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Identification of the Immunophilins Capable of Mediating Inhibition of Signal Transduction by Cyclosporin A and FK506: Roles of Calcineurin Binding and Cellular Location

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The immunosuppressants cyclosporin A (CsA) and FK506 appear to block T-cell function by inhibiting the calcium-regulated phosphatase calcineurin. While multiple distinct intracellular receptors for these drugs (cyclophilins and FKBPs, collectively immunophilins) have been characterized, the functionally active ones have not been discerned. We found that overexpression of cyclophilin A or B or FKBP12 increased T-cell sensitivity to CsA or FK506, respectively, demonstrating that they are able to mediate the inhibitory effects of their respective immunosuppressants in vivo. In contrast, cyclophilin C, FKBP13, and FKBP25 had no effect. Direct comparison of the K_i of each drug-immunophilin complex for calcineurin in vitro revealed that although calcineurin binding was clearly necessary, it was not sufficient to explain the in vivo activity of the immunophilin. Subcellular localization was shown also to play a role, since gene deletions of cyclophilins B and C which changed their intracellular locations altered their activities significantly. Cyclophilin B has been shown previously to be located within calcium-containing intracellular vesicles; its ability to mediate CsA inhibition implies that certain components of the signal transduction machinery are also spatially restricted within the cell.

The immunosuppressants cyclosporin A (CsA) and FK506 block T-cell function by preventing transcriptional activation of the genes that encode the T-cell growth factor interleukin 2 (IL-2) and the immunologically important T-cell-derived lymphokines following antigenic stiumulation (5, 9, 22). Recent work indicates that complexes of CsA and FK506 with their specific binding protein cyclophilin or FKBP can bind and inactivate the calcium-calmodulin-regulated serine/threonine phosphatase calcineurin in vitro (25). Furthermore, overexpression of calcineurin renders T cells resistant to the actions of the drugs and more sensitive to activating agents, strongly suggesting that calcineurin is both a physiologic mediator of T-cell activation and the major, if not the exclusive, target of CsA-cyclophilin and FK506-FKBP complexes (7, 30). However, the family of proteins homologous to cyclophilin and FKBP is large (12–15, 19, 20, 31, 36) and the identity of the biologically active immunophilins has been a mystery. This has confounded the search for endogenous homologs of CsA and FK506 and made the identification of targets for cyclosporin and FK506 confusing (10). We used a novel assay to define the biologically active immunophilins in T lymphocytes. We also obtained evidence suggesting that to block signal transmission, the immunophilin, when complexed to a drug, must both bind calcineurin and be properly localized within the cell.

MATERIALS AND METHODS

Materials. Fresh bovine brain was obtained from Research 87 (Revere, Mass.). Thrombin, α-chymotrypsin and its peptide substrates, calmodulin, and *p*-nitrophenol phosphate were purchased from Sigma Chemical (St. Louis, Mo.). [γ-^{32}P]ATP was purchased from New England Nuclear (Cambridge, Mass.). Factor Xa was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Glutathione Sepharose 4B was purchased from Pharmacia LKB (Uppsala, Sweden). Cyclophilin C (CypC) was obtained as a glutathione *S*-transferase (GST) fusion protein (GST-CypC) in frozen cell pellets as a generous gift from Jeff Friedman and Irving Weissman (Departments of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, Calif.).

Cells and growth media. TAg Jurkat cells were from J. Northrop (Stanford Medical Center). They were grown in RPMI 1640–10% fetal calf serum (Gemini)–penicillin–Streptomycin (GIBCO). NIH 3T3 cells were grown in Dulbecco modified Eagle medium–10% fetal calf serum–penicillin–streptomycin.

Plasmids. pBJ5stop (provided by D. Mendel, Stanford Medical Center) contains the SRα promoter and the simian virus 40 origin of replication (37). Cyclophilin- and FKBP-coding sequences were inserted into the *Xho*I or *Not*I site in the polylinker downstream of the promoter as detailed below. pBEX1 was derived from pBJ5stop by insertion of the synthetic oligonucleotide (restriction site underlined) AATTCTAGATGGCCATGAATTCTACCCATACGACGT CCCAGACTACGCTTAA into the *Eco*RI site just downstream of the promoter. These nucleotides add the influenza virus hemagglutinin epitope (EFYPYDVPDYA) (23) to the C termini of inserts into the *Xho*I-*Eco*RI sites of the plasmid.

Human CypA cDNA was prepared from cytoplasmic Jurkat cell RNA by reverse transcription and amplified by polymerase chain reaction with the primers CTAGGATCCA TGGTCAACCCCACCGTG and AACGAATTCGAGTTGT CCACAGTC. The polymerase chain reaction product was cloned into expression vectors pBJ5stop and pBEX1 at the sites indicated above. Plasmids containing human CypB cDNAs were from Sutcliffe and Walsh (15, 31). The entire

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open reading frames were excised with *Eco*RI-*Not*I or *Eco*RI-*Hin*dIII restriction endonucleases, respectively, and subcloned into the two expression vectors listed above.

A plasmid containing the murine CypC cDNA was from J. Friedman and I. Weissman (12); the full open reading frame was excised by cleavage with endonucleases *Hin*dIII and *Stu*I and subcloned as described above. Human FKBP12, FKBP13, and FKBP25 cDNAs were cleaved with *Eco*RI-*Hin*dIII, *Eco*RI, and *Bam*HI-*Sal*I, respectively, and cloned into expression vector pBJ5stop.

Polymerase chain reaction derivatives of the cyclophilin cDNAs had *Xho*I and *Eco*RI cohesive ends and were cloned directly into pBJ5stop or pBEX1 cleaved with the same restriction endonucleases. The oligonucleotides used were CC-FL (GACTCGAGACCATGAGCCCGGGTCCC), CC-TV (GACTCGAGACCATGGTGACGGACAAGGTCTTCT TT), B-FL (GACTCGAGAACATGAAGGTGCTC), CC-3E (CTGAATTCTGTCACCAATCAGGGAC), F13-TV (GAC TCGAGACATGACGGGGACCGAGGGCAAAAGG), F13-3E (GAGGAATTCTGGTTACAGCTCAGTTCG), C3E-NS (GCGAATTCCCAATCAGGGACCTC), B3E-NS (GCGAA TTCCTCCTTGGGGATGGC), BCC-Top (GGCCCAAA-GT GACGGACAAGGTCT), BCC-Bot (GTCCGTCAC-TTTGG GCCCCTTCT), CBB-Top (GTCCCTCG-GTCACCGTCAA GGTG), CBB-Bot (GACGGTGAC-CGAGGGACCTCGT), BBC-Top (GAGGTGGTG-CATTCCATTGAACTTCA), BBC-Bot (CAATGGAATG-CACCACCTCCATGCCCTC), CCB-Top (GACTGTGGTA-CGGAAGGTGGAGAGCAC), CCB-Bot (CCACCTTCCG-TACCACAGTCATCCCATC).

Construction of N-terminal deletions of CypC and FKBP13 by the method of Ho et al. (16) was done with the CC-TV and CC-3E or F13-TV and F13-3E oligonucleotides, respectively. Fusion constructs of CypB and CypC were made by the technique of Horton et al. (17): the BCC fusion used oligonucleotides B-FL plus BCC-Bot and BCC-Top plus C3E-NS; CBB fusion used oligonucleotides CC-FL plus CBB-Bot and CBB-Top plus B3E-NS. The 3'-end swap constructs BBC and CCB were made in identical fashion by using the last four oligonucleotides listed above.

Reporter construct SXNFAT was made by ligation of a *Pvu*I-*Hin*dIII fragment containing three tandem NFAT sites and the IL-2 TATA element (from plasmid NFAT-Z (11) to a *Hin*dIII-*Ssp*I fragment from pPL/SEAP (3) containing the secreted placental alkaline phosphatase minigene and to the *Nde*I-*Pvu*I fragment of pSV2Neo containing the simian virus 40 origin, the neomycin resistance gene, and half of the β-lactamase gene. (For that ligation, the *Ssp*I and *Nde*I ends were rendered flush by addition of the large fragment of DNA polymerase I and deoxynucleoside triphosphates.) An equivalent construct, SXOAP, using OAP-Oct-binding sites, was made in a similar fashion by using an enhancer-promoter fragment containing four tandemly linked copies of the NFIL2A site (which binds Oct-I and OAP) and the IL-2 TATA element (38).

Transient transfection assay of immunophilin activity. We transfected 10^7 TAg Jurkat cells with 5 μg of the SXNFAT (or SXOAP) reporter plasmid and 1 to 10 μg of the immunophilin overexpression plasmid or the control plasmid (pBJ5stop) by electroporation as previously described (27). Cells were grown in 10 ml of complete RPMI 1640 at 37°C for 24 h and then collected by centrifugation at 1,000 × *g*. They were suspended in 3.6 ml of complete medium, and 100-μl aliquots were placed into the wells of a 96-well plate. Each well was independently stimulated by addition of phorbol myristate acetate (PMA; Sigma) to 20 ng/ml, ionomycin (Calbiochem) to 0.5 to 2 μM, and CsA (Sandoz) at a final concentration of 0 to 100 ng/ml or FK506 at a final concentration of 0 to 500 pg/ml. After a further 24 h of incubation at 37°C, the plates were heated to 67°C for 1 h to inactivate all endogenous cellular and serum phosphatases and specific phosphatase activity was measured by addition of 100 μl of the supernatant to an equal volume of 2 M diethanolamine (pH 10)–1.2 mM methylumbelliferyl phosphate–20 mM L-homoarginine (an inhibitor of nonsecreted alkaline phosphatases) (all reagents were from Sigma). This method was adapted from reference 3. Fluorescence was measured on a Titertek Fluroskan II plate fluorescence reader by using 355-nm excitation and 460-nm emission wavelengths. The measured signal was generally 10 to 20 times the background level of 80 to 100 U after overnight incubation at 37°C. Duplicates were performed for each level of CsA or FK506, and results were usually within 5% of each other.

To verify the CsA sensitivity of EL4 cells, we transfected 10^7 EL4 cells with a plasmid (NFAT-luc) that directs luciferase synthesis from the NFAT enhancer. The cells were stimulated with PMA (20 ng/ml) and ionomycin (0.5 μM) with or without CsA for 8 h, and then luciferase levels were measured by standard protocols. NFAT-specific activated transcription was inhibited 96.2% by 20 ng of CsA per ml and 97.8% by 100 ng of CsA per ml.

RNase protection. A ^{32}P-labeled RNA riboprobe was prepared by using T3 or T7 RNA polymerase (Pharmacia) from template DNA containing the central 155-bp *Bam*HI-*Hin*cII fragment from CypC cDNA or a 104-bp fragment from p68 cDNA (28). RNase protection was done as previously described (27), with 10 μg of tRNA, EL4 cytoplasmic RNA, or mouse kidney total RNA.

Construction of bacterial expression vectors for immunophilin genes. The immunophilins were overexpressed as GST fusions. To construct the fusion genes, the cDNA for each immunophilin was amplified by the polymerase chain reaction with the appropriate primers with the indicated flanking restriction sites. The following primers were used with a reverse M13 oligonucleotide sequencing primer (*Eco*RI) because the cDNA template for CypB was inserted in the *Eco*RI sites of pBS(S/K) (Stratagene) (restriction sites are underlined): FKBP13, 5'-GGCCGGATCCGTAATA TGACGGGGGCCGAGGG-3' (*Bam*HI) and 5'-GCTGGCT AACGAATTCTGGTTACAGCTCAGTTCGA-3' (*Eco*RI); FKBP25, 5'-GCGCGGATCCCCATGGCGGCGCCCGTTC CACAGCGG-3' (*Bam*HI) and 5'-GCGCGAATTCTCAAT CAATATCCAC-3' (*Eco*RI); CypB, 5'-CGCGCGGATCC CCGGGGGGGGGCCCAAAGTCACCG-3' (*Bam*HI). The amplified fragments were digested, ligated into pGEX-3X (Pharmacia), and transformed into *Escherichia coli* BL21.

Isolation and purification of immunophilins as GST fusion proteins. The immunophilins were overexpressed as GST fusion proteins in *E. coli* BL21 and isolated with a glutathione-Sepharose affinity matrix by standard methods (34). The fusion proteins were cleaved with Factor Xa, and in the case of CypC with thrombin; this was followed by ion-exchange chromatography on Q-Sepharose or DEAE CL-2B to obtain pure protein (4).

Determination of the K_is of FK506 and CsA for their respective immunophilins. Rotamase assays and calculation of inhibition constants were performed as previously described (24, 26), with minor modifications. The phosphatase assays were performed at 50, 100, and 200 nM calcineurin with a 1 M equivalent of calmodulin. FK506 and CsA were used at 10 μM. Immunophilin concentrations were varied from 50 nM to 1 μM. Kinetic analysis of phosphatase activity and inhibition assumed saturation of immunophilin

by the drug at a concentration of 10 μM, simplifying K_i to a single equilibrium. For R = immunophilin receptor, C = calcineurin, and A = activity of calcineurin, $K_i = \%A([R]_0 - [1 - \%A]\ [C]_0)/(1 - \%A)$. Binding data for FKBP12 and CypA are from reference 24.

Western blot (immunoblot) analysis of transiently transfected proteins. A monoclonal antibody (12CA5) to the influenza virus hemagglutinin epitope was a generous gift from Peter Kolodziej or was prepared from ascites by protein A chromatography. Extracts containing Jurkat cell proteins (6 to 18 μg, depending on the efficiency of transformation, as determined by cotransfection of the SXNFAT reporter) were fractionated on 1-mm-thick sodium dodecyl sulfate (SDS)–15% polyacrylamide gels and analyzed by standard Western blot techniques. The influenza virus epitope antibody was used at 5 μg/ml, and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G antiserum (Zymed) was used at a 1:2,000 dilution. Specific protein bands were visualized with the ECL detection system (Amersham).

Indirect immunofluorescence. NIH 3T3 cells were collected by trypsinization and replated directly into eight-well tissue culture slides (Nunc). After 2 to 24 h of incubation at 37°C, 1 μg of the epitope-tagged cyclophilin expression vector DNA per well was transfected by the $CaPO_4$ precipitation procedure. Cells were grown for a further 24 h, washed with phosphate-buffered saline, and fixed by incubation with 3.7% formaldehyde in phosphate-buffered saline for 15 min. All steps were performed at room temperature. The cells were rinsed in TS (145 mM NaCl, 25 mM Tris [pH 7.5], 5 mM KCl) and permeabilized with solution B (TS, 0.25% gelatin, 0.5% Triton X-100) for 30 min. Antibody 12CA5 was diluted to 4 μg/ml in B* (TS, 0.25% gelatin, 0.05% Triton X-100) and layered on cells for 30 min. Following washes with B*, a second 30-min incubation with a 1:128 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G antiserum (Caltag) was performed and the unbound antibody was removed by three washes with B*. Fluorescence was imaged with a Zeiss Axiophot microscope.

RESULTS

Identification of biologically active cyclophilins and FKBPs by transient overexpression in Jurkat T cells. To determine which of the immunophilins mediate the actions of CsA and FK506, we examined the effects of cyclophilin or FKBP overexpression on the drug sensitivity of T cells. We reasoned that increasing the amount of an immunologically relevant immunophilin should render cells more drug sensitive by increasing the number of drug-immunophilin complexes. To produce levels of expression greater than that of the endogenous immunophilins, we used an expression vector with a T-antigen-dependent replication origin and Jurkat T cells containing a stably integrated copy of T antigen. The expression levels achieved are comparable to those in COS cells (29). Such vectors which encode the known cloned cyclophilin and FKBP proteins were transfected into TAg Jurkat cells. To quantitate T-cell activation, a phosphatase reporter plasmid containing multiple copies of either the NF-AT or NF-IL2A binding sites from the IL-2 enhancer was cotransfected. Both DNA sequences have been shown to direct high-level transcription from the proper initiation site following T-cell activation through the T-cell receptor in normal thymocytes from transgenic mice (40), in T-cell hybridomas (21), and in human Jurkat cells (9, 27, 38). In addition, the activity of NF-AT closely parallels that of the endogenous IL-2-encoding gene in transgenic mice and is completely inhibited by low levels of CsA or FK506 (9, 21, 27, 39).

Overexpression of CypA rendered the cells three- to fourfold more sensitive to CsA following stimulation by PMA and ionomycin compared with cells transfected with a control plasmid (Fig. 1a). To rule out nonspecific causes for the shift, we examined the effect of CypA overexpression on sensitivity to FK506. There was no change in the FK506 dose-response curve (Fig. 1c), demonstrating that the effect is specific for CsA. Transfection of CypB produced an even greater shift of the CsA dose-response curve to the left (Fig. 1b), with no effect on FK506 sensitivity (data not shown), indicating that it too is capable of mediating the CsA inhibitory effect.

CypC was previously postulated to be the immunologically relevant immunophilin because of its limited tissue distribution (12); the other known cyclophilins are found in all tissues. To identify a possible role for CypC in T cells, we analyzed the level of murine CypC mRNA in the mouse EL4 T-cell line, which is exquisitely sensitive to CsA (32). (We verified that our strain of EL4 is inhibited over 96% by CsA at 20 ng/ml, a finding consistent with previously published results [32].) We were unable to detect any CypC mRNA by RNase protection, whereas it was extremely abundant in total kidney RNA (Fig. 2b), as previously reported (12). The message that encodes the ubiquitously expressed calcium-phospholipid-binding protein p68 (28) was used as a positive control for the RNase protection assay to demonstrate mRNA integrity (Fig. 2a). We determined whether CypC could mediate CsA inhibition if expressed in T cells. In contrast to that of CypA or CypB, transient transfection of CypC had no effect on the response to CsA or FK506 (Fig. 1b). Western analysis of cell extracts following transfection with epitope-tagged cyclophilin expression vectors demonstrated that the proteins were correctly synthesized and accumulated to equivalent levels (Fig. 1e). This result confirms that CypC is not necessary to mediate the CsA immunosuppressive process in vivo.

We performed a similar analysis of the known and cloned FKBPs to determine which can mediate the inhibitory actions of FK506. Overexpression of FKBP12 increased sensitivity to FK506 without any change in CsA sensitivity (Fig. 1a and c). In contrast, neither FKBP13 nor FKBP25 had any effect on the CsA or FK506 dose-response curve (Fig. 1d). Therefore, of the available FKBPs, only FKBP12 can mediate the FK506 inhibitory effect.

Determination of drug- and calcineurin-binding affinities of immunophilins in vitro. The proposed mechanism of action of the immunophilins involves formation of an immunophilin-immunosuppressant complex that binds to and inhibits the activity of calcineurin (33). We therefore measured the abilities of these proteins to inhibit calcineurin when complexed to their respective drugs, as judged by the ability to inhibit phosphatase activity. We also determined the affinities of the drugs for the different immunophilin isoforms. As indicated in Table 1, FKBP12 was the only FKBP that participated in calcineurin inhibition, thus explaining the in vivo transfection results. Surprisingly, all three of the cyclophilins participated in binding to calcineurin, while only the A and B isoforms mediated the action of CsA in vivo, as shown above. However, in no case did we identify a protein that was active in our transfection assay yet did not participate in binding to calcineurin, lending additional support to the model of immunosuppressant action in which

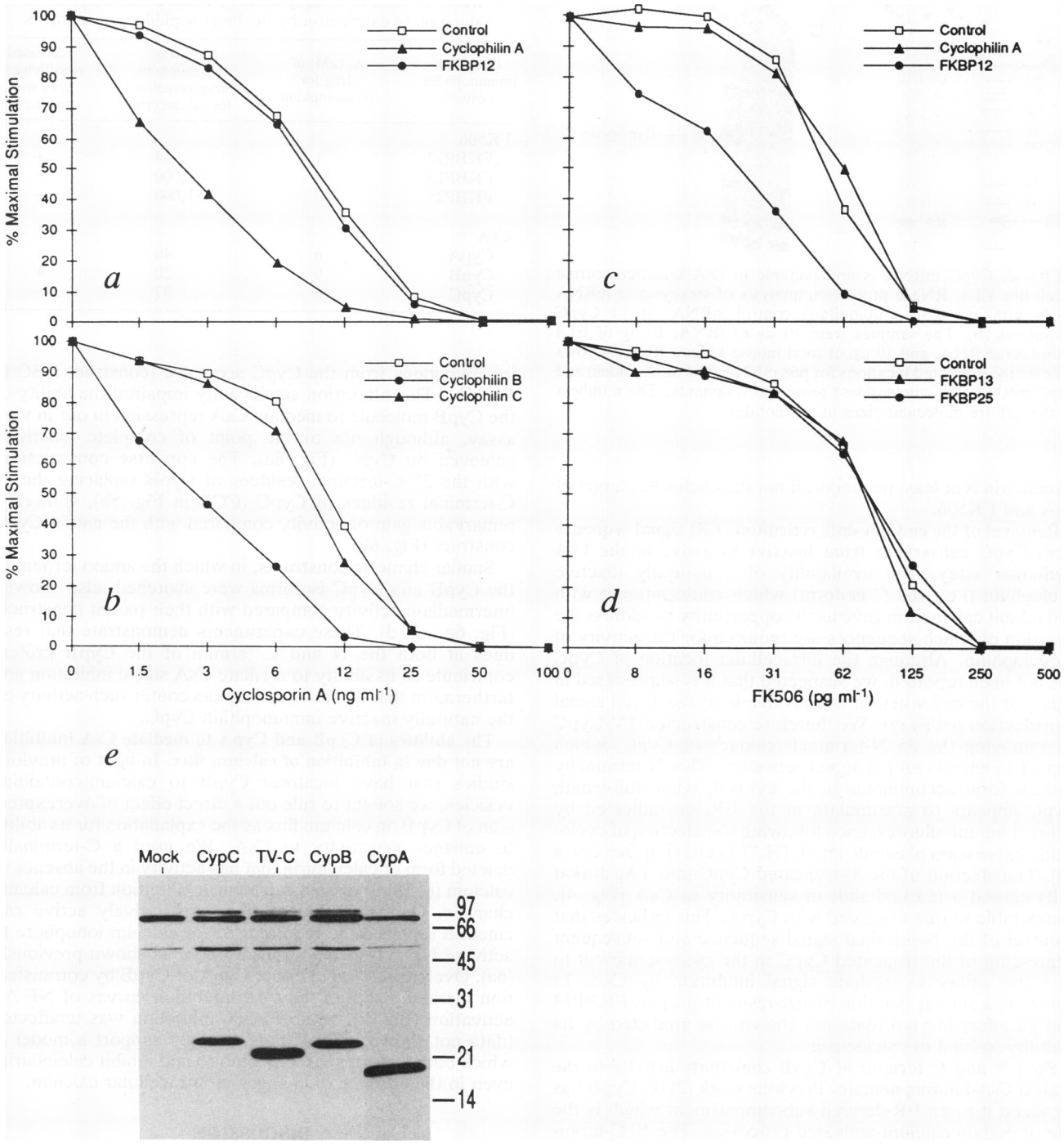


FIG. 1. Effect of overexpression of cyclophilin or FKBP isoforms on T-cell sensitivity to CsA and FK506. TAg Jurkat cells were cotransfected with NFAT-phosphatase reporter plasmid SXNFAT and overexpression plasmids that encode CypA (a and c), CypB (b), CypC (b), FKBP12 (a and c), FKBP13 (d), or FKBP25 (d) or with control plasmid pBJ5. The cells were stimulated with PMA and ionomycin in the presence of the indicated levels of CsA (a and b) or FK506 (c and d), and the amount of induced phosphatase was determined. Similar results were obtained with an NF–IL-2A reporter construct (data not shown). (e) T-antigen-containing Jurkat cells were transfected with epitope-tagged cyclophilin constructs and reporter plasmid SXNFAT. Cells were lysed with Nonidet P-40, and total proteins were analyzed by Western immunoblotting with epitope antibody 12CA5 following electrophoresis on an SDS–15% polyacrylamide gel. Lanes: 1, pBJ5stop; 2, CypC; 3, TV-C (5′-end deletion removing putative ER signal sequence); 4, CypB; 5, CypA. To control for transfection efficiency, the amount of extract loaded was normalized to the level of phosphatase synthesized following stimulation without any CsA or FK506. Addition of the epitope tag EFYPYDVPDYA (23) to the C termini had no effect on the activities of the various constructs in vivo (data not shown). The molecular masses of the epitope-tagged immunophilins are about 20 kDa. The upper bands represent cross-reacting material present in control cells. The numbers to the right are molecular masses in kilodaltons.

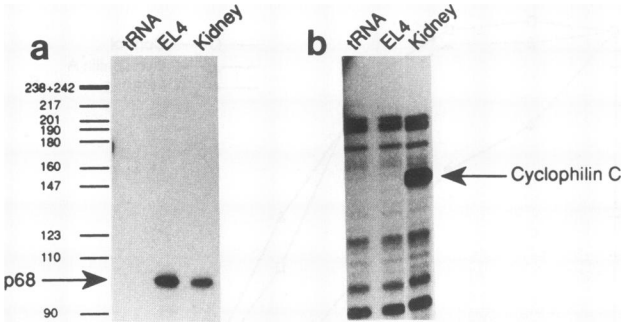


FIG. 2. CypC mRNA is undetectable in CsA-sensitive murine T-cell line EL4. RNase protection analysis of steady-state mRNA levels with p68 (28), a ubiquitous control mRNA (a), or CypC riboprobes (b). The samples were 10 μg of tRNA, 10 μg of EL4 cytoplasmic RNA, and 10 μg of total mouse kidney RNA. Arrows indicate the predicted locations for p68 mRNA (104 nucleotides) and CypC mRNA (155 nucleotides) protected fragments. The numbers on the left are molecular sizes in nucleotides.

calcineurin is at least the major, if not the exclusive, target of CsA and FK506.

Removal of the endoplasmic reticulum (ER) signal sequence from CypC converts it from inactive to active in the CsA mediation assay. The availability of a naturally inactive cyclophilin (i.e., the C isoform) which could interact with and inhibit calcineurin gave us the opportunity to address the question of which sequences are required for the activity of a cyclophilin. Although the intracellular location of CypC has not been reported, we suspected that it is sequestered in a part of the cell where it is unavailable to the T-cell signal transduction pathways. We therefore constructed TV-CypC by removing the 35 N-terminal residues of CypC, which appear to encode an ER signal sequence. This N-terminally deleted form accumulates in the cytosol, while full-length CypC appears to accumulate in the ER, as indicated by indirect immunofluorescence following transfection of cyclophilin expression plasmids into NIH 3T3 cells (Fig. 3c versus 3d). Transfection of the 5′-truncated CypC into TAg Jurkat cells caused a marked shift in sensitivity to CsA (Fig. 4), comparable to that observed with CypA. This indicates that removal of the N-terminal signal sequence and subsequent expression of the truncated CypC in the cytosol allow it to gain the ability to mediate signal inhibition by CsA. In contrast, a similar deletion of ER-resident protein FKBP13 had no effect in vivo (data not shown), as predicted by its inability to bind to calcineurin.

Both N and C termini of CypB contribute activity to the central CsA-binding domain. Previous work (2) on CypB has localized it to an ER-derived subcompartment which is the site of certain calcium-activated processes. The 10 C-terminal amino acids of CypB were shown to be partly responsible for directing it to that site. To assess the importance of the carboxy-terminal extension of CypB, we replaced the 37 C-terminal codons of the CypB cDNA with the corresponding 39 codons from the CypC sequence (construct BBC in Fig. 5b). This alteration significantly impaired the ability of the CypB molecule to mediate CsA repression in our in vivo assay, although not to the point of complete inactivity achieved by CypC (Fig. 6a). The converse construction, with the 37 C-terminal residues of CypB replacing the 39 C-terminal residues of CypC (CCB in Fig. 5b), showed a remarkable gain of activity compared with the native CypC construct (Fig. 6b).

TABLE 1. Binding of drug to its respective immunophilin and inhibition of calcineurin by the immunophilin-drug complex

Drug and immunophilin isoform	K_i (nM) of drug for immunophilin	K_i (nM) of immunophilin-drug complex for calcineurin	Increased sensitivity to drug with transfection
FK506			
FKBP12	1	34	+
FKBP13	55	>1,000	−
FKBP25	180	>1,000	−
CsA			
CypA	6	40	+
CypB	9	20	+
CypC	6	37	−

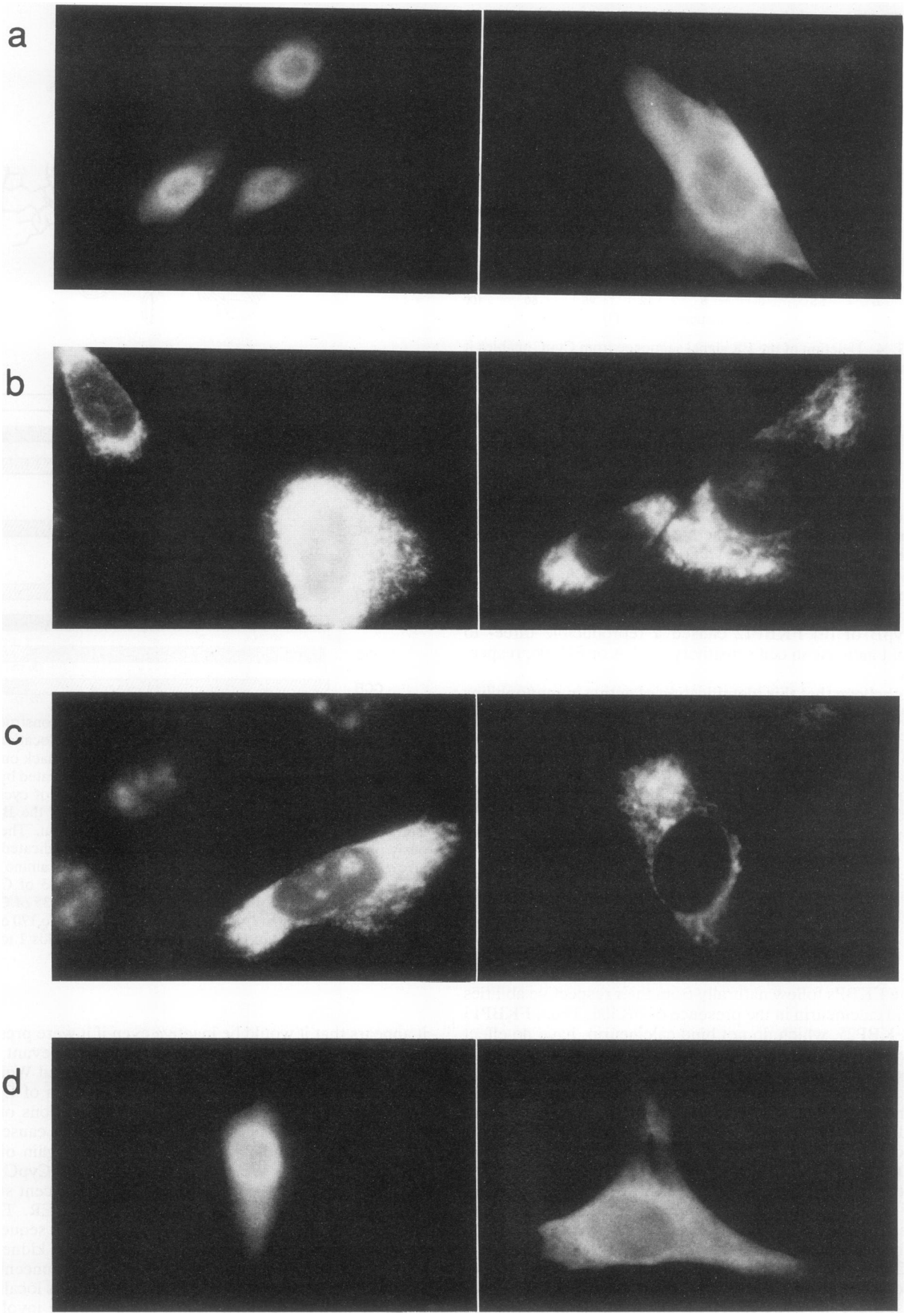
Similar chimeric constructs, in which the amino termini of the CypB and CypC isoforms were switched, also showed intermediate activity compared with their parent constructs (Fig. 6c and d). These experiments demonstrate that residues at both the N and C termini of the CypB protein contribute to its ability to mediate CsA signal inhibition and furthermore that these sequences can confer such activity on the naturally inactive immunophilin CypC.

The abilities of CypB and CypA to mediate CsA inhibition are not due to inhibition of calcium flux. In light of previous studies that have localized CypB to calcium-containing vesicles, we sought to rule out a direct effect of overexpression of CypB on calcium flux as the explanation for its ability to enhance sensitivity to CsA. We used a C-terminally deleted form of calcineurin that has activity in the absence of calcium (8, 18) to dissect calcineurin inhibition from calcium changes. Cotransfection of this constitutively active calcineurin replaced the requirement for calcium ionophore to activate NF-AT-specific transcription, as shown previously (6a). Overexpression of either CypA or CypB by cotransfection markedly shifted the CsA inhibition curves of NF-AT activation (Fig. 7), while FK506 inhibition was unaffected (data not shown). These data strongly support a model in which both isoforms directly bind to and inhibit calcineurin, even in the absence of changes in intracellular calcium.

DISCUSSION

Specific immunophilin isoforms mediate immunosuppressant drug action in vivo. The recent demonstration that calcineurin is the target of FKBP-FK506 and cyclophilin-

FIG. 3. Deletion of 35 N-terminal residues from CypC changes its site of accumulation from the ER to the cytosol. Epitope-tagged cyclophilin expression vectors were transfected into NIH 3T3 cells by calcium-phosphate precipitation. After 24 h, cells were fixed and stained with influenza virus hemagglutinin epitope antibody. Nontransfected or mock-transfected cells had a slight degree of nonspecific staining of nuclei (see the two cells to the left in panel c) but were otherwise unstained. Constructs encoded: a, CypA; b, CypB; c, CypC; d, TV-CypC. The diffuse staining of cells in panels a and d is consistent with localization of CypA and TV-CypC in the cytosol, while the punctate, reticular staining of cells in panels b and c is consistent with sublocalization in the ER.


a
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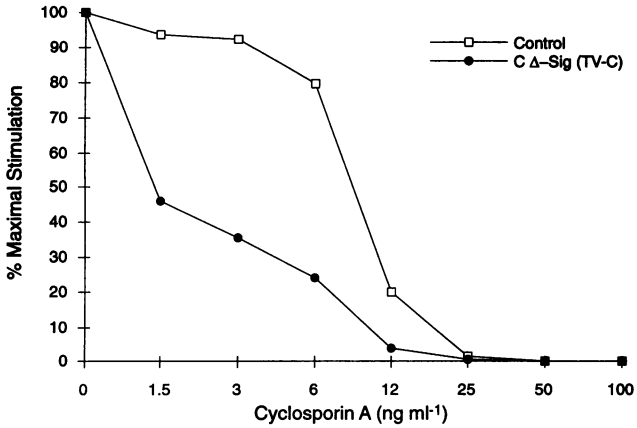

FIG. 4. Deletion of the ER signal sequence from CypC enables it to mediate CsA inhibition. TAg Jurkat cells were transfected with the N-terminally deleted form of CypC (TV-CypC [TV-C]; see map, Fig. 5b) and assayed for sensitivity to CsA as described in the legend to Fig. 1. TV-CypC had no effect on FK506 sensitivity following transfection (data not shown).

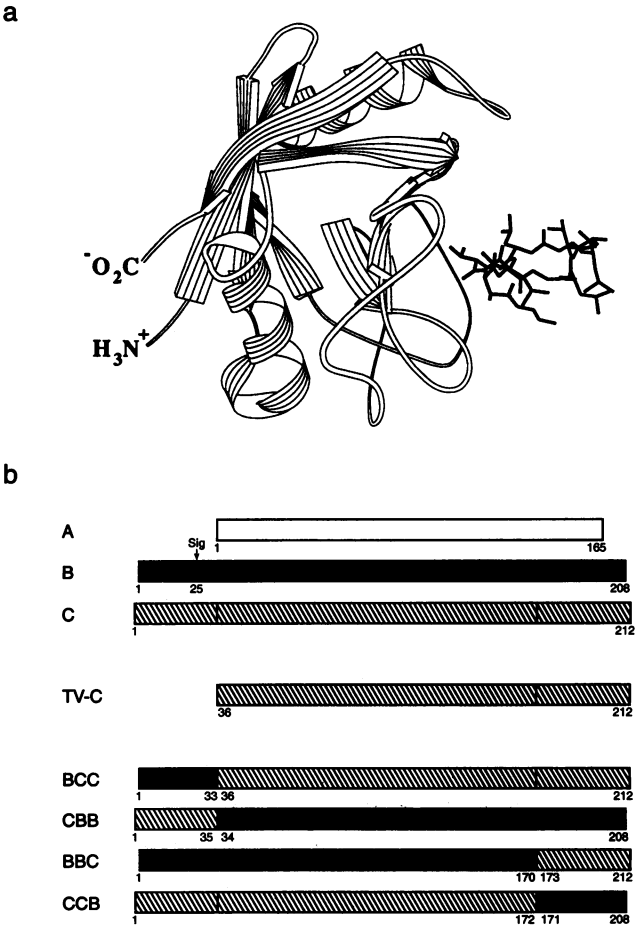

FIG. 5. Structures of cyclophilin and mutant constructs. (a) Three-dimensional structure of CypA-CsA (35). The location of the CsA-binding site is evident from the location of the black outline of CsA to the right. Amino and carboxy termini are indicated by H_3N^+ and O_2C^- to the left of the protein. (b) Schematics of cyclophilin isoform primary structures. The central sections of the B and C isoforms are homologous to the CypA-coding region. The previously proposed signal cleavage site of CypB (2) is indicated by the arrow. N-terminal deletion of CypC (TV-C) removes amino acids 2 to 35. The BCC construct fuses amino acids 1 to 33 of CypB to residues 36 to 212 of CypC. CBB fuses residues 1 to 35 of CypC to amino acids 34 to 208 of CypB. BBC fuses residues 1 to 170 of CypB to residues 173 to 212 of CypC. CCB fuses amino acids 1 to 172 of CypC to residues 171 to 208 of CypB.

CsA complexes (7, 25, 30, 33) raised the question as to which of the various isoforms are active in mediating the immunosuppressive actions of CsA and FK506. We used a novel assay to identify those active isoforms. The approach employed transient transfection to overexpress a test immunophilin in a T cell. We found that overexpression of (i) CypA or CypB or (ii) FKBP12 caused a reproducible three- to fivefold increase in cell sensitivity to CsA or FK506, respectively.

We believe that this identification of active immunophilins is valid for several reasons. (i) The shift in drug response was evident with either NFAT or Oct/OAP reporter constructs (data not shown), both of which are known to be sensitive to these immunosuppressant drugs (9). (ii) The cellular drug response is completely specific for the class of immunophilin transfected (i.e., CsA for cyclophilin and FK506 for FKBP). A nonspecific shift in the drug response curves should affect both drugs equally. (iii) The activity of an immunophilin correlates with its ability to bind calcineurin (see below).

A subset of the known immunophilins mediate drug-induced inhibition of signal transduction. We have demonstrated that (i) CypA and CypB and (ii) FKBP12 can mediate the inhibitory actions of CsA and FK506, respectively. In contrast, CypC, FKBP13, and FKBP25 are inactive. The results for the FKBPs follow naturally from their respective abilities to bind calcineurin in the presence of FK506. Thus, FKBP13 and FKBP25, which do not bind calcineurin, have no effect in the assay, as predicted by the in vitro studies (Table 1) and the postulated mechanism of action of CsA and FK506. The case for the cyclophilins is less clear, since CypC can bind calcineurin but is inactive in vivo. However, in no case did we identify a protein that was active in our assay yet did not participate in binding to calcineurin, lending additional support to the model of immunosuppressant action.

Although CypC was among the three immunophilins initially used to identify the connection between immunosuppressants and calcineurin, we found that it was inactive in our in vivo assay. Our in vitro binding studies indicate that CypC participates in tight binding to calcineurin, with activity comparable to those of the other two cyclophilin isoforms. Since the CypC mRNA is undetectable in T cells and it appears that it would be inactive even if it were present, it is almost certainly not the immunologically relevant cyclophilin, contrary to the proposal of Friedman and Weissman (12). We postulate that the subcellular location of CypC is responsible for its inability to mediate the actions of CsA, since deletion of the N-terminal signal sequence caused both cytoplasmic accumulation of the protein and gain of CsA-mediating activity. We found that full-length CypC transfected into cells had an indirect immunofluorescent staining pattern consistent with its localization in the ER. Thus, it appears that it is inactive by virtue of being sequestered away from calcineurin. The function of CypC in kidney cells has yet to be determined, but the intracellular concentration in that organ is high (12). Assuming that CypC is localized in a similar manner in kidney cells, it may not be involved in

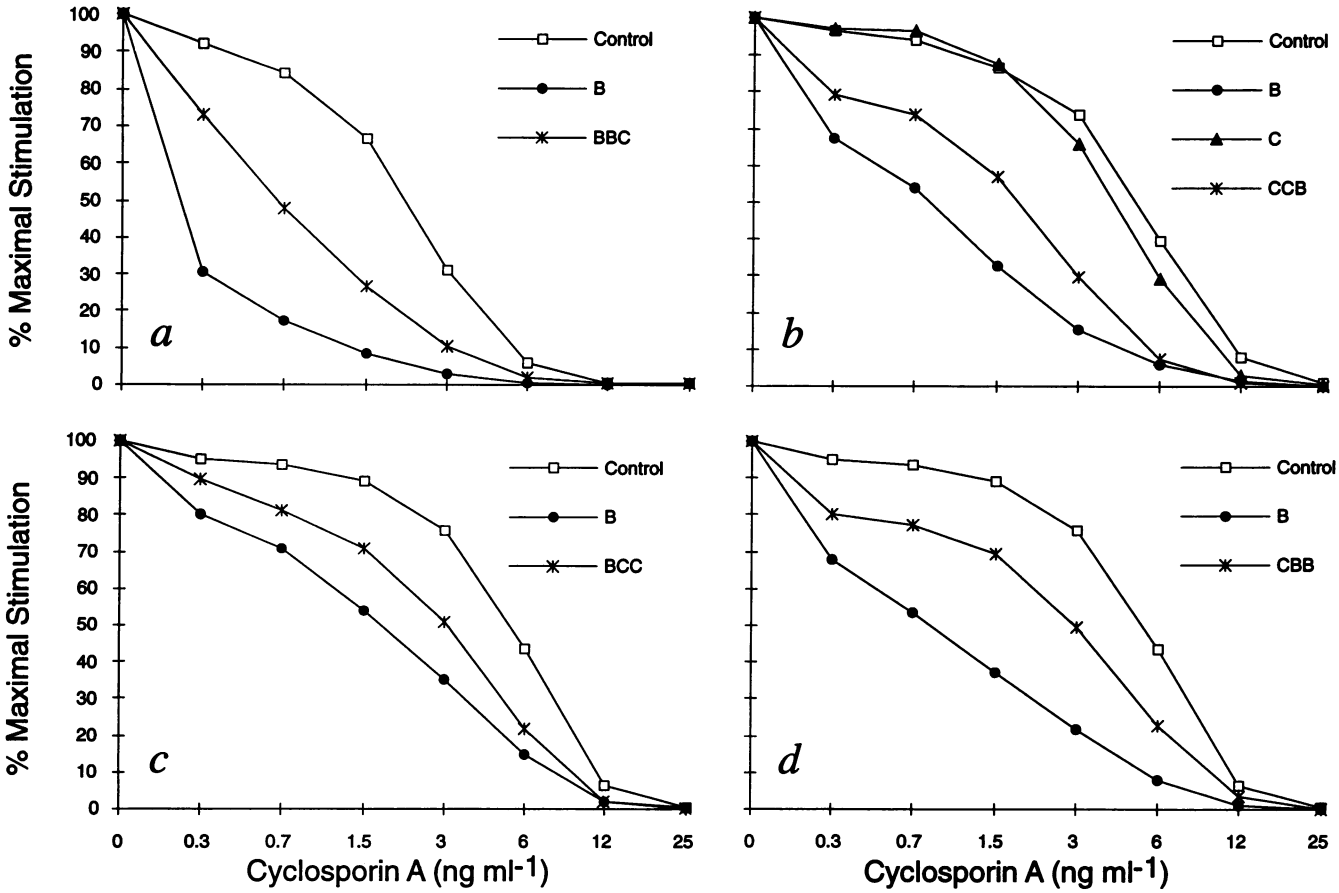

FIG. 6. Transfection assay of chimeric cyclophilin constructs shown in Fig. 5b. TAg Jurkat cells were transfected with overexpression vectors and tested for a CsA mediation effect as described in the legend to Fig. 1. Test constructs were the BBC fusion (a), the CCB fusion (b) and fusions BCC (c) and CBB (d). B, native CypB transfection; C, native CypC transfection. For each experiment, several different amounts of DNA were transfected into different aliquots of cells and analyzed by Western blotting for the amount of immunophilin accumulated. Only samples with equal amounts of recombinant cyclophilin were used for analysis. Fusion constructs had no effect on FK506 sensitivity following transfection (data not shown).

CsA-mediated nephrotoxicity, since calcineurin binding is necessary for inhibition of renal sympathetic nerve activity (33a).

We were initially surprised to discover that CypB is active, considering its known ER location. However, the recent finding that CypB is associated with a specialized part of the ER related to calcium activation events (2) suggests a rational explanation for its activity. Our data suggest that sublocalization within the cell allows CypB to be active, since the carboxy-terminal 37 residues of CypB were able to confer a significant gain of function on the CypC molecule, while the converse construction (replacement of the C terminus of CypB with the 39 C-terminal residues of CypC) lowered the activity of CypB. This region of CypB contains the previously characterized localization signal (2), which could direct a foreign protein to the specialized CypB retention site (the calcium-containing ER substructure or "calciosome"). In contrast, we propose that targeting of CypC to a different part of the ER (or exclusion from the calciosome) may prevent it from participating in inhibition of T-cell activation. Interestingly, we also found that combining the N-terminal 33 amino acids of CypB with the rest of the CypC protein enabled CypC to gain activity. The converse construction (N-terminal 35 residues of CypC on the body and C terminus of CypB) reduced the activity of CypB. These results demonstrate that part of the activity-determining information resides at the amino terminus of CypB as well.

Examination of the three-dimensional structure of CypA (Fig. 5a) reveals that both ends of the protein are closely juxtaposed in space, on one side of the protein directly opposite the CsA-binding site. Since our results indicate that both the surface-exposed C and N termini are involved in CypB activity, we postulate that they create a composite binding site, or surface patch, that localizes the CypB molecule on a structure, possibly the calciosome, involved in the T-cell activation pathway.

One alternative explanation for the activity of CypB is that it could be inefficiently transported into the ER when overexpressed in Jurkat cells. We believe this to be unlikely for two reasons. (i) The migration of newly synthesized CypB on denaturing SDS-polyacrylamide gels exactly corresponds to the predicted size of the cleaved polypeptide, indicating that most of the protein was transported into the ER space. There was a faintly detectable band (making up approximately 2% or less of the total protein) with slower migration corresponding to the expected size of the full-length precursor CypB. A similar faint precursor band was visible in

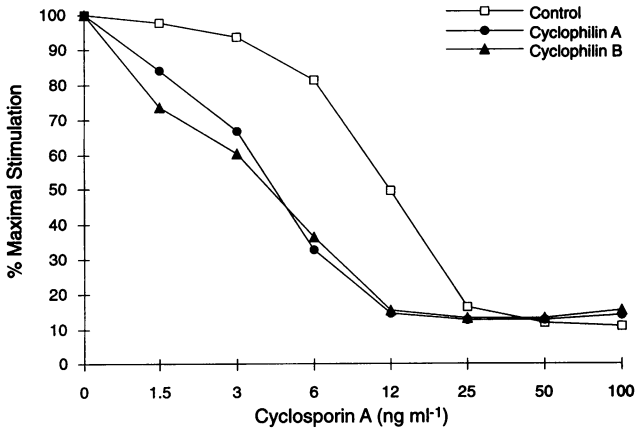

FIG. 7. Effects of full-length CypA and CypB on drug response of T cells activated by cotransfection of a constitutive calcineurin. TAg Jurkat cells were transfected with vectors that encode a calcium-independent truncated calcineurin and CypA or CypB or with a control plasmid. Cells were stimulated with PMA in the presence of the indicated concentrations of CsA. There was no effect on the response to FK506 (data not shown). The total amount of NFAT-specific phosphatase was measured by fluorescence assay. Normalized data from two averaged measurements are shown for each point. In the absence of the constitutive calcineurin, there was no stimulation of NF-AT when cells were treated with PMA alone (data not shown).

CypC transfectants but not in cells transfected with CypA or TV-CypC. (ii) Exchange of the C-terminal residues of CypB and CypC would not be expected to affect their transport into the ER (since, typically, signal recognition particle recognition and initiation of translocation occur before translation has completed), and therefore, the observed activities of the BBC and CCB chimeric constructs cannot easily be explained by changes in slight amounts of cytosolic accumulation.

We cannot rule out the possibility that CypB is released into the cytosol following translation and translocation. Alternatively, there may be a small proportion of calcineurin that is translocated into the CypB-containing calciosome. Either of these possibilities could explain the marked difference in behavior between CypB and CypC.

This study demonstrated that the intracellular concentration of an immunophilin influences the sensitivity of the cell to the immunosuppressant drug CsA or FK506 and identified a subset of immunophilins capable of mediating the drug inhibition effect. CypC cannot mediate such an effect, unless it is altered to accumulate in the cytosol. CypA and FKBP12 are naturally cytosolic and are active in this assay. The specialized sublocalization of CypB, coincident with calcium-activated proteins, appears to cause its activity in our assay, although we cannot rule out alternative explanations for its activity. We suspect that this sublocalization brings it into close proximity to activated calcineurin to inhibit signal transduction. Consistent with this hypothesis are the previous findings that calcineurin contains a myristylation site on the B subunit (1) and has been shown to move upon stimulation of the cell from freely cytosolic to membrane bound (6). This colocalization would bring CypB in close proximity to a part of the cell where activation of calcineurin by Ca^{2+} occurs most rapidly. One hypothesis derived from this work, therefore, is that part of the signal transduction machinery of T cells may also be spatially restricted.

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