FKBP12 Binds the Inositol 1,4,5-Trisphosphate Receptor at Leucine-Proline (1400–1401) and Anchors Calcineurin to this FK506-like Domain*

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The immunophilin FKBP12 is one of the most abundant and conserved proteins in biology. It is the primary receptor for the immunosuppressant actions of the drug FK506 in whose presence FKBP12 binds to and inhibits calcineurin, disrupting interleukin formation in lymphocytes. The physiologic functions of FKBP12 are less clear, although the protein has been demonstrated to physiologically interact with the inositol 1,4,5-trisphosphate receptor (IP₃R), the ryanodine receptor, and the type 1 transforming growth factor β receptor. We now report that FKBP12 binds the IP₃R at residues 1400–1401, a leucyl-prolyl dipeptide epitope that structurally resembles FK506. We further demonstrate that binding to IP₃R at this site enables FKBP12 to interact with calcineurin, presumably to anchor the phosphatase to IP₃R and modulate the receptor’s phosphorylation status. We propose that FK506 promotes an FKBP12-calcineurin interaction by mimicking structurally similar dipeptide epitopes present within proteins that use FKBP12 to anchor calcineurin to the appropriate physiologic substrates.

The immunophilins are proteins that bind the immunosuppressant drugs cyclosporin A (CsA), FK506, and rapamycin with high affinity and are responsible for their therapeutic actions (for review, see Refs. 1 and 2). Cyclosporin A, a cyclic undecapeptide, binds to members of the cyclophilin family, whereas the structurally unrelated FK506 and rapamycin bind to the family of FK506 binding proteins (FKBPs). Although the cyclophilins and FKBPs lack an amino acid sequence homology, both classes of proteins display peptidyl-prolyl isomerase activity, which is inhibited by their respective immunosuppressant ligands. However, inhibition of this rotamase activity does not explain immunosuppression, as some potent ligands of the immunophilins inhibit rotamase activity but lack immunosuppressant effects (3). Immunosuppression appears to stem from the binding of the drug-immunophilin complex to the calcium-activated phosphatase calcineurin (CN) to inhibit catalytic activity resulting in an accumulation of phosphorylated CN substrates (4). One of these substrates, the transcription factor NFAT (nuclear factor of activated T-cells) in its unphosphorylated state passes from the cytoplasm to the nucleus to stimulate interleukin-2 formation. Following treatment with immunosuppressant drugs, phosphorylated levels of NFAT accumulate in the cytoplasm and are unable to enter the nucleus with the associated decrease in interleukin-2 formation being involved in immunosuppressant actions (5, 6).

Whereas pharmacologic actions of immunosuppressant drugs are readily explained by the above model, the physiologic roles of the immunophilins remain obscure despite the fact that they are among the most abundant and conserved proteins in biology. A few proteins, such as collagen and transferrin, have been shown to serve as substrates for immunophilin rotamase activity (7, 8). However, it is unclear whether these are the sole or principal physiologic substrates for the rotamase activity of these proteins. Moreover, it is possible that the immunophilins bind to and regulate intracellular proteins without altering their tertiary structure by rotamase influences.

Recently physiologic interactions of FKBP12 have been demonstrated with the two major intracellular calcium channels, the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor (IP₃R) (9, 10). In both instances FKBP12 is tightly associated with the channel and appears to be a physiologic subunit of the channel protein complex. Dissociating FKBP12 from either channel perturbs the physiologic calcium flux of the channel (10–12).

We recently demonstrated a ternary complex between IP₃R, FKBP12, and CN (13). Within the complex, CN dephosphorylates IP₃R especially when it has been phosphorylated by protein kinase C. The cycle of phosphorylation-dephosphorylation of IP₃R in this complex appears to modulate the calcium flux of the channel. Thus, in this instance FKBP12 appears to anchor calcineurin to IP₃R with regulation of IP₃R function determined by phosphorylation and dephosphorylation and possibly not via a rotamase effect of FKBP12 upon IP₃R. Evidence that rotamase activity of FKBP12 is not crucial to influences upon calcium channel function comes from studies by Fleischer and associates (14) showing that FKBP12 mutants that lack rotamase activity nonetheless bind to the ryanodine receptor and influence its calcium flux.

Mechanisms whereby FKBP12, IP₃R, and CN interact have not been established. Because FK506 displaces IP₃R from FKBP12, we speculated that IP₃R functions as an “endogenous FK506,” binding to FKBP12 at the same site as FK506. Although it is a small organic molecule, FK506 serves as “molec-
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**Site-directed Mutagenesis—**Site-directed mutagenesis of IP$_3$R was carried out using the Stratagene Chameleon double-stranded site-directed mutagenesis kit and followed the instructions. Mutating oligonucleotides were synthesized as follows: P1370S, GATGAGAACACGCTCTTCATGACACATCC; P1410S, AACTCTCTGCTCTCCGGTTGAAGGATGCAG; P1410A, AACTCTCTGGCTCGGTTGAAGGATGCAG; P1410C, AAATGAGGCTCGGTTGAAGGATGCAG; P1410S, GAGGAGGCTCGGTTGAAGGATGCAG; P1416A, GAGGAGGCTCGGTTGAAGGATGCAG; and to knock out the AATT site as described: TAATGAAGTAACCGGTCGAGCTCAAAGC.  

**Yeast Three-hybrid Assay—**A third yeast expression vector, pA6D (referred to as pHYB in Fig. 5 and has been described previously (18)), was obtained and used for three-hybrid assay studies. The pA6D vector contains the URA3 gene (orotidine-5-phosphate decarboxylase) allowing for selection of coexpressed transformed yeast colonies on Leu-Trp-Ura-plates. PJ69–4A strain but not Y190 are Ura- and were thus used for three-hybrid assays. Three constructs were expressed instead of two as above, and co-transformants were assayed for their ability to grow on Leu-Trp-Ura-His-plates via the liquid β-galactosidase assay as described above. FKBP56 was included in the agar plates at 1 μM where indicated.

**RESULTS**

**Leucyl-Proline 1400–1401 in IP$_3$R Mediates Binding to FKBP21—**IP$_3$R is one of the largest membrane proteins in biology comprising 2,749 amino acids. The receptor is suggested to encompass six transmembrane domains in the carboxyl-terminal portion of the molecule that participates in the formation of the calcium ion pore, whereas a very large N-terminal portion of the molecule is free in the cytoplasm (for review, see Ref. 19). We systematically truncated IP$_3$R into overlapping successively regional domains and examined its interactions with FKBP21 in the yeast two-hybrid system. An initial series of truncations reveals the binding domain to be in the central modulatory portion of IP$_3$R, amino acids 942–1770 (Fig. 1, A and B). Further truncations of this region localize the binding site to 112 amino acids, 1349–1460 (Fig. 1, C and D). It is possible to speculate where FKBP21 binds within this 112 amino acid portion of the IP$_3$R based on extensive x-ray crystallographic modeling and in vitro studies of FKBP21 substrate interactions. FK506 has been proposed to mimic protein ligands to, or substrates of FKBP12 with the pyranose rings, o-homoalanine moieties, and homoprolyl moieties of the natural product showing structural similarities to the transition state structures for cis-trans isomerization of leucyl-prolyl and valyl-prolyl substrates, which are optimal rotamase substrates for FKBP12 (20, 21). The 1349–1460 region of IP$_3$R contains three proline residues, only one of which is preceded by either a leucine or a valine residue. Proline 1401 is preceded by a leucine. Mutations of the other two prolines contained within this region, which occur at the 1370 and 1416 positions and are preceded by a serine and an isoleucine, respectively, have no effect upon FKBP12 binding activity, whereas four distinct mutations of proline 1401 eliminate the IP$_3$R-FKBP12 interaction (Fig. 2).

We wondered whether the IP$_3$R sequence responsible for FKBP12 binding is conserved in the other proteins known to 

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*Quantitative Liquid β-Galactosidase Assay—*Liquid β-galactosidase assays were performed as described (2). Briefly, transformed yeast were grown to saturation overnight and diluted the following morning. Diluted yeast were grown to an optical density (A$_{600}$) of 1 and pelleted. Yeast were resuspended in 1 ml of Z buffer (prepared as described) and then diluted in Z buffer at 1:10 and 1:90. Yeasts were lysed with chloroform and SDS and incubated with 4 μg/ml o-nitrophenyl-β-galactoside at 30 °C until they turned yellow. The reaction was stopped with 1 M Na$_2$CO$_3$, and an A$_{420}$ was measured. β-Galactosidase activity was computed as described.

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Fig. 1. Narrowing of FKBP12 binding region within IP$_3$R. A series of constructs were made to represent overlapping regions of the soluble portions of the IP$_3$R. Of these regions, FKBP12 showed affinity for only aa 942–1770, the central, “modulatory domain” of the receptor (A). A quantitative liquid assay of β-galactosidase enzymatic activity confirms a protein–protein interaction between FKBP12 and aa 942–1770 of IP$_3$R, here designated IP$_3$R construct 3. These experiments were repeated four times with the same results (B). Further dissection of IP$_3$R construct 3 reveals that FKBP12 has affinity for IP$_3$R aa 1223–1613 and aa 1349–1460 (C). A liquid β-galactosidase assay confirms that FKBP12 interacts with IP$_3$R, aa 1349–1460, here designated IP$_3$R construct 3e. These experiments were repeated four times with the same results (D).

bind FKBP12. Besides IP$_3$R and the ryanodine receptor, the type 1 TGF-β receptor has been shown to interact with FKBP12 (22). Three subtypes of IP$_3$R, the ryanodine receptor, and type-1 TGF-β receptors possess the proline corresponding to Pro-1401 of IP$_3$R (Fig. 3). Type 2 TGF-β receptors lack the SGSSGGLP motif present in type 1 receptors (23) and also fail to interact with FKBP12 (22, 24). In the ryanodine receptor this proline is preceded by a valine, whereas in the other receptors it is preceded by a leucine. In all of the receptors that bind FKBP12, the proline is followed by a leucine.

Because FKBP12 seems to associate with and regulate calcium flux of the IP$_3$R and the ryanodine receptor in a similar fashion, one might expect FKBP12 to bind to the two channels at an analogous site. The ryanodine receptor comprises 5,037 amino acids and is the largest ion channel currently known. We constructed a 114-amino acid fragment of the ryanodine receptor (aa 2, 407–2, 520) comprising the domain that corresponds to the portion of IP$_3$R that binds to FKBP12. This small fragment binds robustly to FKBP12 in a yeast two-hybrid assay (Fig. 3).

Rotamase Activity Is Not Required for FKBP12-IP$_3$R Interactions—The finding that FKBP12 binds to IP$_3$R at a discrete leucyl-proline dipetide is consistent with the observed in vitro rotamase substrate specificity of FKBP12 (21). This would suggest that FKBP12 regulates IP$_3$R channel function by altering the conformation of the protein via rotamase influences. However, Fleischer and colleagues (14) found that FKBP12 mutants devoid of rotamase activity retain the ability to associate with and modulate the Ca$^{2+}$ flux properties of the ryanodine receptor. We examined whether the observed association of FKBP12 and IP$_3$R requires FKBP12 rotamase activity. We compared wild-type FKBP12 with four previously described FKBP12 mutants, one that is devoid of rotamase activity and another that displays half that of wild-type FKBP12. Each FKBP12 mutant interacts robustly with the IP$_3$R regardless of its peptidyl-prolyl rotamase activity (Fig. 4). Expression of these FKBP12 mutants and subsequent analysis of their ability to interact with and modulate IP$_3$R that has been purified and “stripped” of endogenously associated wild-type FKBP12 is currently underway.

Demonstration of the FKBP12-IP$_3$R-CN Ternary Complex in a Yeast Three-hybrid Assay System—In our earlier study (13) establishing a ternary complex of FKBP12, IP$_3$R, and CN, the sites of interaction of the three proteins were unclear. It was not established in that study whether CN was associating with the IP$_3$R-FKBP12 complex via an FKBP12 anchor or at an allosteric site on IP$_3$R. We set out to distinguish between these two possibilities using the yeast two-hybrid system and a modification of it. We first generated a CN-A construct consisting of amino acids 1–394 that contains the FKBP12 binding portion of the molecule (26, 27). We tested the ability of CN to interact directly with IP$_3$R using this construct and observe no direct CN-IP$_3$R interaction with any of our IP$_3$R constructs including IP$_3$R aa 1349–1460 (data not shown). CN likewise fails to interact with FKBP12 in the yeast two-hybrid system until FK506 is added to the agar plates during preparation (Fig. 5) as previously observed (28).

We obtained a third vector suitable for expressing proteins in one of the yeast strains that we were using in our two-hybrid assay (see “Experimental Procedures”). With this vector, however, expressed proteins are not synthesized as part of a GAL4 transcription factor fusion protein. We attempted to recreate a three protein interaction in the yeast system using a known ternary complex: the CN-PKA-AKAP (A-kinase-anchoring protein) complex described by Scott and associates (29). CN has no direct affinity for PKA in a traditional yeast two-hybrid system assay (data not shown), but the two molecules are brought together in the presence of the anchoring protein AKAP79 (Fig. 5). We next investigated whether the IP$_3$R, aa 1349–1460, mimicks FK506 by promoting an FKBP12-CN interaction in this yeast three-hybrid system. Replacing FK506 with the 112 amino acid domain of IP$_3$R that binds FKBP12 results in a robust FKBP12-CN interaction (Fig. 5).

Mutants of FKBP12 have been developed that vary in their ability to participate in binding CN (15, 16). Some mutants

3 M. T. DeCenzo, S. T. Park, B. P. Jarrett, R. A. Aldape, O. Futer, M. A. Murcko, and D. J. Livingston, submitted for publication.
with almost no rotamase activity still bind CN tightly in the presence of FK506, i.e. FKBP(W59A), whereas others retain rotamase activity but are unable to bind CN when FK506 is present, i.e. FKBP(R42K/H87V) (Fig. 5). We examined the ability of certain of these mutants to support the ternary complex of FKBP12, CN, and IP3R. A mutant previously demonstrated to be almost devoid of rotamase activity, but binds CN, interacts robustly in our yeast three-hybrid assay (Fig. 5). This further supports the finding that rotamase activity is not crucial for the ternary complex. On the other hand, a mutant that is known not to interact with CN, although it possesses rotamase activity, fails to bind in the yeast three-hybrid system (Fig. 5).

**DISCUSSION**

In the present study we have localized the exact peptide sequence within IP3R that binds to FKBP12 as a leucyl-proline dipeptide. An analogous leucyl-prolyl or valyl-prolyl sequence is conserved throughout all subtypes of IP3R, the ryanodine receptor, and the type 1 TGF-β receptors shown to interact with FKBP12 indicating that this sequence may represent a universal ligand selective for FKBP12 binding. The leucyl-proline and valyl-prolyl sequences correspond well with the substrate specificity that Schreiber and associates (21) have shown to be optimal for in vitro FKBP12-peptide interactions.

There are several limitations to our current study utilizing the yeast two- and three-hybrid interaction trap assays. First, although it is now routine to use this assay to map interacting domains within proteins known to associate, this method does not provide for quantification of the affinity of the interaction being studied. Some insight into this question has been attained in previous studies in which relatively high concentrations of the drug FK506 were required to disrupt the FKBP-IP3R interaction (EC50 10–100 nM), and in the case of the FKBP-ryanodine receptor interaction, Fleischer and colleagues (14) have predicted an EC50 of 0.30 μM (13). Likewise, although the FKBP-IP3R protein-protein interaction has been demonstrated in previous reports by traditional protein biochemistry methodology including co-purification, co-immunoprecipitation, and direct binding assays (10, 13), the current study does not repeat those techniques when identifying the site of interaction. Rather the yeast two-hybrid assay is employed to identify the site of interaction, and noninteracting portions of these proteins and mutant constructs are used to ensure the specificity of the interaction being studied. Some insight into this question has been attained in previous studies in which relatively high concentrations of the drug FK506 were required to disrupt the FKBP-IP3R interaction (EC50 10–100 nM), and in the case of the FKBP-ryanodine receptor interaction, Fleischer and colleagues (14) have predicted an EC50 of 0.30 μM (13). Likewise, although the FKBP-IP3R protein-protein interaction has been demonstrated in previous reports by traditional protein biochemistry methodology including co-purification, co-immunoprecipitation, and direct binding assays (10, 13), the current study does not repeat those techniques when identifying the site of interaction. Rather the yeast two-hybrid assay is employed to identify the site of interaction, and noninteracting portions of these proteins and mutant constructs are used to ensure the specificity of the interaction. Ideally, the entire 310-kDa IP3R would be expressed as a recombinant protein with and without mutations at proline 1401 and tested for its ability to interact with FKBP in a biochemical assay. These ambitious experiments are currently underway to confirm the data obtained in the present round of yeast two-hybrid experiments. Finally, our data reported here do not speak to how small a domain of IP3R is required to mediate FKBP binding. We have shown that a 112-amino acid truncation of IP3R including proline 1401 is sufficient for FKBP binding and that proline 1401 is necessary.
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Rotamase activity, % wt

| Peptide          | Activity | Interaction with IP₃R, 1349-1460? |
|------------------|----------|----------------------------------|
| FKBP,(wt)        | 100%     | +                                |
| FKBP,(W59A)      | 3%       | +                                |
| FKBP,(R42I)      | 58%      | +                                |
| FKBP,(R42K,H87V) | 102%     | +                                |
| FKBP,(Y80F)      | 100%     | +                                |

**FIG. 4. Interaction of FKBP12 with IP₃R is not dependent on its rotamase activity.** FKBP12 point mutants and double mutants have been developed that are nearly devoid of rotamase activity as described previously (15, 16). We selected several of these mutants with varying rotamase activity and tested their ability to interact with IP₃R. Each of these mutants interacted with IP₃R in a fashion indistinguishable from wild-type FKBP12. These interactions were confirmed using the liquid β-galactosidase assay and repeated three times.

**FIG. 5. Use of a yeast “three-hybrid” system confirms IP₃R-FKBP12-CN ternary complex.** FKBP12 is unable to interact with CN in our yeast two-hybrid system in agreement with previous studies (28). However, inclusion of FK506 in the agar plates on which the yeast grow permitted an observable interaction between FKBP12 and CN. CN has no affinity for PKA and is unable to interact with PKA in a yeast two-hybrid assay, but the two proteins both bind to AKAP79. This link is observable in a yeast three-hybrid assay system in which AKAP79 is co-expressed in the yeast using a third expression plasmid (see “Experimental Procedures”). Similarly, expression of IP₃R aa 1349–1460 promoted the interaction of FKBP12 and CN in a yeast three-hybrid assay. FKBP(W59A) is a mutant with low rotamase activity that binds to IP₃R. This mutant is able to interact with CN in the presence of FK506 and will bind CN when IP₃R aa 1349–1460 is co-expressed in a three-hybrid assay. FKBP(W59A) is a mutant with high rotamase activity that binds IP₃R aa 1340–1460. This mutant is unable to bind CN in the presence of FK506. This mutant does not interact with CN in a three-hybrid assay when IP₃R aa 1349–1460 is co-expressed. These interactions were confirmed using a liquid β-galactosidase assay and were repeated four times with the same results.

In an attempt to further narrow down how many residues surrounding Pro-1401 are required for FKBP interaction, we have generated an 11-amino acid peptide consisting of IP₃Ra 1396–1406. This peptide was not able to support the FKBP-CN interaction in a binding assay, implying that three-dimensional structural motifs contained with the surrounding 112 amino acids also participate in the IP₃R-FKBP-CN complex (data not shown).

The crucial leucyl-proline sequence in IP₃R implies that FKBP12 regulates IP₃R by its rotamase activity. However, we showed that rotamase activity is not required for FKBP12 binding to IP₃R, and Fleischer and colleagues (14) have likewise shown that this rotamase activity is not necessary for the association of FKBP12 with or modulation of the ryanodine receptor.

More important than rotamase activity in regulating IP₃R function is the ability of FKBP12 to serve as a scaffold linking CN to IP₃R. Because of the numerous similarities of the ryanodine receptor and IP₃R, we suggest that FKBP12 also regulates the ryanodine receptor function by a link to CN. FKBP12 has been proposed to play such an anchoring role when it associates with the type 1 TGF-β receptor (24). Previous findings from our laboratory demonstrated that calcineurin complexed to IP₃R via FKBP12 was catalytically active (13). Interestingly, recent x-ray crystallographic evidence suggests that FK506-FKBP12 binds to calcineurin at a site removed from the phosphatase active site of the enzyme and therefore would not be likely to inhibit phosphatase activity (26). Rather, binding of FK506-FKBP12 seems to sterically block the association with and subsequent dephosphorylation of some substrates (i.e. NFAT) while not affecting or actually promoting the dephosphorylation by calcineurin of others (4). If calcineurin is shown to be the cytoplasmic protein associated with the TGF-β receptor-FKBP12 complex as proposed then the recent data of Wang et al. (22, 24) indicate calcineurin would be catalytically active in such a complex as well.

Although FKBP12 has been studied extensively, its physiologic substrates have not been well characterized. No major normal function of a protein has been shown to be regulated by the rotamase activity of FKBP12. Thus, it is possible that the anchoring function of FKBP12 represents its major physiologic role. Anchoring of CN and perhaps other phosphatases and
kinases to appropriate substrates might regulate phosphorylation-dephosphorylation events. Such a model is reminiscent of the AKAP, which anchors CN to appropriate substrates at the postsynaptic density via an FKBP-like domain within its sequence. Besides anchoring PKA and CN to their appropriate subcellular locales, AKAP serves as a scaffolding anchor for protein kinase C (25). The AKAP complex thus includes both phosphatase and kinase enzymes. By analogy, there might exist a physiologic quaternary complex including a protein kinase with FKBP12, IP3R and CN. In support of this model, our earlier study showed that calcium flux of IP3R was most strikingly regulated by interactions between calcineurin and protein kinase C (13). Conceivably protein kinase C exists in a quaternary complex with IP3R, FKBP12, and CN.

Within the ternary complex, IP3R seems to mimic the role of FK506 in promoting the association of calcineurin with FKBP12. Indeed, it is remarkable that a small organic molecule such as FK506 could contain two distinct domains to participate in binding to two different proteins, something that one would expect a protein such as IP3R to accomplish more efficiently. Our experiments with various truncations of IP3R establish that the domain of IP3R that is responsible for FKBP12 binding also enables bound FKBP12 to associate with calcineurin.

The high affinity and selectivity of FK506 binding to FKBP12 has suggested to many investigators the existence of an endogenous FK506-like ligand, conceivably a small peptide, analogous to the enkephalins serving as endogenous ligands for the opiate receptor. Since the chemical structure of FK506 resembles a leucyl-proline dipeptide, it would not be surprising if the endogenous FK506-like ligand would comprise such a structure. Indeed, our results indicate that the leucyl-proline dipeptide within IP3R represents that endogenous ligand, except it is buried within a large protein. Situating the endogenous ligand within a large protein enables it to carry out a bridging function linking calcineurin via FKBP12 to an appropriate cellular substrate of its phosphatase activity (Fig. 6).

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