Charged Surface Residues of FKBP12 Participate in Formation of the FKBP12-FK506-Calcinneurin Complex*

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The mechanism of FK506 immunosuppression has been proposed to proceed by formation of a tight-binding complex with the intracellular 12-kDa FK506-binding protein (FKBP12). The FK506-FKBP12 complex then acts as a specific high-affinity inhibitor of the intracellular protein phosphatase PPI2B (calcineurin), interrupting downstream dephosphorylation events required for T-cell activation. Site-directed mutagenesis of many of the surface residues of FKBP12 has no significant effect on its affinity for calcineurin. We have identified, however, three FKBP12 surface residues (Asp-37, Arg-42, and His-87) proximal to a catalytic domain of calcineurin (17) with the FKBP12 fusion protein requires the presence of FK506 and Ca++. Further experiments have demonstrated that the complex forms with a very high affinity (K = 6 nM, this report) and that FKBP12 is the only known FKBP capable of forming a tight-binding complex with calcineurin in the presence of FK506 and Ca++. At least one FKBP12 analog, 15-O-demethyl-FK520, which is known to bind tightly to FKBP12 and to be both immunosuppressive, appears to form a substantially weaker trimeric complex than that mediated by FK506 itself (15).

Although the key substrates of calcineurin in T-cell activation pathways need to be identified, the experiments reported to date suggest that a more detailed structural model of the FK506-FKBP12-calcineurin complex will be essential in explaining how FK506 can modulate this protein-protein interaction. It is important to delineate the regions of both the FKBP12 and its ligands that form the calcineurin-binding surface. It has been suggested (18) that FKBP12 acts as an intracellular “presenter” of the “effector domain” of FK506 to an intracellular target. Alternatively, the surface residues of the FKBP12 protein may themselves contribute to the high affinity interaction with calcineurin in the presence of FK506, i.e. form part of the effector domain. We report herein a structure-function analysis of the FK506-FKBP12 complex which demonstrates the critical role of charged surface residues of FKBP12 and an exposed segment of the FK506 macrolide in high-affinity binding of calcineurin.

MATERIALS AND METHODS

Methods for expression of mutant FKBP12s from mutagenized cDNA inserts (19, 20) and for purification of mutant proteins to homogeneity have been described previously (11). FKBP12 mutants in partially purified form were prepared from 10-ml bacterial cultures induced with isopropyl-1-thio-ß-D-galactopyranoside, grown for 15-18 h post-induction, and centrifuged for 5 min at 2000 x g. Bacterial pellets were washed with ice-cold 0.5 M Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, to which 6.4 µl of a 10 mg/ml lysozyme solution was added. This suspension was held at room temperature for 20 min and sonicated for 30 s with a Heat Systems sonicator with a microtip probe. Lysates were spun for 5 min, and the supernatant fraction was passed over a 3-ml syringe column of DE53 anion-exchange resin (Whatman) preequilibrated in 50 mM Tris, pH 7.5. Following application of the lysate, the column was washed with cold equilibration buffer, and 0.5-ml fractions were collected. Fractions 2-4 usually contained the maximum amounts of mutant FKBP12. Concentrations of mutant FKBP12 in partially purified lysates were estimated this enzymatic activity, whereas still unclear, is being examined by disruption of the genes encoding the FKBP1s (12, 13). Studies of FK506 analogs have provided convincing evidence that inhibition of PPIase activity is insufficient to cause interruption in T-cell signaling (14, 15). Instead, immunosuppression may occur as a consequence of formation of an FKBP-FK506 complex, which in turn antagonizes other enzymic processes required in T-cell activation. Liu et al. (16) provided evidence for the identity of the cognate enzyme by affinity experiments with Jurkat T-cell extracts. When such extracts are incubated with FK506 and a glutathione-S-transferase/FKBP12 fusion protein, and adsorbed to glutathione-Sepharose, the protein phosphatase calcineurin (PP2B) is tightly bound (16). The interaction of calcineurin (17) with the FKBP12 fusion protein requires the presence of FK506 and Ca++. Further experiments have demonstrated that the complex forms with a very high affinity (K = 6 nM, this report) and that FKBP12 is the only known FKBP capable of forming a tight-binding complex with calcineurin in the presence of FK506 and Ca++. At least one FKBP12 analog, 15-O-demethyl-FK520, which is known to bind tightly to FKBP12 and to be both immunosuppressive, appears to form a substantially weaker trimeric complex than that mediated by FK506 itself (15).

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from specific activities in the PPlase assay (10, 11).

The calcineurin assay used was essentially that described by Hubbard and Klee (21). Radiolabeled phosphorylated peptide substrate, derived from the serine phosphorylation site sequence of the RII kinase, was prepared using bovine heart 3',5'-cyclic AMP-dependent protein kinase (2500 units, Sigma) and 300 nM of [γ-32P]ATP (Du Pont-New England Nuclear) with a specific activity of 0.5 Ci/nmol after carrier dilution were used to phosphorylate 150 nM of synthetic peptide substrate (Peninsula Laboratories). The serine phosphate assay was performed in 60 μl of buffer containing 20 mM Tris, pH 8.0, 0.1 M NaCl, 6 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM diethiothreitol, and 0.1 mg/ml bovine serum albumin (21). The following ordered additions were made for the assay: 500 nM okadaic acid (Kamiya Biomedical), 200 nM FKBP12 (partially purified), 540 nM FK506, 160 nM bovine calmodulin (Sigma), and 30 nM bovine brain calcineurin (Sigma). 32P-Phosphorylated peptide was added to 1–2 μM final concentration, followed by a 15-min incubation at 30 °C. Reactions were quenched with 540 μl of 0.1 M potassium phosphate, 5% trichloroacetic acid (w/v). Cation exchange columns (Dowex AG1-X8, 0.6 ml) for separation of free [32P]P, were prepared as described previously (21). The quenched reaction mixtures (0.6 ml) were applied to the columns, followed by a 0.6-ml H₂O wash, and the eluants were collected in scintillation vials and counted with 5 ml of scintillation mixture (Beckmann Liquiscint). All assays were performed in duplicate.

To determine fractional activity of calcineurin remaining in the presence of partially purified FKBP mutants, measured disintegrations/min of released [32P]P, in the presence of FK506 were divided by disintegrations/min in the absence of FK506. Disintegrations/min due to [32P]P, released in the background reaction (all additions except calcineurin) were subtracted from numerator and denominator. Fractional activities were then converted to percentages (Table I).

Protein concentrations of purified mutant proteins were determined by absorbance at 280 nm (A₂₈₀ = 0.81 for a 1 mg/ml protein solution). Specific activities of FKBP12 mutants in the PPlase reaction (Table II) were computed using this concentration. Affinity for FK506 was determined by inhibition of the PPlase reaction, and Kₐ values calculated using nonlinear fitting to an equation for competitive inhibitors (22) using the program KineTic v 3.0 (Biokin, Ltd.), running on a Macintosh Quadra 700 computer. Affinity of the mutant FKBP12-FK506 complexes for calcineurin was determined by varying the concentrations of mutant FKBP12 and FK506 at 30 °C, using a drug:FKBP12 ratio of 1.35:1 for mutants with high affinity for FK506 (i.e., comparable with wild-type FKBP12). FK506:FKBP12 ratios were increased appropriately for the lower affinity mutants to ensure saturation of the mutant FKBP12 with drug. Control reactions at 30 °C were run in the presence of the same concentration of dimethyl sulfoxide (0.1%, v/v) as reactions containing FK506. Kₐ values for calcineurin inhibition were determined under the same assay conditions described above, omitting okadaic acid. The data were fit to the equations derived by Liu et al. (15) for all of the equilibria that describe the interactions of FK506, FKBP12, and calcineurin. These equations were used to calculate the concentration of free FK506 and FKBP12. Equation 7 from Liu et al. (15) can be rearranged to

\[
I/(1 - I) = [\text{FK506}]_{\text{free}}[\text{FKBP12}]_{\text{free}}/(K_{d}, K_{a})
\]

where I is the fractional inhibition of calcineurin, (1 – I) is the fractional activity remaining, and Kₐ is the Kₐ for calcineurin inhibition by the FKBP12-FK506 complex.

RESULTS

Recombinant mutant FKBP12s can be detected in DEAE-purified cell lysates by the PPlase assay. More than 95% of the PPlase activity in these preparations is suppressed by FK506, confirming that E. coli cyclophilin is retained on the DEAE column (11, 20). The concentration of wild-type FKBP12 in the peak DEAE-purified fractions is ~25 pg/ml. Mutant FKBP12 concentrations in post-DEAE peak fractions range from 10 to 240 µg/ml.

Analysis in the calcineurin assay of a DEAE-purified fraction from lysates of E. coli not expressing FKBP12 showed interference by endogenous phosphatases. Okadaic acid, a general phosphatase inhibitor that does not affect calcineurin at sub-micromolar concentrations (23), suppressed the interfering phosphatase activity significantly. Under these conditions, we determined a Kₐ value for calcineurin inhibition with partially purified wild-type human FKBP6 of 6 nM, a value identical to that determined for the purified protein (Table II). The affinity of each FKBP12 variant for FK506 (Kₐ) is greater than the affinity of the FKBP12-drug complex for calcineurin (Kₐ). The Kₐ, therefore, describes the dissociation of the FKBP12-FK506-calcineurin ternary complex to the FKBP12-FK506 and free calcineurin species.

Forty mutant FKBP12s were characterized in partially purified form as calcineurin inhibitors at 20 nM FKBP12 in the presence and absence of 540 nM FK506 (Table I). Four point mutants (D37V, R42I, R42K, and H87V) were observed to be much less inhibitory than the wild-type protein. Subsequently

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* Determined at 15 °C by inhibition of PPlase activity by FK506.

³ Measured at 30 °C by inhibition of calcineurin activity of the FKBP-FK506 complex.
we constructed a double mutant, R42K/H87V, which inhibits calcineurin with an affinity significantly lower than either single mutant (Table II).

The mutant proteins displaying the weakest inhibition of calcineurin at 200 nM FKBP12-FK506 complex were prepared in larger quantities and purified to homogeneity. Specific activities in the PPIase reaction were determined for each mutant FKBP12 (Table II). Enzymatic activities and affinity for FK506 for the R421, R42K, H87V, and R42K/H87V mutant FKBP12s are similar to the parent protein (Table II). Although the D37V mutant is an efficient catalyst of the PPIase reaction, it is 10-fold lower in specific activity than the wild-type protein and binds FK506 580-fold less tightly.

The measurements of fractional inhibition of calcineurin by partially purified FKBP12 mutants were found to be predictive of $K_i$ determined for the homogeneous proteins. FKBP12 variants which inhibit calcineurin more poorly at a single protein concentration indeed display higher $K_i$ values for phosphatase inhibition (Table II). The most dramatic point mutation is R421, with a decreased affinity for calcineurin 175-fold with respect to the wild-type FKBP12. Even the more conservative Arg-42 to Lys mutation at this position decreases calcineurin affinity 107-fold. When the latter mutation is combined with the H87V mutation, the double mutant was found to have a calcineurin affinity 3 orders of magnitude lower than the parent protein, with essentially no change in FK506 affinity or PPIase activity (Table II).

DISCUSSION

To explore the required inter-protein contacts of the FKBP12-FK506-calcineurin complex, we needed to select point mutations that were most likely to affect the binding energy of the complex without altering the global tertiary structure of FKBP12 or its affinity for FK506 (Fig. 1). Since high-resolution x-ray crystallographic and solution phase NMR structures have been determined for native FKBP12 and its complex with FK506 (24–26), we were guided by our knowledge of which residues are on the solvent-exposed surface of FKBP12. Such residues would be most likely to function as contact points with calcineurin. We analyzed the inter-residue interactions of these surface residues and eliminated from consideration most residues that are involved in stabilizing localized structure through multiple electrostatic or hydrogen-bonding interactions. Given the many charged residues in the FKBP12-FK506 complex proximal to FK506 and the known importance of the disruption of salt bridges (27), we chose to focus primarily on those residues. However, we also altered several neutral surface residues (Gln-53, Ala-84, Thr-85) that are situated proximal to FK506 (26).

The possibility that FK506 forms part of the interacting surface with calcineurin was suggested by investigations with FK506 analogs (14, 15). 15-O-Demethyl-FK520, an analog with an ethyl for allyl substitution at position 21 of FK506 and a 15-methoxy group altered to hydroxyl (Fig. 2), forms an FKBP12 complex with a 50-fold lower affinity for calcineurin than the FKBP12-FK506 complex (15). In contrast, FKBP12 complexes of the FK506 analog with a methyl substitution at position 21 alone show only a 7-fold decreased affinity for calcineurin versus FKBP12-FK506 (15). These data can be explained, however, either by a direct interaction of the solvent-exposed section of the ligand with calcineurin or by more indirect effects, such as changes in the protein surface conformation of FKBP12 or differential solvation effects on ternary complex formation.

Another approach to determining whether the exposed regions of FK506 comprise the calcineurin-binding surface, and whether FKBP12 surface residues contribute as well to the binding energy of the FKBP12-FK506-calcineurin complex, is to observe the effect of mutating FKBP12 surface residues. If altering FKBP12 residues which are proximal to FK506 affects calcineurin binding, strong evidence is provided that FK506 itself and the surrounding FKBP12 residues form the calcineurin interface. Thirty mutations of residues distributed over the solvent-exposed surface of FKBP12 (Fig. 2) result in no significant effect on calcineurin affinity and maintenance of PPIase activity (Table I). Site-directed mutagenesis of surface residues, therefore, does not cause a global disruption of protein conformation which might have affected these properties for a wide range of mutant FKBP12s.

Indeed four substitutions, R421, R42K, H87V, and D37V, dramatically affect calcineurin affinity of the FKBP12-FK506 complex.
complex. Only one of these (D37V) affects FK506 affinity or the specific activity of PPIase catalysis (Table II). The very conservative R42K mutation results in a 107-fold decrease of calcineurin affinity and strongly suggests a key role of Arg-42 in forming the calcineurin complex. The identical affinity of this mutant for FK506, with respect to the parent protein, proves that an indirect effect of loosening the FK506 binding to FKBP12 is not responsible for the decrease in calcineurin affinity. Inspection of the structure of the FKBP12-FK506 complex does not suggest that this mutation would trigger changes in local protein conformation. Furthermore, molecular dynamics simulations on the FK506 complex of R42K predict that the replacement will not result in significant structural perturbations and that the mutant will retain the salt bridge to Asp-37 observed in the parent protein (26). Determinations by x-ray crystallography are in progress of high-resolution structures of mutant FKBP12s with replacements at Arg-42.

A recently identified member of the FKBP family, FKBP25, has a Lys residue at the sequence position homologous to Arg-42 in FKBP12 (9). It has been hypothesized that the 500-fold lower affinity of FKBP25 for FK506 compared with FKBP12 has a direct contact residue for calcineurin, or the effect on calcineurin 195-fold compared with the C15-methoxy group of FK506 (26). This is the only region where such a difference is observed crystallographically. The crystal structures of the complexes of Asp-37, Arg-42, and His-87 mutations (Fig. 2) encompasses the C15-methoxy group of FK506 (Fig. 2), as might have been predicted from the substantial effect of substitutions at the C15 position on calcineurin affinity (15). In contrast, the allyl substituent at C21 of FK506 is more peripheral to this surface patch. The calcineurin-binding surface of FKBP12-FK506 we propose, therefore, is consistent with the relative effects of FK506 substitutions on immunosuppression and calcineurin binding (15). A detailed analysis of the calcineurin-interacting surface of this immunophilin-ligand complex suggests strategies for design of novel ligands of FKBP12 that may function as immunosuppressants.

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REFERENCES