; lane 3, calcineurin with buffer A and 1 μM calmodulin; lane 4, calcineurin with buffer B, 1 μM calmodulin, and 20 mM EGTA; lane 5, 1 μM calmodulin in buffer A; lane 6, 2 μg of bovine brain calcineurin used in experiments shown in lanes 1–5. CaM = calmodulin; CnA = calcineurin A; CnB = calcineurin B.

ments with authentic calcineurin purified from bovine brain. Calcineurin was found to be retained by GFK–FK506 and GCyP–CsA, but not by GFK, GCyP, or GFK–rapamycin. The GFK–FK506 complex binds directly to calcineurin in the presence of Ca^{2+} without calmodulin (Figure 6, lane 1), and the binding is dependent on Ca^{2+} and Mg^{2+} (lane 2). In the presence of calmodulin, however, increased amounts of calcineurin (both A and B subunits) appear to be adsorbed by the same amount of GFK–FK506 complex (Figure 6, lane 3), and calmodulin is retained as well. The binding of calcineurin–calmodulin by GFK–FK506 was abolished by EGTA (lane 4). Calmodulin alone does not bind to the GFK–FK506 complex in the presence of calcium (lane 5). These experiments demonstrate that the immunophilin–drug complexes bind directly to calcineurin and only indirectly, via calcineurin, to calmodulin.

Calcineurin is known to be a Ca^{2+}- and calmodulin-dependent protein phosphatase (Stewart et al., 1982). Using a phosphorylated peptide fragment from the regulatory subunit of cAMP-dependent kinase as a substrate, the phosphatase activity of authentic calcineurin was assayed in the presence of the immunophilins, the individual drugs, and their respective complexes with or without calmodulin. As shown in Figures 7A and 7B, both the intrinsic Ca^{2+}-dependent and Ca^{2+}- and calmodulin-stimulated phosphatase activities of calcineurin are potently inhibited by FKBP12–FK506 and cyclophilin A–CsA complexes, in agreement with the glutathione–Sepharose adsorption experiments (Figure 6). It is worth noting that complexes of FKBP–rapamycin and FKBP–506BD do not significantly inhibit the phosphatase activity (Figures 7A and 7B), in full agreement with the competitive binding assay (Figure 2) and the previous observations that rapamycin inhibits different, calcium-independent signaling pathways (Dumont et al., 1990b) and that 506BD, although a potent rotamase inhibitor of FKBP12, is a very weak inhibitor of TCR-mediated signaling (Bierer et al., 1990b).

Discussion

CsA and FK506, despite their lack of structural similarities, have highly similar biological properties. Both molecules interfere with the TCR-mediated signaling pathway that results in the transcription of early T cell activation genes, although FK506 is able to do so at 100-fold lower concentrations (Tocci et al., 1989). The transcriptional activation and (to a lesser degree) DNA-binding properties of the transcription factors NF-AT, AP-3, and (partially) NF-κB are potently inhibited by both immunosuppressants (Emmel et al., 1989; Mattila et al., 1990). Their site of action has been localized to the cytoplasm: they act following early membrane-associated events but prior to nuclear events. Without interfering with membrane-associated signaling processes, both molecules inhibit an IgE receptor–mediated signaling pathway that results in the exocytosis of secretory granules in mast cells (Hultsch et al., 1991). This cytoplasmic event is sensitive to the actions of CsA and FK506 at concentrations similar to those required to inhibit transcription in T cells. A common feature of CsA- and FK506-sensitive signaling pathways identified to date is their dependence on Ca^{2+}; conversely, several Ca^{2+}-independent signaling pathways have been studied that are resistant to the actions of CsA and FK506 (Lin et al., 1991).

In addition to their similar effects on cell function, both CsA and FK506 bind with high affinity to abundant cytosolic receptors (immunophilins) that catalyze the interconversion of cis- and trans-rotamers of peptidyl-prolyl amide bonds of peptide and protein substrates. The rotamase activity of cyclophilin A (CsA-binding protein; the "A" descriptor refers to the originally discovered isoform [Handschumacher et al., 1984], whereas cyclophilin C refers to

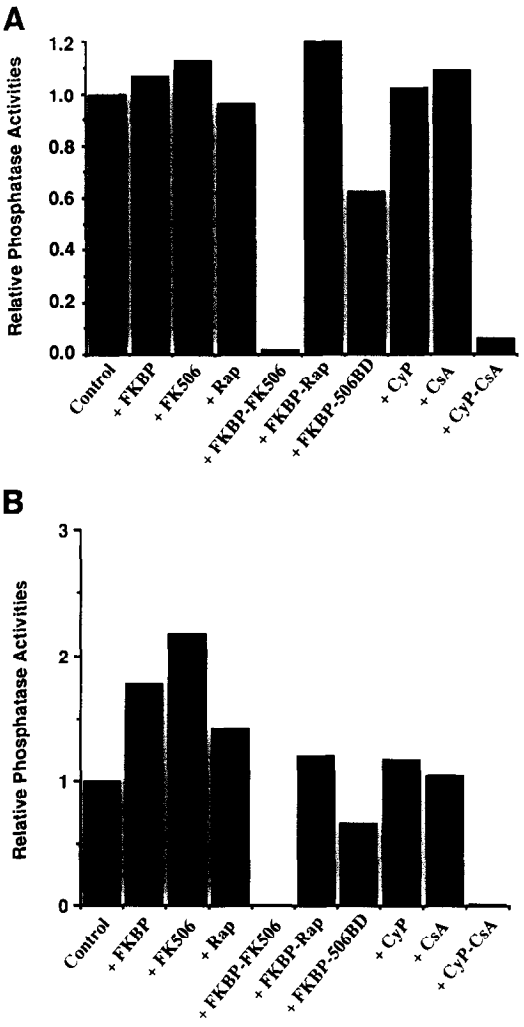

Figure 7. Inhibition of Ca^{2+}- or Ca^{2+}- and Calmodulin-Dependent Phosphatase Activity of Calcineurin toward a Phosphopeptide Substrate by FKBP12–FK506 and Cyclophilin A–CsA Complexes

Under the assay conditions (see Experimental Procedures), the amounts (cpm) of $^{32}P_i$ released from the phosphopeptide were taken as a measure of phosphatase activity without further conversion into initial velocities of the reactions. The final concentrations for different components were as follows: 40 nM calcineurin; 200 nM each FKBP12 and cyclophilin A; 300 nM each FK506, rapamycin, and CsA; and 2.3 μM 506BD. (A) Phosphatase assay in the presence of calmodulin (80 nM). (B) Phosphatase assay in the absence of calmodulin. The presence of immunophilins, their ligands, and the corresponding complexes is indicated below the bars.

the isoform described by Friedman and Weissman [1991]) is inhibited by CsA (K_i = 6 nM) (Fischer et al., 1989; Takahashi et al., 1989). The rotamase activity of the FK506- and rapamycin-binding protein FKBP12 (the "12" descriptor refers to the originally discovered 12 kd isoform [Siekierka et al., 1989; Harding et al., 1989]; other isoforms [Fretz et al., 1991], including the 13 kd FKBP13 [Jin et al., 1991], have been described) is inhibited by FK506 (K_i = 0.4 nM) and rapamycin (K_i = 0.2 nM) (Bierer et al., 1990a). Although sequence-related members of both the cyclophilin and FKBP families exist, there are no similarities in sequence between the two families (Standaert et al., 1990). The atomic structure of cyclophilin A has not been reported, but a preliminary investigation (J. Kallen, M. D. Walkinshaw, and M. Zurini, personal communication) suggests its structure differs significantly from the recently reported atomic structures of FKBP12 (Michnick et al., 1991; Moore et al., 1991) and the FKBP12–FK506 complex (Van Duyne et al., 1991).

Recent investigations have shown that the inhibition of the rotamase activity of immunophilins is an insufficient requirement for mediating the actions of immunosuppressants (Bierer et al., 1990a). For example, a potent, nonnatural rotamase inhibitor (506BD, which inhibits the rotamase action of FKBP12 with K_i = 5 nM) does not inhibit TCR or IgE receptor–mediated signaling pathways at low concentrations (Bierer et al., 1990b). On the other hand, 506BD was found to potently inhibit the actions of both FK506 and rapamycin. Similar results have recently been reported with cyclophilin ligands (Sigal et al., 1991). Binding of immunosuppressant to immunophilin results in a gain, rather than a loss, of function. This is evident in genetic studies in Saccharomyces cerevisiae that provide strong support for a role for immunophilin–drug complexes. For example, deletion of yeast FKBP12 results in rapamycin-resistant strains that are returned to rapamycin sensitvity following transfection of human FKBP12 (Koltin et al., 1991; Heitman et al., 1991b). Related results were obtained with cyclophilin A and CsA (Tropschug et al., 1989) and FKBP12 and FK506 (Heitman et al., 1991a). The role of immunophilins in the absence of drug is an active area of investigation: Friedman and Weissman (1991) describe the association of an immunophilin (cyclophilin C) with specific target proteins, and a cyclophilin variant has been shown to be necessary for the proper trafficking of rhodopsin in Drosophila (Stamnes et al., 1991). The immunophilin ligands CsA, FK506, and rapamycin are equipped with structural elements that provide for high affinity interactions with their immunophilin receptors (in the case of FK506 and rapamycin, these FKBP-binding elements are very similar) and effector elements that, when presented by immunophilin, determine the target (Bierer et al., 1990a, 1990b).

The difference in response to rapamycin and 506BD distinguishes the otherwise inseparable biological actions of CsA and FK506, suggesting that their immediate receptors are distinct (cyclophilin and FKBP) but that these complexes may eventually converge on a common target. The simplest possibility is that the common target binds directly to both the cyclophilin–CsA and FKBP–FK506 complexes. The experiments reported herein identify calcineurin, a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase (Klee and Krinks, 1978; Klee et al., 1979; Stewart et al., 1982) previously shown to be the predominant calmodulin-binding protein in T lymphocytes (Kincaid et al., 1987), as a common target of these immunophilin–drug complexes.

Calcineurin, which is a complex of 61 kd calcineurin A and 19 kd calcineurin B, and calmodulin were retained on a cyclophilin C or FKBP12-based matrix only when it had been preloaded with CsA or FK506, respectively (Figure

1). The observation that CsA is required for the cyclophilin C matrix to retain the calcineurin A is in accord with the results of Friedman and Weissman (1991). The additional protein of 57 kd retained from the tissue extracts used in these studies is very likely the known proteolytic fragment resulting from cleavage of the C-terminus of calcineurin A (Hubbard and Klee, 1989). Affinity matrices based on cyclophilin C, FKBP12, FKBP12–rapamycin (Figure 1), CsA, FK506, and rapamycin (Fretz et al., 1991) do not retain any of these proteins. Elution of the proteins from either immunophilin–drug matrix was also achieved with soluble cyclophilin A–CsA or FKBP12–FK506 complexes (Figure 2). These results suggest the two immunophilin–drug complexes bind competitively to calcineurin and also indicate that two different forms of cyclophilin (A and C) are able to present CsA to the common target. The Ca^{2+}-dependent binding of the immunophilin–drug complexes to calcineurin is in keeping with the correlation of CsA and FK506 actions on Ca^{2+}-dependent signaling pathways (Figure 2).

Treatment of the eluted proteins with Ca^{2+} prior to gel electrophoresis resulted in a gel mobility shift for the M_r 15,000 and 17,000 bands that is characteristic of myristoylated calcineurin B and calmodulin (Klee et al., 1979), respectively (Figure 3). Blotting experiments with anti-calcineurin antibodies (Figure 4) and $^{45}Ca^{2+}$ (Figure 5) provide further support for the identification of the M_r 61,000, 57,000, 17,000, and 15,000 proteins as calcineurin A, the C-terminal peptide cleavage product of calcineurin A, calmodulin, and calcineurin B, respectively. In addition, the primary immunophilin–drug interaction site within the target calcineurin–calmodulin complex was shown to reside within calcineurin by affinity experiments with authentic calcineurin samples that lacked calmodulin. In these experiments only the cyclophilin C–CsA and FKBP–FK506 matrices are able to retain authentic calcineurin (Figure 6). Finally, after the initial review of this article, 25 and 20 amino acid fragments derived from the M_r 61,000 and 15,000 proteins obtained from the affinity experiments described above were sequenced by automated Edman degradation and shown to be 100% identical to sequences in calcineurin A (SQTTGFPSLITIFSAPNYLDVYNNK) and calcineurin B (IYDMDKDGYISNGELFQVLK), respectively.

The influence of immunophilins or immunophilin–drug complexes on the phosphatase activity of authentic calcineurin in the presence of Ca^{2+} and calmodulin was assayed with both p-nitrophenyl phosphate and a phosphopeptide substrate (H_2N-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser(OPO_3^{2-})-Val-Ala-Ala-Glu-CO_2H). In accord with the binding studies, a specific effect is seen with the complexes of both cyclophilin A–CsA and FKBP12–FK506. Whereas these complexes induce a slight increase in the phosphatase activity of calcineurin–Ca^{2+}–calmodulin (by a factor of ~2–3) toward p-nitrophenyl phosphate (data not shown), they potently inhibit activity toward the phosphopeptide substrate in the presence (Figure 7A) or absence (Figure 7B) of calmodulin. These results suggest that the biological function of the immunophilin–drug complexes may be to inhibit the phosphatase activity of calcineurin, but that this may be achieved by binding to a site adjacent to the active site, rather than to the active site. The small p-nitrophenyl phosphate reagent presumably interacts with calcineurin nearly exclusively at active-site residues, whereas the phosphopeptide, which is composed of a sequence derived from the phosphorylation site on the RII subunit of cAMP-dependent protein kinase (a calcineurin substrate) (Blumenthal et al., 1986), is presumed to make extensive contact with the enzyme. In both phosphatase assays, little or no effect was observed with cyclophilin A, FKBP12, CsA, FK506, or rapamycin alone, or with the FKBP12–rapamycin and FKBP12–506BD complexes (Figures 7A and 7B).

Whereas an affinity matrix based on cyclophilin C–CsA was used in the initial adsorption protocol, recombinant cyclophilin A–CsA was found to elute calcineurin–calmodulin from the FKBP12–FK506 affinity matrix and to inhibit the phosphatase activity of calcineurin. Although the relative affinity of different cyclophilin–CsA complexes is unknown, these preliminary studies provide evidence for redundancy in the cyclophilin component. The ability of cyclophilin C to participate in calcineurin binding supports the possibility that other cyclophilin isoforms mediate the actions of CsA (Friedman and Weissman, 1991; Price et al., 1991).

These biochemical investigations suggest that the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin is a common "downstream" biological target of CsA and FK506. As these agents exhibit specificity for activation pathways that induce an increase in intracellular Ca^{2+} concentration, such as those mediated by the TCR and the IgE receptor, calcineurin may be involved in regulating the phosphorylation state of a downstream component of these signaling pathways. The cellular specificity of the actions of CsA and FK506 may be related to their selective interactions with specific isoforms of calcineurin or due to the existence of cell-specific calcineurin substrates.

The competitive binding of cyclophilin A–CsA and FKBP12–FK506 to calcineurin is surprising in light of the absence of apparent structural similarities between the immunophilins cyclophilin A and FKBP12 and their ligands CsA and FK506. It is possible that different binding elements within the same binding site on calcineurin–calmodulin are used by these distinct immunophilin–drug complexes. Although the final resolution of this dilemma may require detailed structural analyses of multimeric ensembles, recent progress in the determination of the structure of immunophilins (Michnick et al., 1991; Moore et al., 1991) and their drug complexes (Van Duyne et al., 1991) and in the understanding of the domain structure of calcineurin (Hubbard and Klee, 1989), together with the availability of genetic systems that lack specific immunophilins (and thus present the characteristic drug-resistance phenotype) (Koltin et al., 1991; Heitman et al., 1991a, 1991b), provides significant opportunities for research in this area.

Our studies define the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin as a common and specific target of cyclophilin–CsA and FKBP–FK506 complexes in vitro. Biological studies should soon determine whether cal-

cineurin is the relevant target of these immunosuppressants in vivo and is thus a key molecule in signal transmission pathways emanating from both the TCR in T lymphocytes and the IgE receptor in mast cells.

Experimental Procedures

Materials

Fresh calf thymus, brain, and spleen were obtained from Research 87 (Revere, MA). Bovine brain calcineurin and calmodulin were purchased from Sigma Chemical (St. Louis, MO). $^{45}CaCl_2$ was purchased from New England Nuclear (Cambridge, MA). ^{32}P-labeled phosphorylated peptide substrate, bovine brain calcineurin, and rabbit anti-calcineurin IgG were generous gifts from Dr. Claude B. Klee (Department of Biochemistry, National Cancer Institute). Goat anti-rabbit IgG conjugated with alkaline phosphatase and the alkaline phosphatase substrates BCIP and NBT were obtained from Pierce (Rockford, IL). Glutathione–Sepharose 4B was from Pharmacia LKB (Uppsala, Sweden). 506BD was prepared by Thomas J. Wandless and Patricia K. Somers in the Harvard laboratory.

Preparation of Fusion Proteins GCyP and GFK

The construction of a glutathione S-transferase–cyclophilin C fusion gene and purification of GCyP were achieved as described (Friedman and Weissman, 1991). To construct the GFK fusion gene, FKBP12 was amplified by the polymerase chain reaction from an FKBP12 coding plasmid (pRFS) using two primers: 5′ primer, 5′-CAGGACACAGGATC-CATGGGC GTGCAGGTGGA-3′; 3′ primer, 5′-GCTGGCTAACGAATT-CAAGGGAGGAGGCCATTCCTGTCAT-3′. The amplified fragment was purified by phenol–chloroform extraction and ethanol precipitation. It was then digested with EcoRI and BamHI and cloned into pGEX-2T, which had been linearized with the same restriction enzymes. The fusion construct pGFK12 was transformed into E. coli XA90, in which the expression of GFK can be induced with IPTG.

To purify GFK, a 1 liter LB culture of XA90/pGFK12 was incubated at 37°C. At an OD_{595} of 0.65, the culture was induced with 1 mM IPTG. The cells were harvested 6 hr after induction, resuspended in 20 ml of 20 mM Tris–HCl (pH 7.8) containing 1 mM PMSF, and lysed by two passes through a French press at 12,000 psi. The nucleic acids were precipitated by addition of 0.2 vol of neutralized 2% protamine sulfate solution to the crude lysate followed by centrifugation (20,000 × g, 20 min). The crude cell extract was then fractionated with ammonium sulfate, and the 40%–80% protein pellet was resuspended in 30 ml of 20 mM Tris–HCl (pH 7.8) and dialyzed against 4 liters of the same buffer.

The dialyzed protein solution was first purified with glutathione–Sepharose as described previously (Smith and Johnson, 1988). The glutathione (5 mM) eluate from the glutathione–Sepharose affinity column was directly loaded onto a DE-52 column that had been equilibrated with 5 column vols of 20 mM Tris–HCl (pH 7.8). The column was then washed with another 5 column vols of the buffer, and the fusion protein was eluted with a step gradient of 0–200 mM KCl. GFK-containing fractions were collected and used directly.

Preparation of Tissue Extracts of Bovine Brain, Thymus, and Spleen

Fresh bovine brain, thymus, and spleen were homogenized (1:3, wt/vol) in 20 mM Tris–HCl (pH 6.8), 0.25 mM NaCl, 2 mM β-mercaptoethanol, 0.02% NaN_3, 1 mM PMSF, and 5% glycerol. The homogenate was centrifuged at 8,000 × g for 4 hr. The supernatant was separated, and the pellet was resuspended in an equal volume of the same buffer. Centrifugation at 8,000 × g for 4 hr gave a second supernatant. The two supernatants were mixed (1:1, vol/vol) and centrifuged at 30,000 × g for 45 min. The supernatant was then filtered through a 0.45 μm filter and kept at 4°C before use.

Adsorption of Calcineurin-Calmodulin with GFK-FK506 and GCyP-CsA Using Glutathione-Sepharose

The crude tissue extracts were preincubated with glutathione–Sepharose (about 1:100 to 1:200 dilution of the Sepharose) at 4°C for 1–3 hr to remove the endogenous glutathione-binding proteins, including glutathione S-transferases. A typical incubation mixture has a total volume of 0.2 ml, consisting of buffer A (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$), 2 μM GFK (or GCyP), 20 μM FK506 (or rapamycin or CsA), and 0.05 to 0.1 ml of tissue extract. After incubation at 4°C on a Nutator for 1.5 hr, 25 μl of 50% (vol/vol) glutathione–Sepharose in buffer A was added, and incubation was continued for 0.5 to 2 hr. The Sepharose beads were precipitated by centrifugation in a microcentrifuge at 8,000 × g for 2 min. The glutathione–Sepharose beads were washed three times with 0.5 ml of buffer A containing 0.2% Triton X-100. The washed glutathione–Sepharose was then resuspended in 25 μl of 2× SDS sample buffer, heated in boiling water for 3 min, and centrifuged for 2 min. The supernatant was subjected to SDS–PAGE followed by silver staining. For purification of the target proteins from calf thymus and brain extracts, each of the components was scaled up proportionally and the proteins were eluted with 20 mM EGTA in 50 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol after three washes with buffer A containing 0.2% Triton X-100.

Western Blot Analysis of Calcineurin, Gel Mobility Shift of Calmodulin and Calcineurin B, and Detection of Calcineurin B by ^{45}Ca Autoradiography

For the Western blot analysis of calcineurin A and B, the proteins were subjected to 12% SDS–PAGE followed by electroblotting onto nitrocellulose using the Bio-Rad Mini-blotting apparatus. Development of the blot with rabbit anti-calcineurin IgG and alkaline phosphatase–conjugated goat anti-rabbit IgG was performed as previously described (Burnette, 1981). To detect the calcium-dependent gel mobility shift of calmodulin and calcineurin B, Ca^{2+} (1 mM) or EGTA (5 mM) was added to the protein solution in SDS sample loading buffer before loading the gel. The $^{45}Ca^{2+}$ binding to calcineurin B and calmodulin and subsequent autoradiography were carried out as previously described (Maruyama et al., 1984).

Phosphatase Assay of Calcineurin

The assay was carried out as described by Manalan and Klee (1983). The substrate used, provided by C. B. Klee, was a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (DLDVPIPGRFDRRVSVAAE), which was phosphorylated with ^{32}P-labeled ATP at the serine residue. The assay buffer consists of 40 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, and 0.5 mM dithiothreitol. The assay mixture (60 μl) contained 40 nM calcineurin, 80 nM calmodulin (when present), and 2 μM phosphopeptide in addition to the assay buffer. It was found that the presence of methanol (3%) inhibits the phosphatase activity significantly. Therefore, the drug solutions were prepared in DMSO as follows. DMSO stock solutions of the drugs were prepared (3000× final concentration) and then diluted 100× with the assay buffer; 2 μl was added to the incubation mixture, giving a final concentration of DMSO of less than 0.04% in the final assay mixture. The incubations were carried out at 30°C for 10 min before the reaction was stopped by addition of the stop solution (5% trichloroacetic acid, 0.1 M potassium phosphate) and loaded onto 0.5 ml Dowex AG 50W-X8 (200–800 mesh; Bio-Rad) columns. After the $^{32}P_i$ was eluted from the column, it was mixed with 12 ml of ScintiVerse II (Fisher Scientific) and counted on a Beckman LS1801 Liquid Scintillation System.

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