A Rapamycin-Selective 25-kDa Immunophilin[†,Δ]

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Received August 7, 1991; Revised Manuscript Received November 21, 1991

ABSTRACT: FKBP25, a previously uncharacterized 25-kDa FK506- and rapamycin-binding protein, was purified to homogeneity from calf thymus, brain, and spleen, and the sequence of a 215 amino acid (aa) 24-kDa C-terminal peptide was established. The N-terminal domain (101 aa) is unrelated to any known protein, is hydrophilic, and is predicted by circular dichroism spectroscopy to be largely α-helix. The C-terminal domain (114 aa) is homologous to FKBP12 and other FKBPs but has a potential nuclear targeting sequence and a unique insertion of seven amino acids in one of its loops. FKBP25 displays the rotamase activity characteristic of FKBPs; the activity is inhibited by the immunosuppressants rapamycin (K_i = 0.9 nM) and FK506 (K_i = 160 nM), but not cyclosporin A. The protein, its rapamycin selectivity, and the potential nuclear targeting sequence are discussed in terms of the structure of hFKBP12.

FK506- and rapamycin-binding proteins (FKBPs)[1] constitute a family of receptors for the two immunosuppressants (Figure 1) which inhibit T cell proliferation by arresting two distinct cytoplasmic signal transmission pathways (Kay et al., 1989, 1991; Dumont et al., 1990a,b; Bierer et al., 1990b). Affinity chromatography using cell or tissue extracts with FK506 and rapamycin affinity matrices has revealed at least five proteins that bind the ligands or perhaps a complex of ligand with an FKBP (Fretz et al., 1991). Of these, the best characterized is the archetype, a cytosolic protein of $M_r \approx 12\,000$ (11 778, bovine; 11 819, human), termed FKBP or FKBP12 (Siekierka et al., 1989; Harding et al., 1989);[2] it is found in many tissue types (Siekierka et al., 1990), and homologous proteins have been identified in a variety of organisms (Figure 2). FKBP12 displays rotamase activity with peptide substrates, and this activity is potently inhibited by both FK506 (Siekierka et al., 1989; Harding et al., 1989; Harrison & Stein, 1990) and rapamycin (Bierer et al., 1990b). However, it has been shown that inhibition of the rotamase activity of FKBP12 is not the cause of the drugs' antiproliferative effects on T cells; rather, the complex of drug and an FKBP is the active entity (Bierer et al., 1990a,b; Liu et al., 1991).

We have undertaken a systematic effort to isolate and characterize additional members of the FKBP family; recently, the isolation and molecular cloning of FKBP13, a heavy-membrane associated protein, was reported (Jin et al., 1991). Herein, we present the purification and nearly complete amino acid sequence of FKBP25, a rapamycin-selective 25-kDa FKBP from bovine tissue. Further, the enzymatic and spectroscopic properties of FKBP25 are described, and its sequence is discussed in light of homology to other FKBPs, the structure of hFKBP12, and a potential nuclear targeting sequence.

Experimental Procedures

Materials. Bovine brain, thymus, and spleen were from Arlene and Sons (Hopkinton, MA). Iodoacetamide, poly(ethylene glycol), α-chymotrypsin for the rotamase assay, and Suc-Ala-Ala-Pro-Phe-pNA were obtained from Sigma, Suc-Ala-Leu-Pro-Phe-pNA was from Bachem, sequencing grade trypsin, chymotrypsin, and endoproteinases AspN and GluC were from Boehringer Mannheim, dithiothreitol was from Calbiochem, HPLC grade trifluoroacetic acid and reagents for automated sequencing and analysis were from Applied Biosystems, HPLC grade acetonitrile and water were from Burdick & Jackson, 6 N HCl was from Pierce, and Vydac HPLC columns were from The Nest Group (Southboro, MA). DE-52 was from Whatman. Sephacryl S-100, the Mono S FPLC column, pI markers, Ultrodex, ampholytes for preparative IEF, and IEF gels were obtained from Pharmacia. Molecular weight markers for SDS–PAGE were from Bio-Rad. Other reagents were purchased in the highest grade available.

Isolation and Purification of FKBP25. Fresh tissue (brain, thymus, or spleen; 400 g) was minced, frozen in liquid nitrogen, dry-homogenized with a Waring blender, and dispersed in 1.5–2 L of buffer containing 250 mM NaCl, 20 mM Tris (pH 7.2), 5 mM β-ME, 0.02% NaN_3, and 1 mM PMSF. Bulk debris was removed by centrifugation for 30 min at 8000*g*, and the supernatant was centrifuged at 37000*g* for 3 h at 4 °C. After filtration through a prefilter (Millipore type AW) and a 0.45-μm membrane filter (Millipore type HAWP), the high-speed supernatant was ultrafiltered through a 100 000 MWCO sulfone exclusion membrane (Millipore PTHK00001) and concentrated to about 500 mL on a 10 000 MWCO sulfone exclusion membrane (Millipore Pellicon cassette PTCG-0005). The protein solution was further concentrated in a 6000–8000 MWCO dialysis bag against powdered poly(ethylene glycol) (MW $\approx$ 10 000) and finally dialyzed against 50 mM phosphate (pH 6.8) containing 5 mM β-ME and 1

[†] This work was supported by a grant from the National Institute of General Medical Sciences (GM-38627, awarded to S.L.S.).

[Δ] The sequence of FKBP25 has been deposited in the Protein Identification Resource databank under the Accession Number A40050.

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[1] Abbreviations: FKBP, FK506- and rapamycin-binding protein; aa, amino acid(s); β-ME, β-mercaptoethanol; CCF, cross-correlation coefficient; CD, circular dichroism; CsA, cyclosporin A; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; MWCO, molecular weight cut-off; NTS, nuclear targeting sequence; PMSF, phenylmethanesulfonyl fluoride; pNA, *para*-$O_2NC_6H_4NH$–; PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Suc, succinyl; SVD, singular value decomposition; TFA, trifluoroacetic acid.

[2] Numerical suffixes used to differentiate FKBPs refer to their size in kilodaltons. The prefixes b and h, for bovine and human, are used when reference to a particular species is needed. FKBP12 has in the past been referred to as FKBP.

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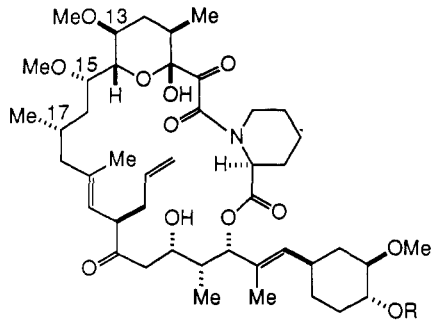
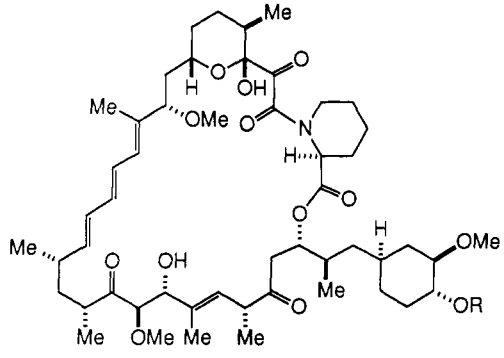


FK506



rapamycin

FIGURE 1: Structures of the immunosuppressants FK506 and rapamycin.

mM PMSF. The supernatant was loaded onto a 5 × 70 cm column of DE-52 equilibrated with 50 mM Tris and 2 mM β-ME (pH 7.5) and was eluted with the same buffer at 40 mL/h. FKBP25 eluted in the first peak detected by A_{280nm}. Column fractions were analyzed by SDS–PAGE, and those containing FKBP25, which migrates as a 30-kDa protein, were pooled and concentrated against a 10000 MWCO membrane (Amicon YM-10). The combined DE-52 fractions were then mixed with 4 g of Ultrodex and 5 mL of a 1:1 (v/v) mixture of pH 6.5–9 and 9–11 ampholytes. The mixture was subjected to preparative IEF in a 12 × 24 cm bed (8 W, 15 mA, 800 V for 22 h at 10 °C). Proteins positioned between pI 7.5 and 11 were pooled and refocused in 2 g of Ultrodex with 2 mL of pH 9–11 ampholytes. The bed was fractionated at 0.2 pI intervals, and the fractions were packed in columns and eluted with 100 mM NaCl, 20 mM phosphate, and 1 mM EDTA (pH 7.2). FKBP25-containing fractions (pI 9.7–10.2) were concentrated on a 10000 MWCO membrane (YM-10), chromatographed on a 1.6 × 100 cm column of Sephacryl S-100 HR, and reconcentrated. Final purification was performed on a 0.8 × 10 cm Mono S FPLC column, using a buffer of 20 mM phosphate and 2 mM β-ME (pH 7.0). A flow rate of 0.5 mL/min was used, and a 40 min gradient from 0 to 1M NaCl was applied beginning 11 min after injection. FKBP25 elutes at approximately 0.55 M NaCl. The yield from 400 g of brain (the best source) was 100–150 μg.

Gel Electrophoresis and Blotting. One-dimensional SDS–PAGE was performed using the Laemmli (1970) buffer system, and proteins were generally visualized by silver staining (Blum et al., 1987). The eluate from a rapamycin affinity column was electroblotted onto Immobilon-P (0.45-μm pore size, Millipore) for N-terminal sequencing as described by Matsudaira (1987) and onto nitrocellulose for in situ tryptic digestion as described by Aebersold et al. (1987); blotted proteins were visualized with Ponceau S. Analytical IEF was performed in pI 3–10 gels.

Reduction and Alkylation of FKBP25. A sample of FKBP25 was prepared for proteolytic cleavage by reduction and *S*-carboxamidomethylation as described by Stone et al. (1989). The protein was dissolved in 50 μL of 8 M urea/0.4 M NH_4HCO_3, 5 μL of 45 mM dithiothreitol was added, and the sample was heated to 50 °C for 15 min. Cysteine residues were then alkylated with 5 μL of 100 mM iodoacetamide at room temperature for 15 min. Subsequent enzymatic cleavage was carried out without desalting or transfer.

Proteolytic Digestion of FKBP25. The *S*-carboxamidomethylated FKBP25 was diluted 4-fold with water but was not otherwise processed (Stone et al., 1989). Protease (trypsin, chymotrypsin, AspN, or GluC) was added to achieve an FKBP25 to protease ratio of 25:1 (w/w), and the mixture was incubated at 37 °C for 20 h. One N-terminally blocked fragment from an AspN digest was digested with a GluC after reducing the volume to 1–2 μL in vacuo and reconstituting with 50 μL of 1% NH_4HCO_3. Samples were stored at −20 °C until chromatographed.

Narrow-Bore Reverse-Phase HPLC Separation of Peptides. Peptides were chromatographed on a Hewlett-Packard 1090 HPLC equipped with a model 1040 diode array detector and a Vydac 2.1 × 150 mm C_{18} column. The gradient employed was a modification of that described by Stone et al. (1989). Briefly, where buffer A was 0.06% trifluoroacetic acid/H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile, a sequence of linear gradients from 5% B at 0 min to 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min was used with a flow rate of 150 μL/min. Absorbance was monitored at 210, 277, and 292 nm, and UV spectra from 209 to 321 nm were acquired for each peak. Peaks detected by absorbance at 210 nm were collected manually into 1.5-mL microcentrifuge tubes and stored immediately without drying at −20 °C prior to sequence analysis. Peaks of sufficient absorbance were rechromatographed.

Estimation of Peptide Length. HPLC fractions with $A_{292nm}/A_{277nm} > 0.5$ were predicted to contain tryptophan, and, conversely, those with $A_{292nm}/A_{277nm} < 0.25$ were predicted to contain tyrosine (Fasman, 1989). Absorbance ratio factors, derived empirically from HPLC profiles of peptides of known length containing tryptophan or tyrosine, were used to approximate peptide length prior to sequencing. The relations are peptide length/tryptophan $\approx 0.80\ A_{210nm}/A_{292nm}$ and peptide length/tyrosine $\approx 0.48\ A_{210nm}/A_{277nm}$.

Amino-Terminal Peptide Sequence Analysis. Sequences were determined by automated sequential Edman degradation. Samples for amino-terminal sequence analysis were applied directly to a polybrene precycled glass-fiber filter and placed in the reaction cartridge of an Applied Biosystems model 477A protein sequencer equipped with an on-line model 120 HPLC. The standard program was modified for faster cycle time (36 min) by raising the reaction cartridge temperature to 53 °C during coupling and reducing the duration of the three R2 delivery steps from 400 to 250 s.

Circular Dichroism Spectroscopy. The CD spectra were recorded with an AVIV model 60 DS spectrometer. Spectra were measured with thermostated, quartz, square cuvettes (0.1 and 0.2 cm) in 20 mM phosphate/2 mM β-ME (pH 7.5) at 10 °C. Data were averaged over five scans. The averaged CD spectra were baseline-corrected and subjected to polynomial smoothing. Protein concentration was estimated by absorbance, taking $\epsilon_{278nm} = 33000\ M^{-1}\ cm^{-1}$. The mean residual ellipticity Ω was calculated assuming a mean residual weight of 112 and is expressed in deg $cm^2\ dmol^{-1}$. CD spectra were analyzed with the PROTEN program (written by A.G.), which

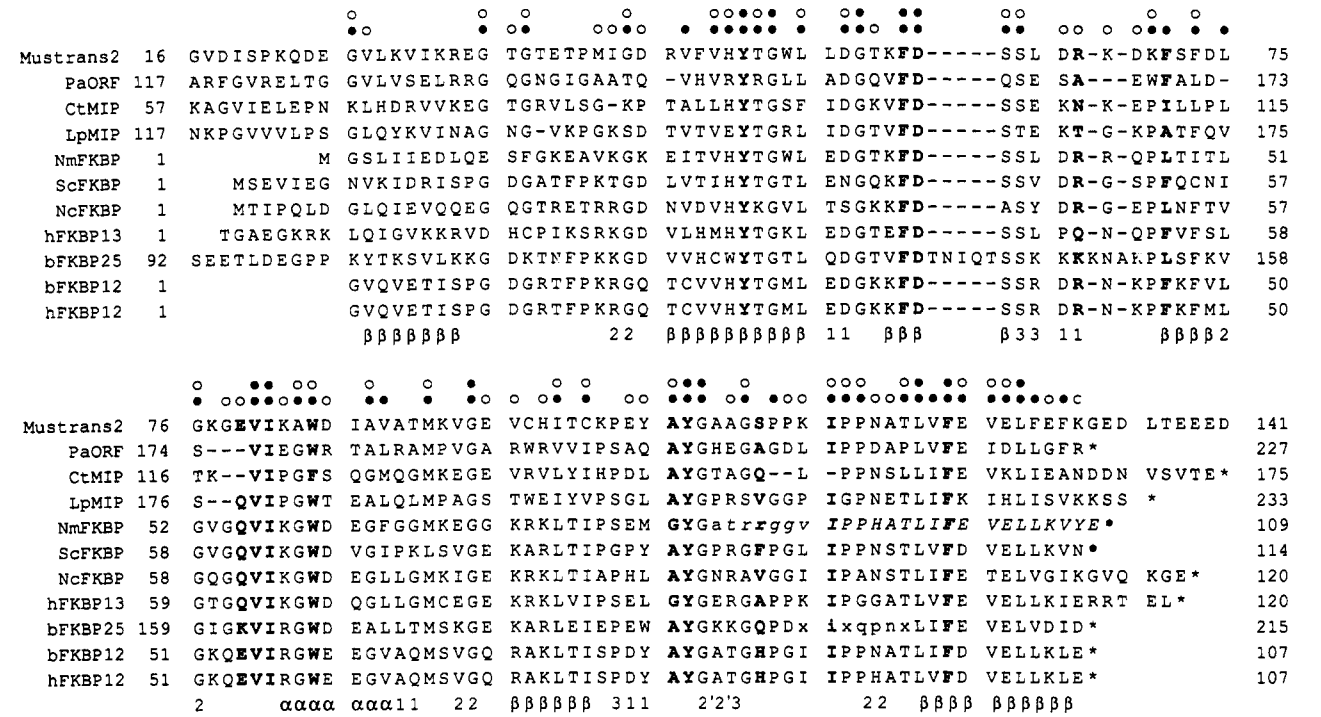


FIGURE 2: Sequence alignments of known FKBPs. Sequences were aligned manually, and only the homologous region of each protein is shown. Numbers at the ends of each row are the position of the nearest amino acid in the full protein. Gaps inserted in the sequences are represented by hypens, C-termini are marked with stars, and uncertain assignments are in lowercase type. Residues in boldface correspond to residues of hFKBP12 that contact FK506 in the X-ray crystal structure (Van Duyne et al., 1991a). Degrees of conservation are indicated by solid and open circles above the sequence as follows: ●●, completely conserved; ●○, strong preference for one amino acid (at least seven occurrences); ●, conservative substitutions; ○, mostly similar amino acids (e.g., charged, polar, basic, or hydrophobic). Beneath the sequences, the secondary structure of hFKBP12 in the complex with FK506 is shown; α-helix is marked with α, antiparallel β-strand with β, and β-turns with numbers corresponding to type (1 for type I, 2′ for type II′, etc.) beneath the second and third positions. Dihedral values of turns are within 40° of ideal (Venkatachalam, 1968). The following sequences are included: hFKBP12 [human, 12 kDa (Standaert et al., 1990; Maki et al., 1990)], bFKBP12 [bovine, 12 kDa (Lane et al., 1991)], bFKBP25 [bovine, 25 kDa (this work)], hFKBP13 [human, 13 kDa (Jin et al., 1991)], NcFKBP [*N. crassa* (Tropschug et al., 1990)], ScFKBP [*S. cerevisiae* (Wiederrecht et al., 1991; Koltin et al., 1991; Heitman et al., 1991)], NmFKBP [*N. meningitidis* (Perry et al., 1988; Standaert et al., 1990)], LpMIP [*L. pneumophila* macrophage infectivity potentiator (MIP) (Engleberg et al., 1989)], CtMIP [*C. trachomatis* (Lundemose et al., 1991)], PaORF [open reading frame in the *P. aeruginosa algR2* gene (Kato et al., 1989; Wiederrecht et al., 1991)], and Mustrans2 [homologue of mouse transition protein 2 (Nelki et al., 1990)]. Tropschug et al. (1990) first noted the homology of the *L. pneumophila* protein. The *N. meningitidis* protein sequence reflects the addition of a single nucleotide gap to the DNA sequence; affected amino acids (86–109) are italicized. Here, as in Standaert et al. (1990), the gap was positioned to give optimal alignment with the hFKBP12 cDNA sequence. Residues that could be changed by reasonable, alternative placements of the gap are shown in lowercase. The numbering of Mustrans2 differs from that of Nelki et al. (1990), who assigned Met42 as the initiator because it was the first methionine in the open reading frame of the cDNA. As Met42 is internal to the putative FKBP domain, does not fall in the usual context of initiators (Kozak, 1986), and is not preceded by any in-frame stop codons, the cDNA is probably incomplete.

utilizes a variable selection method (Manavalan & Johnson, 1987) and singular value decomposition (Forsythe et al., 1977) to fit the calculated CD spectrum to the experimental band envelope. SVD values were obtained using a database containing 21 proteins.

Secondary Structure Analysis. Secondary structure predictions were made with the SEQPRO program (written by A.G.) using the method of Chou and Fasman (1974, 1979). The predictions were constrained to yield the overall secondary structure content derived from CD. Hydrophobicity profiles were calculated with the same program using a seven-residue window and the Kyte–Doolittle hydrophobicity scale (Kyte & Doolittle, 1982). Polarity and hydration potential were calculated using data cited by Argos et al. (1983) and smoothed by Fourier transform. Cross-correlation coefficients (CCF) for hydrophobicity, polarity, hydration potential, and predicted secondary structures of FKBP25 were calculated with eq 1, where X_j and Y_j are the parametric values of sequence stretches of length L, and X and Y are the mean values of these parameters (Jones, 1975).

$$\mathrm{CCF} = \sum_{j=1}^{L}(X_j - X)\sum_{j=1}^{L}(Y_j - Y)/[\sum_{j=1}^{L}(X_j - X)^2\sum_{j=0}^{L}(Y_j - Y)^2]^{1/2} \quad (1)$$

Database Searches and Motif Searches. The University of Wisconsin GCG software package (version 7.0; Devereux et al., 1984) was used to search the GenBank (release 67.0, 3/91, plus updates to 6/91), EMBL (release 26.0, 2/91), PIR (release 27.0, 12/90), and SwissProt (release 17.0, 2/91) databases. A motif search using the PROSITE dictionary (release 6.0, 11/90) was performed with the GCG program MOTIFS; in addition, SEQPRO was used to search the MIPS database (release 27.0, 2/91), CLARK (Lupas et al., 1991) was used to search for the coiled-coil motif, and ALOM (Klein et al., 1985) was used to search for potential membrane-spanning segments.

Rotamase Assays and Calculation of K_i. The rates of cis → trans isomerization of peptide substrates Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Leu-Pro-Phe-pNA were measured by a variation of the method of Fischer et al. (1984, 1989). Assays were conducted at 10.0 °C using a Hewlett-Packard 8452A diode array UV/visible spectrophotometer equipped with a water-jacketed cell holder, a stirring attachment, and a personal computer; the sample compartment was covered and purged with dry nitrogen as required to prevent condensation. Temperatures of solutions in the cuvette were measured directly with a digital thermocouple thermometer and were constant within ±0.1 °C. A 1 × 1 cm glass cuvette containing a stir bar was charged with 1.4 mL of a solution of substrate

Table I: Peptides from Proteolytic Digests of FKBP25

peak[a]	sequence[b]	position	yield[c]
T1	**ETKSEETLDEGPPKYTk**	89–105	13
	FKGTeSISK	64–72	9
T2	**AWTVEQLR**	1–8	30
T3	FLQDhGSDSFLAExK	20–34	16
T4	**GWDEALLTMSKGEK**	166–179	33
	TANKDHLVTAYNHLFESK	45–62	13
T5	**KGDVVHCxYTGTLQDGTVFDTNIQ**	119–142	15
T6[d]	**LIFEVELVDID**	205–215	4
Y1	**KGTESISKVSEQVKNVKLNEDKPKETKSEETLxeg**	65–99	41
Y2	**LQDHGSDSFLAEHKl**	21–35	11
	TMSKGEKARLEIEPExAYg	173–191	8
Y3	**KVGIGKVIRGWDEAL**	157–171	15
Y4[e]	**TMSKGEKARLEIEPEWAYGKKGQPDxixgpn**	173–203	25
	SFKVGIGKVIRGWDEAl	155–171	14
E1	**HKLLGNIKNVAKTANK**	33–48	4
	IEPExAYG	184–191	4
E2	ALLTMsKg	170–177	3
	SKRFKGTExI	61–70	2
D1	**DVVHCWYTGTLQ**	121–132	9
D2	**DEGPPKYTKSVLKKGDKTNFPKKGDVVHCwYT**	97–128	28
D3	**DTNIQTSSKKKKNAKPLSFKVGIgk**	138–162	13
DE1	**QLPKKDIIKFLQDhGsDsFla**	11–31	3
DE2	**QLRSEQLPkkDiikFLQdxg**	6–25	5

[a] Peaks are numbered in order of elution on reverse-phase HPLC. Peptides were derived from digestion with trypsin (T), chymotrypsin (Y), AspN (D), GluC (E), and AspN followed by GluC (DE). [b] Peptide sequences in boldface are the minimum required to elucidate the primary structure of FKBP25. Lowercase assignments denote less than full confidence; x = no assignment. [c] Values are initial yields in picomoles of PTH-amino acid. [d] C-terminal peptide. [e] Contaminant sequence coeluted (see Results).

(55 μM) in a buffer containing 40 mM Na-HEPES (pH 7.9 at 22 °C), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and 0.015% (w/v) Triton X-100. FKBP25 (25 μL of a 2.2 μM solution containing ca. 50 μM CsA) was added, the cuvette was placed in the spectrophotometer, and stirring was begun. Inhibitors, in 25 μL of 50% (v/v) methanol, or a solvent blank were then added. When the temperature of the solution reached 10.0 °C, a precooled solution of α-chymotrypsin (30 μL of a 20 mg/mL in 1 mM HCl) was added, and the absorbance (average of 408, 410, and 412 nm) was measured at 0.5-s intervals until hydrolysis was complete (5–10 min). First-order rate constants were calculated using the KORE program (Swain et al., 1980). Data from the initial, rapid rise in absorbance produced by hydrolysis of the trans peptide were ignored; the time threshold for inclusion of data was varied for each assay to provide the minimum standard deviation in the rate constant (typically, a threshold that included the last 7–8% of the total rise in absorbance met this criterion). Inhibition data were fit using the KINMIN program (written by A.G.) to a tight-binding inhibitor model (Williams & Morrison, 1979) modified to include the contribution of uncatalyzed isomerization to the rate constant (eq 2, where k_{obs} is

$$k_{obs} = k_{uncat} + 0.5(k_{cat}/K_m)\{[(K_i + I - E)^2 + 4EK_i]^{1/2} - (K_i + I - E)\} \quad (2)$$

the observed first-order rate constant, k_{uncat} is the first-order rate constant for the uncatalyzed isomerization, E is the enzyme concentration, and I is the inhibitor concentration). All four parameters, i.e., k_{uncat}, k_{cat}/K_m, K_i, and E, were optimized to provide the minimum relative standard deviation between the calculated and observed data using the Powell method of conjugate gradients (Powell, 1965) or, alternatively, using the Levenberg–Marquardt algorithm (Marquardt, 1963).

Fluorescence Binding Studies. Fluorescence measurements were made with 1 × 1 cm cuvettes in a Perkin-Elmer MPF4 fluorimeter. The cell was kept at 10 °C, the excitation wavelength was 287.5 nm, and the maximum fluorescence was observed at 336 nm. Protein was at a concentration of 50 μg/mL in a buffer of 20 mM phosphate and 2 mM β-ME (pH 7.2). Upon addition of varying amounts of rapamycin and FK506 in 50% (v/v) methanol/water, the emission spectra were recorded.

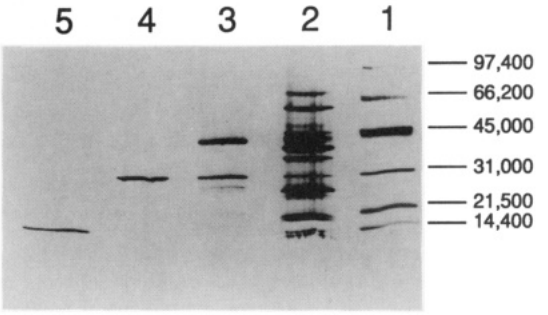


FIGURE 3: SDS–PAGE analysis of the purification of FKBP25 from calf brain. Samples were electrophoresed on an 8–18% gradient gel and visualized with silver stain. (Lane 1) Molecular mass markers: 14.4, 21.5, 31.0, 45.0, 66.2, 97.4, and 200 kDa. (Lanes 2–4) Samples taken after chromatography on DE-52, Sephacryl S-100 HR, and Mono S columns, respectively (see Experimental Procedures). (Lane 5) Recombinant hFKBP12.

Matrix-Assisted, Laser Desorption, Time-of-Flight Mass Analysis. Approximately 0.5 pmol of FKBP25 was mixed with 0.5 μL of 3,5-dimethoxy-4-hydroxycinnamic acid and spotted onto a gold-plated target. The mass spectrum was obtained by summing the data from 26 laser pulses on a Finnigan Lasermat mass spectrometer.

RESULTS

FKBP25, first detected in the eluates from rapamycin and FK506 affinity columns, has been purified to homogeneity from bovine tissue by a combination of ion-exchange and size-exclusion chromatography aided by preparative IEF (Figure 3). The purified protein has an approximate pI of 9.8–9.9 (IEF) and migrates as a 30-kDa protein on SDS–PAGE; however, the M_r of the protein, measured by mass spectrometry, is 25 235 ± 1%. As FKBP25 proved to be N-terminally blocked, we were unable to determine its entire amino acid sequence by Edman degradation, but we were able to assemble a 215 aa 24-kDa C-terminal peptide by sequencing

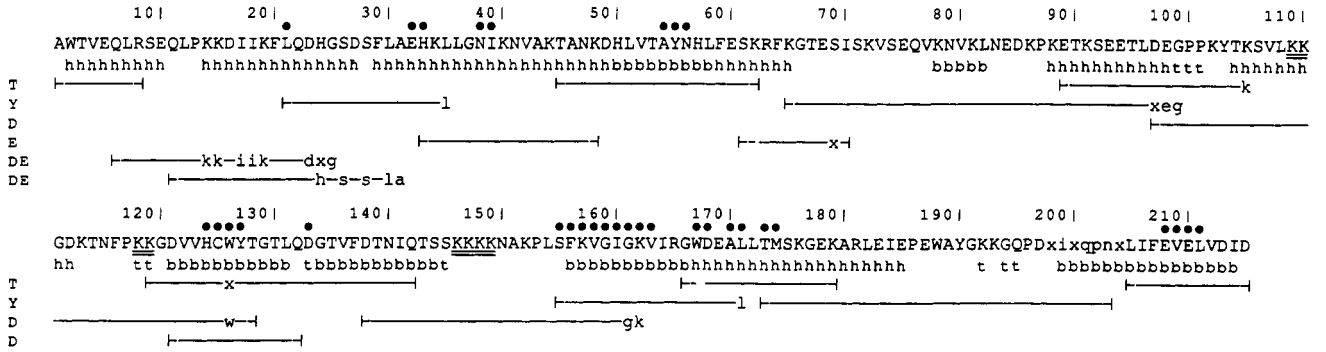


FIGURE 4: Partial amino acid sequence of bovine FKBP25 (single-letter code). Numbering begins with the first known amino acid, which is not the N-terminal amino acid of FKBP25. Solid circles above the sequence mark hydrophobic regions; specifically, each circle is the center of a seven-amino acid window having a positive average Kyte–Doolittle hydrophobicity. Three segments of a hypothetical nuclear targeting sequence are double-underlined (see the text and Figure 6). Immediately below the sequence is the predicted secondary structure (h, α-helix; b, β-sheet; t, β-turn). The remaining lines below the sequence denote peptide fragments of FKBP25 obtained after cleavage with trypsin (T), α-chymotrypsin (Y), AspN (D), GluC (E), and AspN plus GluC (DE). Only the fragments required to establish the sequence are shown. Lowercase letters within the fragments denote assignments of less than full certainty. Vertical bars mark the beginning and end of each fragment, but they are omitted where a lowercase letter is present.

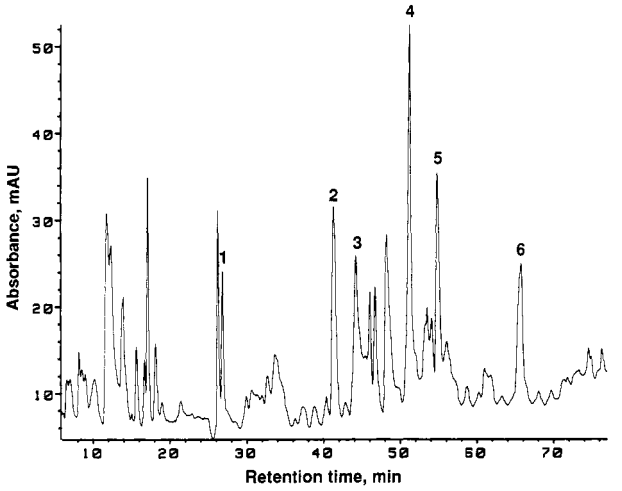


FIGURE 5: HPLC separation of fragments from an in situ tryptic digest of FKBP25 (see Experimental Procedures). Detection was by absorbance at 210 nm, and peak numbers are as in Table I.

proteolytic fragments (Figures 4 and 5; Table I). An aspartate-terminated tryptic peptide yielded the C-terminus. Several peptides coeluted on HPLC, and, in such cases, sequences were assigned as primary or secondary on the basis of relative yield of phenylthiohydantoin and were confirmed by unambiguous overlap with other proteolytic fragments. One peptide (Thr173–Asn203) used to assemble the sequence was isolated from a digest of an impure sample of FKBP25; it could be attributed to FKBP25 on the basis of overlap with fragments from pure samples. The sequence was determined at high confidence except for the region 198–204; three of these seven amino acids could not be assigned, and the other four could be assigned only with low confidence (Figure 2). There was no overlap at position 204, meaning that this position could represent a polypeptide fragment, but we have assumed it is a single amino acid on the basis of molecular weight and homology to other FKBPs; it is expected to be lysine or arginine because it falls at a site of tryptic cleavage.

The length of the unknown N-terminal peptide can be estimated as follows. Subtracting the molecular weight of the known sequence (assuming a residual weight of 110 for the unassigned residues) from the molecular weight of FKBP25 as determined by mass spectrometry leaves 900 ± 250, which corresponds to 8 ± 3 aa (neglecting the mass of the blocking group). Data consistent with this conclusion are provided by a blocked and presumably N-terminal peptide isolated in good yield from the AspN/GluC digest. The peptide displays an A_{210nm}/A_{292nm} ratio indicative of a tryptophan content of 1 in 9 ± 3 residues. If it is assumed that this peptide overlaps the known sequence, that the absorbance at 292 nm is from Trp2 (numbered from the first known amino acid), and that the C-terminus of the peptide is Glu5 (cleavage at this position was efficient), then the length of the unknown region is estimated to be 4 ± 3 residues. If the peptide does not overlap the known sequence, the gap between its C-terminus and the N-terminus of the known sequence must be 5 aa or less to agree with the mass spectral data, and the whole unknown region must be 11 aa or less.

Experiments were undertaken to determine whether FKBP25 binds FK506 and rapamycin. We were unable to observe binding of [^{3}H]dihydroFK506 in Sephadex LH-20 assays (Handschumacher et al., 1984) using the concentrations of protein and ligand appropriate for FKBP12, i.e., 1 nM protein and 0.1–10 nM ligand. Raising the protein concentration to 100 nM was of no avail, and we had insufficient quantities of protein and ligand to pursue even higher concentrations. An alternative method of assessing the interaction of the immunosuppressive ligands with FKBP25 is kinetic analysis, and FKBP25 possesses the requisite rotamase activity with peptide test substrates. We first ascertained that FKBP25 has different substrate specificity than FKBP12, isomerizing Suc-Ala-Ala-Pro-Phe-pNA 35% faster than Suc-Ala-Leu-Pro-Phe-pNA, the preferred substrate for FKBP12 (Harrison & Stein, 1990; Albers et al., 1990). With its preferred substrate, bFKBP25 has $k_{cat}/K_m = 0.8 \times 10^6$ M^{-1} s^{-1}, making it slightly less active than recombinant hFKBP12 ($k_{cat}/K_m = 2.2 \times 10^6$ M^{-1} s^{-1}; Albers et al., 1990; Bierer et al., 1990b). We next investigated the inhibition of catalysis by FK506 and rapamycin (Figure 6) and determined that FK506 was an inhibitor (K_i = 160 nM) and that rapamycin was a much better one (K_i = 0.9 nM). CsA had no effect, and the assays were in fact conducted in the presence of 1 μM CsA to prevent interference from traces of cyclophilin, a more efficient rotamase (Fischer et al., 1989; Harrison & Stein, 1990; Liu et al., 1990). Fluorescence binding studies provided qualitative confirmation of the kinetic data. Upon binding FK506 or rapamycin, the tryptophan fluorescence of FKBP25 is quenched by 36% or 57%, respectively.

Discussion

We report the purification, properties, and nearly complete amino acid sequence of a previously uncharacterized immu-

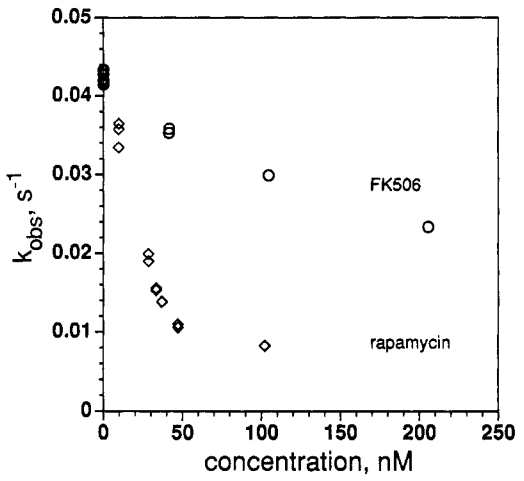


FIGURE 6: Inhibition of rotamase activity by FK506 and rapamycin. The observed rate constant for cis → trans isomerization of Suc-Ala-Ala-Pro-Phe-pNA at 10 °C in the presence of 38 nM FKBP25 is plotted against added inhibitor concentration (FK506, K_i = 160 nM, circles; rapamycin, K_i = 0.9 nM, diamonds). Additional data for FK506 (concentrations from 425 nM to 8.5 μM) are not shown but were used in the calculation of K_i. The horizontal asymptote of 0.0075 s^{-1} is the rate constant for uncatalyzed isomerization.

nophilin, a 25-kDa FKBP. An estimated 8 ± 3 unknown aa and an unknown blocking group precede the sequence given in Figure 4, and seven other residues could not be assigned with confidence. Two domains are clearly discernible in the sequence, an N-terminal domain of 101 aa and a C-terminal peptide of 114 aa. The N-terminal domain is not homologous to any known protein; it is rich in charged residues (Asp + Glu + His + Lys + Arg = 38), particularly lysine (Lys = 15), and free of cysteine and methionine, but its composition is otherwise unremarkable. The C-terminal domain is homologous to bFKBP12 (42% identical, counting gaps as mismatches and omitting the seven uncertain amino acids; CCFs 0.27–0.66), but less so than are the *Neurospora crassa* (47%, 0.18–0.46), *Neisseria meningitidis* (48%, 0.17–0.53), and *Saccharomyces cerevisiae* (57%, 0.46–0.68) proteins; FKBP25 is unique among the FKBPs in having an insertion of seven amino acids in one of its loops. Three bacterial FKBPs are similar in size to FKBP25 (*Legionella pneumophila* and *Pseudomonas aeruginosa*, 25 kDa; *Chlamydia trachomatis*, 27 kDa), but their N-terminal domains are unrelated to its. Like FKBPs 12 and 13, FKBP25 is basic (pI 9.8–9.9) and is a rotamase. Its activity is comparable to that of hFKBP12, but its substrate specificity differs, with Suc-Ala-Ala-Pro-Phe-pNA preferred over Suc-Ala-Leu-Pro-Phe-pNA. Further, it is inhibited preferentially by rapamycin (K_i = 0.9 nM) over FK506 (K_i = 160 nM). Circular dichroism provided evidence that FKBP25 is approximately 40% α-helix. As hFKBP12 contains only a single α-helix of seven residues, and the C-terminal domain of FKBP25 is homologous, it appears that the N-terminal domain is largely α-helix. Such a conclusion is also consistent with Chou–Fasman calculations, which predict the helical arrangement for 62 of the 101 known amino acids of the N-terminal domain (Figure 4). Thus, FKBP25 probably falls in the α + β class of proteins.

Several trends are evident in the sequences of FKBPs. About 38% of the residues are strongly conserved (7 or more of 11 occurrences of the same amino acid at a given position), but the homology is weak in the region corresponding to the first 24 amino acids of FKBP12. Thirteen residues are perfectly conserved, and their significance can be appreciated from the structure of FKBP12 (Michnick et al., 1991; Van Duyne et al., 1991a,b; Moore et al., 1991). Seven of them map onto the drug-binding site (also the active site) of hFKBP12 (Van Duyne et al., 1991a), but six do not. Of the latter, four are glycines (Gly28, Gly33, Gly69, and Gly83) and two are leucines (Leu97 and Leu103). The two leucines lie opposite each other near the ends of the central strand of the β-sheet, and their side chains have extensive contacts within the protein, suggesting they might serve as nuclei for the packing of the hydrophobic core. The glycines serve more obvious purposes. In the complex of FKBP12 with FK506, Gly33, Gly69, and Gly83 all assume (ϕ,ψ) values not favorable for any other amino acid. Gly28 is essential not for its flexibility but for its size; in both free and bound (to FK506) hFKBP12, the Gly28 *pro-S* hydrogen lies in contact with two of the conserved aromatics (Phe36 and Phe99) of the binding pocket, and the conservation of this residue suggests that no other amino acid would fit in its position. Thus, the six residues, though not part of the binding site, are conserved because they are essential to the protein fold.

Whereas hFKBP12 binds FK506 and rapamycin with comparable affinities (K_d = 0.4, 0.2 nM, respectively; Bierer et al., 1990b), FKBP25 has a pronounced selectivity for rapamycin, which is manifested as impaired FK506 binding rather than enhanced rapamycin binding (K_i = 160 and 0.9 nM, respectively). Because the drug-binding site and the active site are the same, K_i and K_d should be equivalent measures of affinity, and affinity chromatography provides qualitative support of the difference in binding. FKBP25 is well-retained by rapamycin affinity columns, but not by FK506 affinity columns (Fretz et al., 1991), and it is sometimes undetectable in eluates from the latter. Fluorescence binding studies suggest a difference in the modes of binding of the two drugs to FKBP25. Rapamycin quenches tryptophan fluorescence more effectively than does FK506, which implies that one or more tryptophans may pertain to rapamycin selectivity. As the FKBP domain of FKBP25 is only 42% identical to bFKBP12, any of the other 60+ differences, alone or in concert, might also contribute to rapamycin selectivity, as might the N-terminal (non-FKBP) domain, but the residues of the drug-binding site are likely to play an important role.

Sequence comparisons and examination of the X-ray crystal structures of the hFKBP12/FK506 and hFKBP12/rapamycin complexes (Van Duyne et al., 1991a,b) draw attention to one region of FKBP25 that may be relevant to rapamycin selectivity, the region containing the seven aa loop insertion (residues 139–151). The loop modification necessitates a change in the conformation of the protein in this region if the overall fold of FKBP12 is to be maintained, and there is a contact with FK506 that could be lost, either because of the conformational change or because of an amino acid substitution within the loop. Although the binding domains of FK506 and rapamycin assume nearly identical conformations when bound by hFKBP12, there are small differences in the protein–drug interactions. In hFKBP12, Arg42 (corresponding to Lys148 in FKBP25) is one of two residues, Ile91 being the other, that contact FK506 but not rapamycin; the π-face of its guanidinium packs in van der Waals contact with the C15 methoxy and C17 methyl groups of FK506 (see Figure 1). As lysine would not be expected to participate in this type of interaction, the seemingly conservative Arg → Lys change might adversely affect FK506 binding. Rapamycin binding, which does not involve an analogous contact, might not be affected as strongly. The significance of the Arg42–FK506 interaction, and of the loop modifications, remains to be determined by structural studies and site-directed mutagenesis.

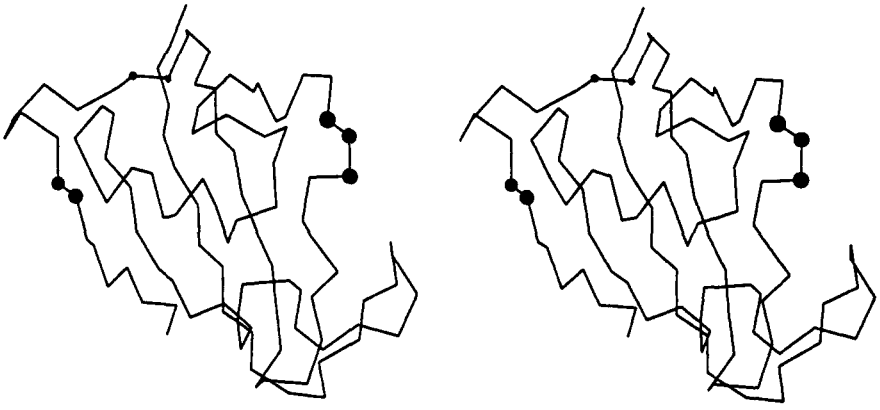


FIGURE 7: Stereoview of an α-carbon trace of hFKBP12. One structure generated from NMR constraints (Michnick et al., 1991) is shown. Residues corresponding to a hypothetical bipartite nuclear targeting sequence in FKBP25 are marked with balls, the different sizes of which result from depth-cueing: Left, residues 8 and 9; top, residues 17 and 18; right, residues 40–42. For the analogous residues of FKBP25, it is proposed that one or the other of the first two parts could combine with the third to form the NTS. In the average solution structure of free hFKBP12, the corresponding separations (shortest C_α–C_α distance) are 22 and 24 Å for the first and second parts, respectively, from the third. In the complex of hFKBP12 with FK506, the values are 17 and 24 Å, respectively (Van Duyne et al., 1991a).

Examination of the sequence of FKBP25 for motifs in the PROSITE dictionary revealed possible phosphorylation sites for protein kinase C (Kishimoto et al., 1985; Woodgett et al., 1986) and casein kinase II (Marin et al., 1986; Kuenzel et al., 1987); however, FKBP25 is not detectably phosphorylated in stimulated or unstimulated Jurkat cells (Fretz et al., 1991). Clearly absent from the FKBP25 sequence are the common metal-, nucleotide-, and DNA-binding motifs, and the coiled-coil motif (Lupas et al., 1991). Manual inspection of the sequence revealed the only motif of obvious consequence, a potential nuclear targeting sequence (Robbins et al., 1991) located in the FKBP domain (double underline in Figure 4). Such signals are bipartite, one part being two consecutive basic residues and the second having at least three of five residues basic. The spacer is 10 aa in the consensus, but longer ones are allowable; for instance, extension of the spacer in nucleoplasmin from 10 to 22 amino acids had no effect on nuclear translocation (Robbins et al., 1991). In FKBP25, the sequence $KK(X)_7KK(X)_{26}KKKK$ begins at position 110.[3] For comparison, the corresponding sequence in FKBP12 is $SP(X)_7KR(X)_{21}RDR$ (residues 8–42). It is not apparent which of the two KK sequences might belong to the hypothetical NTS. Inspection of the solution and crystal structures of hFKBP12 reveals that all of the corresponding regions are exposed to solvent and suggests that the KK sequence beginning at position 110, despite the greater number of intervening amino acids, is closer to the KKKK sequence (Figure 7).

An abundance of sequences and structural data does not constitute an understanding of function. Efforts to characterize the FKBPs, FKBP12 in particular, have outpaced efforts to uncover their biological roles, but function has not been ignored. The available data are sufficient to construct a plausible model for the effects of FK506, rapamycin, and CsA on T cell activation [recent reviews: Sigal et al. (1990) and Schreiber (1991)] and, further, to imply roles for the immunophilin–drug complexes in the signaling pathways of mast cells (Hultsch et al., 1991), yeast (Koltin et al., 1991; Heitman et al., 1991b), and *N. crassa* (Tropschug et al., 1989). Moreover, recent work has identified calcineurin, a Ca^{2+}, calmodulin-dependent protein phosphatase, as a common target of the cyclophilin-/CsA and FKBP12/FK506 complexes (Liu et al., 1991). As regards FKBP25, no role for it, either alone or complexed with drug, is yet evident. Further, the role of its N-terminal domain, the extent of its interaction with the C-terminal domain, and how it affects or is affected by the drugs are unknown. A rapamycin-selective and possibly nuclear FKBP might account for some of the differing effects of FK506 and rapamycin, and it might also account for an unrecognized effect.

[3] Standard one-letter codes are used here for compactness.

ACKNOWLEDGMENTS

We thank Renee A. Robinson for her expert technical assistance in the fragmentation and HPLC separation of FKBP25. We are grateful to Tom Oglesby (Finnigan MAT) for the mass spectral analysis of FKBP25, to Sherman Lehrer (Boston Biomedical Laboratories) for time on CD and fluorescence equipment, to Andrei Lupas and Jeff Stock for their computer program CLARK, used to search for the coiled-coil motif, to Mark Albers for critical review of the manuscript, and to Mark Albers, Stephen Michnick, Mike Rosen, and Tom Wandless for helpful discussions.

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