A Novel Class of Dual-family Immunophilins*

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Immunophilins are protein chaperones with peptidyl-prolyl isomerase activity that belong to one of two large families, the cyclosporin-binding cyclophilins (CyPs) and the FK506-binding proteins (FKBPs). Each family displays characteristic and conserved sequence features that differ between the two families. We report a novel group of dual-family immunophilins that contain both CyP and FKBPs domains, for which we propose the name FCBP (FK506- and cyclosporin-binding protein). The FCBP of Toxoplasma gondii, a protozoan parasite, contains N-terminal FKBPs and C-terminal CyPs domains joined by tetratricopeptide repeats. Structure-function analysis revealed that both domains were functional and exhibited family-specific drug sensitivity. The individual domains of FCBP inhibited calcineurin (protein phosphatase 2B) in the presence of the appropriate drugs. In binding studies, FCBP recruited calcineurin in the presence of FK506 and a putative target of rapamycin homolog in the presence of rapamycin. Two additional FCBP sequences in Flavobacterium and one in Treponema (spirochete) were also identified in which the CyPs and FKBPs domains were in the reverse order; T. gondii growth was inhibited by cyclosporin and FK506 in a moderately synergistic manner. The knockdown of FCBP by RNA interference revealed its essentiality for T. gondii growth. Clearly, the FCBPs are novel chaperones and potential targets of multiple immunosuppressant drugs.

The immunophilin superfamily consists of highly conserved proteins with rotomase or peptidyl prolyl cis-trans-isomerase (PPIase) activity that act as chaperones by accelerating the isomerization of X-Pro-peptide bonds (1, 2). They belong to two major families that bind specific immunosuppressant molecules of fungal origin (3). The immunophilins (CyPs) bind cyclosporin A (CsA), a cyclic undecapeptide (4, 5), and the FKBPs (FK506-binding proteins) bind macrolides such as FK506 (tarcolimus) (6) and rapamycin (sirolimus) that are structurally unrelated to CsA. In both families, the drugs bind to and inhibit the respective PPIase domains that are roughly 100 amino acids long. Thus, the PPIase domain is essentially synonymous with the drug-binding domain. For instance, the FKBP PPIase domain is often called FK506-binding domain (FKBD) (Figs. 1 and 2). Despite the common PPIase activity, there is no significant sequence homology between these two families (1). Furthermore, no immunophilin has been characterized to date that contains both CyPs and FKBPs domains (1).

In the generally accepted nomenclature, the immunophilins are named with a prefix of one or two letters indicating the species followed by a number for the calculated molecular mass in kDa. Thus, the archetypal 12-kDa human FKBP is hFKBP12 or Homo sapien FKBP12. As a rule, the small immunophilins (e.g. hFKBP12) contain a single PPIase domain, whereas the larger ones may contain additional domains important for protein-protein interaction (1) such as WD40, calmodulin (CaM)-binding domain, and tetratricopeptide repeat (TPR) (Fig. 1). The TPR motif is a degenerate 34 amino acid sequence that folds into a pair of anti-parallel α-helices (7). In immunophilins containing three TPR motifs as in FKBP52 and CyP40 (Fig. 1), TPR1 and TPR2 are separated by 15–16 amino acid residues, whereas TPR2 and TPR3 are closer and separated by zero to one residue. Interestingly, the sequences of the TPR are more conserved within the family, i.e. a FKBP TPR is more similar in sequence to another FKBP TPR than to a CyP TPR (1).

The immunosuppressive activity of CsA and FK506 does not correlate with PPIase inhibition but involves a mechanism in which the CsA-CyP or the FK506-FKBP complex binds to and inhibits cytosolic calcineurin (CN), a Ca2+-CaM-dependent protein phosphatase, also known as protein phosphatase 2B or protein phosphatase 3 (3, 5, 8, 9). The rapamycin-FKBP complex, in contrast, inhibits a protein named TOR (target of rapamycin) (10).

In this communication, we report a novel class of immunophilins, which contain both CyP and FKBPs domains. We suggest that they be called FCBPs (FK506- and cyclosporin-binding protein), in keeping with the existing single family names such as FKBP and CyP. We found the first FCBP in T. gondii, a prototype protozoan parasite of the Apicomplexan family, to which the lethal malaria parasite, Plasmodium falciparum, also belongs. A sequence search subsequently led to the characterization of three additional FCBPs, two in Flavobacterium johnsonii, a bacterium with gliding motility, and one in Treponema denticola, an oral spirochete bacterium that causes periodontal disease.

EXPERIMENTAL PROCEDURES

Materials—Ethyl-FK506 (ascomycin) was purchased from Calbiochem, cyclosporin A and bovine cyclophilin were from Sigma, and monoclonal His-tag antibody was from Novagen. Geldanamycin was a kind gift from NCI, National Institutes of Health (11). The peptide N-succinyl-Ala-Leu-Pro-Phe-nitroanilide was from Bachem. Synthetic peptides 15CGGSDKVDVDPAS123 and 500TDKSNDRFKPGVDQIVDC168 corresponded to the N- and C-terminal ends of TrFCBP57. Mice antibodies against these peptides were made as described previ...
calmodulin (1/H9262 domains (final concentration 20 nM) and was followed at 10 °C by this solution with 2.2 ml of buffer A containing recombinant FCBP or its foreskin fibroblast cells and purified as recommended (13). The internal TPR deletion mutants were done through the ClustalW program.

The chaperone assay involved refolding of RNase T1 (18). In this condition, RNAse T1 (50 μM) was first unfolded by incubating at 25 °C for 3 h in buffer A (50 mM Tris-Cl (pH 8.0), 1 mM EDTA) containing 6M EDTA, 5 mM MgCl₂, 2 mM ATP, and 5 mM glutathione. Electroporation at 1.5 kEV pulse; 50 ohms; 50 microfarads; and 1 ms with a 0.2-cm electrode gap in the Bio-Rad Gene Pulser. 

Reactions were performed at 10 °C, and the rise in A₂₆₈ nm in a Hitachi UV-visible spectrophotometer. Phosphatase assay for calcineurin was done in a 96-well plate format using the RII phosphopeptide as substrate, and the liberated phosphate was measured by malachite green assay according to the instructions of the manufacturer (CalBiochem). Phosphatase reactions were routinely initiated with the addition of substrate. Where indicated, the following additions were made at the indicated concentrations 10 min prior to initiating the reaction: Ca²⁺; Mg²⁺; Mn²⁺ (2 mM each); calmodulin (1 μM); purified recombinant FCBP or its deletion mutants (1 μM); and the indicated amounts of CsA or ethyl-FK506 (19). Reactions were followed with time to ensure linearity, and the results were corrected by subtraction of the corresponding values from an enzyme-free reaction.

**RESULTS**

Characterization of the Dual-Family Immunophilins—We and others (21, 22) recently identified a FKBp sequence in *P. falciparum* that was 35 kDa in size and contained three TPRs. This FKBp ortholog showed the unique ability to inhibit the plasmodial calcineurin in the absence of drugs. Because the characterization of parasitic drug targets is a major interest in our laboratory, these results prompted us to search for FKBp orthologs in another clinically significant member of the *Apicomplexa* family, namely *T. gondii*. As described under “Experimental Procedures,” homology query with the prototype human FKBp12 as well as CyP40 identified the 521 amino acid long putative *T. gondii* sequence in which the FKBp and CyP homology domains were at the N and C termini, respectively (Figs. 1 and 2), and a TPR domain containing three TPR motifs linked the two. We named the dual-family immunophilin FCBP. Because of its theoretical molecular mass of 57 kDa, the *T. gondii* protein was named TgFCBP57. Subsequent GenBank searches led us to two entries (TgTigr10011 and TgTigr10012) and named antibodies A and B, respectively. Similar antibodies against a peptide corresponding to the catalytic (CnA) and regulatory (CnB) subunits of *T. gondii* calcineurin were similarly expressed and purified. The internal TPR deletion mutants were done through the ClustalW program.

**RNA Interference Analysis of TgFCBP57—**

RNAi studies were carried out essentially as described previously (20). Using the appropriate primers, a 500-bp segment of the TgFCBP57-coding sequence (nucleotides 484–883) was amplified by PCR and cloned into HindIII and SacI sites of pGEM-4 (Promega). The resultant clone was transcribed using a mixture of T7 and SP6 polymerases, and the double-stranded RNA(dsRNA) was treated with calf-intestinal phosphatase. dsRNA was electroporated into 5 x 10⁶ *T. gondii* RH parasites in 400 μl of electroporation buffer containing 120 mM KCl, 0.15 mM CaCl₂, 10 mM KH₂PO₄/KH₂PO₄ (pH 7.6), 25 mM HEPES (pH 7.6), 2 mM EDTA, 5 mM MgCl₂, 2 mM ATP, and 5 mM glutathione. Electroporation conditions were as follows: 1.5 kEV pulse; 50 ohms; 50 microfarads; and 1 ms with a 0.2-cm electrode gap in the Bio-Rad Gene Pulser.

**Fig. 1. Representative domain arrangements of FKBPs, Cyps, and the dual-family FCBPs.** The "single-family" enzymes (FKBP, CyP) are shown for comparison only. The proteins are named by the initials of the organism (*Dm*, *Drosophila melanogaster*; *Hs*, *Homo sapiens*; *Td*, *T. denticola*; *At*, *Arabidopsis thaliana*) followed by the domain name(s) and the theoretical molecular mass in kDa. The accessory domains, i.e. TPR, WD40, and the putative CaM-binding domains, are also indicated. CyP domains are black rectangles, and FKBPs are white.
with anti-His antibody and was ~2 kDa larger due to the tag (data not shown).

In Fig. 2, we compared the deduced primary structures of these FCBPs with representative FKBPs and Cyps, including the only FKBP in *P. falciparum* (PfFKBP35). Whereas the high sequence conservation is obvious in each domain, there are a number of differences between the eukaryotic and the prokaryotic FCBPs. The prokaryotic FKBP domains have variable insertions after the invariant FD-dipeptide (Fig. 2). In the CyP domains, the eukaryotic ones are more similar to each other than to their prokaryotic counterparts as evidenced by the gaps. The SIY-tripeptide, for example, is present in the human and *T. gondii* domains but not in the bacterial ones. Most importantly, the relative order of the two immunophilin domains is reversed. In the prokaryotic FCBPs, the CyP domain is N-terminal, whereas in the eukaryotic FCBP, the FKBP domain is N-terminal (Figs. 1 and 2). The prokaryotic FCBPs have a much shorter linker with no TPR between CyP and FKBP domains. Thus the N-terminal half of TgFCBP57, containing the FKBD followed by the three TPRs, appears very similar to the same domains in PfFKBP35 (Fig. 1), perhaps indicating a close evolutionary relationship.

**PPIase and Chaperone Activities of the FCBPs—** Recombinantly expressed TgFCBP57 (Fig. 3A) and both the flavobacterial enzymes (Fig. 5) displayed PPIase function independent of PPIase activity (23, 24). However, direct measurement of refolding of RNase T1, a traditional substrate for folding assay, revealed that the full-length TgFCBP57 as well as its FKBP and CyP domains had protein-folding activity (Fig. 3B). When compared on a molar basis, the individual domains contained approximately half of the activity of the full-length FCBP, matching the PPIase results above. Together, these results establish the modular nature of the FCBP and suggest that the individual FKBP and CyP domains, although quite diverse in sequence, both contribute to the activity of the full-length FCBP.

**Drug Sensitivity of the FCBPs—** As mentioned before, the PPIase activities of the CyPs and FKBPs are inhibited by cyclosporins and FK506, respectively. A number of amino acids known to be important for interactions with these drugs are in fact conserved in the corresponding domains of the FCBPs (Fig. 4). One important exception is His-474 of TgFCBP35, which is Trp in the other homologs (Fig. 2). Available evidence has

![Fig. 2. Sequence homology of the dual-family immunophilins of Tg, Fj, and *T. denticola* (Td). The nomenclature has been described in Fig. 1. Highlighted residues are important for various functions, including PPIase activity, substrate binding, and interaction with immunosuppressant drugs (CsA for the CyP domain and FK506 for the FKBP domain). A, homology of the FKBP domains. The prototype human FKBP (hFKBP12) and the *P. falciparum* FKBP35 (21, 22) are included for comparison. B, homology of the CyP domains. The prototype human CyP (hCyP18) is included for comparison. Residues important for CN binding are underlined. The W (for Trp), marked with overhead circle, is especially important for CsA binding but is replaced by H (for His) in *Toxoplasma*, which explains the relative CsA resistance of the latter (Fig. 4). Note how the residue numbers differ between the *Toxoplasma* and the bacterial FCBPs because of the reversal of the domain arrangement.](http://www.jbc.org/Downloaded from)
shown that this change leads to CsA resistance. In three CyPs, namely E. coli Cyp20, human Cyp40, and a CyP from Brugia malayi (a causative agent of human filaria), the equivalent residue is His and changing it to Trp greatly increased the CsA affinity (25–27). For hCyp40, the values for the His → Trp mutant in fact approached the corresponding values for hCyp18 (25), which naturally contains a Trp in this position (Fig. 2). Therefore, it was important to investigate the drug sensitivity of a FCBP and its two PPIase domains. We first set out to determine the IC$_{50}$ of CsA and FK506 on the full-length TgFCBP57; however, as high as a concentration of 10 μM of either inhibitor failed to reduce the PPIase activity by half (Fig. 4A). A simple explanation is that each drug only inhibited its cognate PPIase domain. For example, FK506 inhibited the FKBP domain only and not the CyP domain (5). To test this directly, we expressed each PPIase domain of TgFCBP57 combinatorially and determined their drug sensitivity. Indeed, the FKBP domain was highly sensitive to FK506 with an IC$_{50}$ of 70 nM while being fully resistant to CsA (Fig. 4B). Similarly, the CyP domain was sensitive to CsA, although its IC$_{50}$ was considerably higher at 750 nM (Fig. 4C). Based on the foregoing, we mutated the His-474 of the CyP domain to Trp, which resulted in a dramatic lowering of IC$_{50}$ of CsA to 40 nM. As expected, the CyP domain (the wild type as well as the H474W mutant) was fully resistant to FK506. Thus each PPIase domain of TgFCBP57 individually exhibited sensitivity to the family-specific drug, further authenticating their membership in the respective families.

We next tested the dual drug sensitivity of the FCBPs. The results (Fig. 5) show that CsA and FK506 additively inhibited all of the FCBPs. Thus the relative positioning of the CyP and FKBP domains did not affect drug-PPIase interaction in each domain, further confirming the modular nature of the FCBPs. The higher sensitivity of the bacterial enzymes toward CsA is easily explained by the conserved Trp corresponding to the His-474 in T. gondii enzyme (Fig. 2).

**Inhibition of Calcineurin by Individual FCBP Domains—**

Because the biochemical dissection experiments revealed the modular nature of the FCBPs, it was logical to ask whether each domain also maintained the ability to inhibit calcineurin in the presence of the cognate drug (4–6). We took purified T. gondii calcineurin and measured its phosphatase activity in the presence of the two recombinant domains of TgFCBP57 and either CsA or FK506. The CyP and FKBP domains clearly inhibited calcineurin in the presence of CsA and FK506, respectively, but not in the reciprocal combination (Fig. 6). Therefore, it is obvious that the modular arrangement of the FCBP extends to calcineurin inhibition as well.

**Inhibition of T. gondii by CsA and FK506—**
The exact physiological target of CsA or FK506 in T. gondii is not known. However, because the immunophiIins are potential pharmaceutical targets, an obvious question was whether these drugs would have anti-parasitic activity. Using an established [3H]uridine incorporation assay that is specific for the salvage pathway of the parasite and absent in the host, both drugs were found to inhibit T. gondii growth with IC$_{50}$ values at 0.7–0.8 μM extracellular drug concentration (Fig. 7A). When tested together, the two drugs showed a slightly synergistic inhibition (Fig. 7B).

**Knockdown of TgFCBP57 by RNAi Inhibits T. gondii Growth—**RNAi has emerged as an important tool to knock down specific gene expression (28). Recently, double-stranded RNA and short interfering RNA have been shown to trigger an
TOR significantly competed with the binding of TgTOR to the responding to the central region of the putative FRBD of Tg-TOR. A synthetic peptide, TFRETLFLQKYGRELENAYT, corresponded to the phosphatidylinositol 3-kinase-like domain of humanTOR, also known as FKBP-rapamycin-associated protein. A roughly 290 kDa, close in size to the mammalian homolog of CsA, although the affinity with CsA was considerably lower. In contrast, rapamycin led to the recruitment of a protein of viral P protein (GenBank accession number P12579) had no affinity with CsA and CsA. Note that the experimental line is slightly concave, indicating modest synergism, in contrast to the hypothetical straight line (dotted) that would have been obtained if there were no interaction between the drugs. The numbers on both axes are drug concentrations expressed as fractions of their individual IC50 values. Results represent average of three experimental sets with mean ± S.E.

**Drug-dependent Binding of TgFCBP57 to Specific Targets**—As the previous results implicated TgFCBP57 in the anti-parasitic activity of the immunosuppressant drugs, we initiated studies of the downstream targets to gain some insights into the mechanism. His-tagged recombinant TgFCBP57 was immobilized on the Ni2+ column, and soluble T. gondii lysate was passed through in the presence or absence of the drugs. Analysis of the bound proteins (Fig. 9) clearly revealed that TgFCBP57 associated with CN in the presence of FK506 and CsA, although the affinity with CsA was considerably lower. In contrast, rapamycin led to the recruitment of a protein of roughly 290 kDa, close in size to the mammalian homolog of TOR, also known as FKBP-rapamycin-associated protein. A homology search of the predicted proteins of T. gondii indeed revealed a prospective TOR sequence (TgTigrScan 2443 in ToxoDB), which is being characterized further (Fig. 10). Henceforth referred as TgTOR, the sequence showed striking similarity to the FKBP-rapamycin-binding domain (FRBD) as well as the phosphatidylinositol 3-kinase-like domain of human TOR. A synthetic peptide, TFRRETFLQKYGRELENAYT, corresponding to the central region of the putative FRBD of Tg-TOR significantly competed with the binding of TgTOR to the FCBP-rapamycin complex (Fig. 9, last lane), suggesting the authenticity of the sequence. An irrelevant peptide corresponding to the C-terminal 15 amino acids of respiratory syncytial viral F protein (GenBank™ accession number P12579) had no effect (data not shown), demonstrating specificity. Thus, the TOR pathway may represent an important downstream target of the FCBP-rapamycin complex in T. gondii.

**DISCUSSION**

Results presented here constitute the first report of dual-family immunophilins in which one PPIase domain was derived from the CyP family and the other was derived from the FKBP family. Therefore, the enzymes were tentatively named FCBP (Figs. 1 and 2). It is possible that more FCBPs will come...
to light as new genomes are sequenced and annotated; however, it is already apparent that FCBPs are not as ubiquitous or numerous as CyPs or FKBPs. For instance, we did not find any FCBP in another Treponema sp., namely Treponema pallidum (the causative agent of syphilis), although it contained putative CyP and FKBP orthologs with single family-type PPIase domains (GenBankTM accession numbers NP_219383 and NP_219298). Other spirochetes are also clinically highly significant and include Borrelia (Lyme disease) and Leptospira (leptospirosis); however, a homology search of their complete genome sequence did not reveal any dual-family FCBP. In contrast, CyPs and FKBPs are not only ubiquitous but often have multiple paralogs within the same organism. Humans, for example, have 15 FKBPs and at least 16 CyP-like sequences, whereas the lower eukaryote, Saccharomyces cerevisiae, encodes eight CyPs and four FKBPs (1).

The mechanism of anti-toxoplasma activity of the immunosuppressants calls for further work. Because CsA is a weak inhibitor of TgFCBP57, a more probable target for CsA is CyP. A few CyPs are predicted in the T. gondii genome data base such as TgTwinScan_5726 and TgTwinScan_3965, but none has been characterized. These sequences have Trp corresponding to His-474 of TgFCBP57 and thus are likely to be sensitive to CsA. However, for FK506 and rapamycin, TgFCBP57 appears to be the sole proximal target because no other FKBP domain is to be found in the T. gondii genome.2 As mentioned before, P. falciparum also has only one FKBP sequence, viz. PfFKBP35 (Fig. 1). Further characterization of TgTOR will provide an important direction in rapamycin action. In an analogy to its mammalian homolog of TOR orthologs (10), TgTOR may act as a nutrient sensor, regulating parasitic cell growth and cell cycle progression, probably by regulating translation through the phosphorylation of translation factors. The phosphatidylinositol 3-kinase-like domain of TgTOR (Fig. 10) may well play a role in this process.

In all of the TPR-containing immunophilins (CyP and FKBP) characterized to date, the TPR domain is found downstream (C-terminal) of the PPIase domain (Fig. 1) (1). Thus, we speculate that TgFCBP57 evolved as a chimeric protein generated by the fusion of a FKBP-(TPR)3 sequence, such as PfFKBP35, with a CyP. However, there is an important difference between the

2 B. Adams, A. Musiyenko, R. Kumar, and S. Barik, unpublished observation.

FIG. 9. Drug-specific association of FCBP with downstream proteins. The experiment was done essentially as described previously (38). Purified His-tagged recombinant TgFCBP57 (10 μg) was immobilized on Ni2+-agarose column (60 μl), and 400 μg of T. gondii cell extract was passed through it. Where indicated (last lane), the extract was pre-mixed with 10 μM drug. Where indicated, 0.2 μg of competing TOR peptide (FRBD region) was added to the extract. The column was then washed with 1 ml of wash buffer (Novagen) containing 0.2 mM imidazole. The bound proteins were stripped by boiling in 2× SDS sample buffer and analyzed by SDS-PAGE and silver staining. The catalytic subunit of T. gondii CN was further confirmed by immunoblot (data not shown). See “Results” for details.

FIG. 10. A putative T. gondii TOR homolog. The C-terminal end of the deduced primary structures of human (P42345; hTOR) and T. gondii (TgTOR) proteins are aligned. The TgTOR sequence is based on TgTigrScan_2443 in ToxoDB but was conceptually corrected and partially sequenced. The following domains are marked. Part of the FAT (FKBP-rapamycin-associated protein, ATM, and transformation/transcription domain-associated protein (TRRAP)) domain (smaller box), FRBD (larger box), and phosphatidylinositol 3-kinase-like domain (underlined) is shown. Identical residues are denoted by asterisk, and similar ones are represented by dots. We tentatively estimate the full-length TgTOR to be 2845 amino acids long.
FKBP domains of these two parasites. Whereas PpFkbp35 can inhibit calcineurin in the absence of any drug (21, 22), the FKBP domain of TgFCBP57 requires FK506 for this effect (Fig. 6).

Examples of double enzymatic units in the same polyopeptide are relatively rare in nature. Recently, a double protein domain of TgFCBP57 requires FK506 for this effect (Fig. 6). Inhibit calcineurin in the absence of any drug (21, 22), the FKBP domains of these two parasites. Whereas PfFKBP35 can interact with two client proteins in such a multiprotein complex in human (36, 37). Perhaps the relative positioning of the CyP and FKBP domains in the FCBP allows them to simultaneously interact with two client proteins in such a multiprotein complex. The flexibility of the linker sequence may allow fine-tuning of the orientation, and the TPR domain may promote additional protein-protein interactions (7). Clearly, the determination of the interacting partners will shed light on the function of this remarkable new family of immunophilins.

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