Molecular Cloning, DNA Sequence Analysis, and Biochemical Characterization of a Novel 65-kDa FK506-binding Protein (FKBP65)*

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We have identified a mouse gene encoding a 65-kDa protein (FKBP65) that shares homology with members of the FK506-binding protein (FKBP) class of immunophilins. Predicted amino acid sequence shows that this protein shares significant homology with FKBP12 (46%), FKBP13 (43%), FKBP25 (35%), and FKBP52 (26%). FKBP65 contains four predicted peptidylprolyl cis-trans-isomerase (PPIase) signature domains, and, although smaller in size, is distinct from FKBP52 (also identified as FKBP59, hsp56, or HBI), which contains three FKBP12-like PPIase domains. With N-succinyl-Ala-Ala-Pro-Phe-nitroanilide as the substrate, recombinant FKBP65 is shown to accelerate the isomerization of the prolyl peptide bond with a catalytic efficiency similar to other family members. This isomerization activity is inhibited by FK506 and rapamycin, but is not sensitive to Cyclosporin A. Based on Northern blot analysis, FKBP65 mRNA transcripts are present in lung, spleen, heart, brain, and testis. A polyclonal antibody, raised against a COOH-terminal peptide (amino acid residues 566-581), was used to immunoprecipitate FKBP65 from NIH3T3 cells and demonstrate that FKBP65 is a glycoprotein. In addition, [35S]orthophosphate labeling experiments show that FKBP65 is also a phosphoprotein. These results suggest that FKBP65 is a new FKBP family member.

FK506 and rapamycin are immunosuppressant macrolide antibiotics that mediate their activity in part by binding to members of a ubiquitous family of highly conserved intracellular receptors termed immunophilins (1, 2). Although FK506 and rapamycin are structurally related and bind to a distinct class of immunophilins termed FK506-binding proteins (FKBPs), they exert their immunosuppressive effects by blocking different signal transduction pathways in normal T cells. FK506 selectively inhibits the Ca2+-dependent signal transduction events by specifically binding calcineurin, a Ca2+- and calmodulin-dependent serine/threonine phosphatase (3). Inhibition of calcineurin phosphatase activity interferes with an early event that is essential for T-lymphocyte growth and differentiation (4, 5). In comparison, rapamycin appears to affect Ca2+-independent, IL-2-driven T-cell proliferation (6, 7). A characteristic shared by immunophilins is peptidylprolyl cis-trans-isomerase (PPIase) activity (8, 9), which is inhibited upon drug binding (10). However, the inhibition of PPIase activity in itself does not appear to be responsible for the immunosuppressive effect of the drugs (7, 11).

To date, four FKBP isoforms, named according to their calculated molecular mass: FKBP12 (8, 10), FKBP13 (12), FKBP25 (13-15), and FKBP52 (16, 17), have been identified. This report describes the molecular cloning, sequencing, and biochemical characterization of a novel 65-kDa protein, FKBP65, isolated from a NIH3T3 fibroblast cDNA library. We show that this protein, as well as containing four peptidylprolyl cis-trans-isomerase domains, possesses PPIase activity comparable to other FKBPs and appears to be a new and distinct FKBP family member.

EXPERIMENTAL PROCEDURES

Medium and Reagents—L-[35S]Methionine, [35S]dATP, [α-32P]dCTP, [α-32P]orthophosphate, and protein molecular weight markers were from Amersham. Methionine- or phosphate-free Dulbecco's modified Eagle's medium, heat-inactivated dialyzed fetal bovine serum, and agarose were obtained from Life Technologies, Inc. Penicillin/streptomycin and L-glutamine were from MediaTech (Washington, D.C.).

Antipeptide Antibody Preparation—Peptides corresponding to amino acids 369-386 (Pep 2) and 566-581 (Pep 4) were synthesized and coupled to keyhole limpet hemocyanin (Macromolecular Resources, Colorado State University, Ft. Collins, CO). Rabbits were immunized with Freund's adjuvant in accordance with National Cancer Institute and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cloning and Sequencing of Murine FKBP65 cDNA—Anti-Pep 2 antiserum was used to screen a J B6 murine epidermal cell (18) cDNA expression library (Stratagene). A 2468-bp clone was isolated and sequenced (19). A 1700-bp (EcoRI-Sac1) segment of the 2.4-kb clone was then used to screen a Zap NIH3T3 fibroblast cDNA library (Stratagene). Antipeptide Antibody Preparation—Peptides corresponding to amino acids 369-386 (Pep 2) and 566-581 (Pep 4) were synthesized and coupled to keyhole limpet hemocyanin (Macromolecular Resources, Colorado State University, Ft. Collins, CO). Rabbits were immunized with Freund's adjuvant in accordance with National Cancer Institute and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Northern Blot Hybridization—Total RNA (20 μg) from various mouse tissues (Clontech, San Francisco, CA) was electrophoresed in a 3.9% formaldehyde, 20 mM MOPS, pH 7.6, 1.0% agarose gel, transferred to MagnaNylon Membrane (Micron Separations Inc., Westborough, MA),
and hybridized with the 1.7-kb cDNA probe (described above) in Fast-Pair (Digene, Silver Spring, MD) at 42°C for 48 h. The most stringent wash was in 0.4 × SSPE, 0.1% SDS at 65°C.

Cells—NIH3T3 fibroblast cells were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) heat-inactivated calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin at 37°C, 5% CO2.

Protein Expression, Purification, and Peptidylprolyl cis-trans-Isomerase Assay—A 1600-bp segment of the FKBP65 segment was cloned into the pET21d vector; and protein was expressed with isopropyl-1-thio-β-D-galactopyranoside (Novagen, Madison, WI). Cell pellets were sonicated in 50 mM sodium phosphate, pH 7.4, 10% glycerol, 1% Triton X-100, and dialyzed against 20 mM Tris, pH 8.0, 1 mM β-mercaptoethanol (20). Further purification was by RP-HPLC (μBondapak C18 PrepPak cartridge 10 μm, 125 Å, 25 × 100 mm, Waters, Bedford MA) and verified as FKBP65 by peptide sequencing. Recombinant FKBP65 (rFKBP65) was renatured prior to using in the PPIase assay. The isomerization activity of the renatured rFKBP65 was assayed as described (8, 9) with minor modifications, using N-succinyl-Ala-Ala-Pro-Phe-t-butylamide (Sigma) as substrate at a final concentration of 108 μM, preincubated with rFKBP65 (8–80 nm final concentration) at ambient temperature. α-Chymotrypsin (Sigma) was used at a final concentration of 27 μM. Absorbance readings were taken immediately upon mixing in a Perkin Elmer Lambda 6 spectrophotometer at 395 nm at 0.2-s intervals for 180 s. Drug inhibition studies were performed as described above except various amounts of FK506 or rapamycin (27–200 μM final concentration) were added to the substrate/ FKBP65 mixture using 16 mM FKBP65. The Kd/ka was determined by plotting the observed rate constants (kob) versus FKBP65 concentration (data not shown). The first order rate constant (kob) was calculated from the slope of the plot of the log of the difference between absorbance at steady state and absorbance at time t plotted against time (21, 22).

In Vivo [35S]Methionine Metabolic Labeling and Immunoprecipitations—NIH3T3 cells were washed with phosphate-buffered saline and incubated in methionine-free Dulbecco’s modified Eagle’s medium, 5% dialyzed fetal bovine serum for 1 h at 37°C, 5% CO2. The cells were then washed with phosphate-buffered saline and preincubated with 0.4 unit of Endo-F (endoglycosidase F, Boehringer Mannheim), and incubated for 3 h at 37°C (24). Samples were electrophoresed using the method of Laemmli (25).

Endoglycosidase F Treatment—NIH3T3 cells were labeled in vitro with [35S]methionine and immunoprecipitated as described above, after which the supernates were denatured by boiling, cooled to 37°C prior to adding 0.4 unit of Endo-F (endoglycosidase F, N-glycosidase F-free; Boehringer Mannheim), and incubated for 3 h at 37°C (24). Samples were electrophoresed as described above.

In Vivo [32P]Orthophosphate Labeling—The in vivo [32P]orthophosphate labeling has been described in detail previously (25). Cells were lyzed in TNT, immune-precipitated and electrophoresed (described above), electroblotted onto Immobilon PSM (Millipore), and exposed to film. Western blotting was performed on the membranes after the [32P]orthophosphate labeling.

RESULTS

Cloning, Sequencing, and mRNA Tissue Distribution of FKBP65—A unique 2468-bp clone was isolated from a mouse NIH3T3 fibroblast cDNA library. A 1600-bp segment of the 2.6-kb clone was cloned into the pET21d vector, and protein was induced with isopropyl-1-thio-β-D-galactopyranoside (Novagen, Madison, WI). Cell pellets were sonicated in 50 mM sodium phosphate, pH 7.4, 10% glycerol, 1% Triton X-100, and dialyzed against 20 mM Tris, pH 8.0, 1 mM β-mercaptoethanol (20). Further purification was by RP-HPLC (μBondapak C18 PrepPak cartridge 10 μm, 125 Å, 25 × 100 mm, Waters, Bedford MA) and verified as FKBP65 by peptide sequencing. Recombinant FKBP65 (rFKBP65) was renatured prior to using in the PPIase assay. The isomerization activity of the renatured rFKBP65 was assayed as described (8, 9) with minor modifications, using N-succinyl-Ala-Ala-Pro-Phe-t-butylamide (Sigma) as substrate at a final concentration of 108 μM, preincubated with rFKBP65 (8–80 nm final concentration) at ambient temperature. α-Chymotrypsin (Sigma) was used at a final concentration of 27 μM. Absorbance readings were taken immediately upon mixing in a Perkin Elmer Lambda 6 spectrophotometer at 395 nm at 0.2-s intervals for 180 s. Drug inhibition studies were performed as described above except various amounts of FK506 or rapamycin (27–200 μM final concentration) were added to the substrate/ FKBP65 mixture using 16 mM FKBP65. The Kd/ka was determined by plotting the observed rate constants (kob) versus FKBP65 concentration (data not shown). The first order rate constant (kob) was calculated from the slope of the plot of the log of the difference between absorbance at steady state and absorbance at time t plotted against time (21, 22).

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Endoglycosidase F Treatment—NIH3T3 cells were labeled in vitro with [35S]methionine and immunoprecipitated as described above, after which the supernates were denatured by boiling, cooled to 37°C prior to adding 0.4 unit of Endo-F (endoglycosidase F, N-glycosidase F-free; Boehringer Mannheim), and incubated for 3 h at 37°C (24). Samples were electrophoresed as described above.

In Vivo [32P]Orthophosphate Labeling—The in vivo [32P]orthophosphate labeling has been described in detail previously (25). Cells were lysed in TNT, immune-precipitated and electrophoresed (described above), electroblotted onto Immobilon PSM (Millipore), and exposed to film. Western blotting was performed on the membranes after the [32P]orthophosphate labeling.

Analysis of the 2.6-kb clone, using the University of Wisconsin GCG software analysis program (29), revealed a domain containing significant amino acid sequence similarity to members of the FK506/rapamycin-binding protein (FKBP) family (19). In accordance with the convention for naming FKBP family members corresponding to their calculated molecular weights (17), we have renamed the protein previously designated FKBP25 (19) as FKBP65. Overall, FKBP65 shares 46% and 43% amino acid sequence identity with human FKBP12 and FKBP13, respectively, and 35% and 26% sequence homology with human FKBP25 and FKBP52 (FKBP59, hsp56, or HBI).

Additional sequence analysis of the 2.6-kb FKBP65 clone revealed four peptidylprolyl cis-trans-isomerase (PPIase) signature domains as defined by the Motifs program (GCC, University of Wisconsin, Madison, WI; Ref. 29). These four domains are located at amino acid residues 61–149, 173–261, 285–373, and 398–485 and represent 67% of the protein (Fig. 1B). The domains flanking the four PPIase domains share 30% similarity; however, they appear to be unique as they show no homology with other FKBP family members or any sequences reported in available data bases. The FKBP65 PPIase Domain II conforms to the PPIase consensus motif, whereas the other three domains each contain one mismatch (Fig. 1B).

Crystallographic studies of FK506 bound to human FKBP12 (hFKBP12) have identified two regions within the PPIase domain that appear to be important for FK506-binding interactions. There are five amino acid residues (Tyr26, Phe99, Val33, Ile68, and Trp90) proposed to form the hydrophobic drug-binding cavity and three amino acid residues (Ile166, Asp37, and Tyr82) of hFKBP12 that appear to form hydrogen bonds with FK506 (30, 31). These seven amino acids are conserved in all FKBP that have been identified to date. Sequence alignment of hFKBP12 with the four PPIase domains of FKBP65 reveals that Domains I–III of FKBP65 strictly conserve six of these seven amino acid residues and Domain IV conserves five of the seven amino acids (Fig. 1B).

Unlike the ubiquitously expressed FKBP12 identified to date, FKBP65 mRNA shows restricted expression. Northern blot analysis reveals a unique 2.6-kb RNA band in mouse lung, spleen, heart, and brain that hybridizes to a radiolabeled 1.7-kb (ORF) fragment of the FKBP65 clone (Fig. 2). The testis mRNA appears to contain two transcripts, the 2.6-kb band and an additional 3.5-kb band. Although RNA was visualized in all
lanes, by either ethidium bromide staining of the gel prior to blotting or hybridization with an actin probe (data not shown), no FKBP65-specific hybridization in liver RNA was seen.

Biochemical Characterization of FKBP65 in the Murine NIH3T3 Cells— To determine if FKBP65 possessed PPIase activity, recombinant FKBP65 (rFKBP65) was expressed using the pET21d expression vector (20) and purified by RP-HPLC as described under "Experimental Procedures" (Fig. 3, lane 2). The purified rFKBP65 was renatured and utilized in the coupled peptidylprolyl cis-trans-isomerase assay developed by Fischer et al. (32). The PPIase assay measures the cis to trans isomerization of the prolyl peptide bond in the peptide, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Chymotrypsin readily cleaves the trans form of the peptide, releasing p-nitroanilide, which is quantitated spectrophotometrically (Fig. 3B). The initial burst observed in the progress curves is a result of the large percentage of the peptide that exists in the trans conformation at ambient temperature. When rFKBP65 is preincubated with the peptide substrate (Fig. 3B, curve a), the rate of cis to trans isomerization is significantly higher than the chymotrypsin control (Fig. 3B, curve e). Recombinant FKBP65 appears to be fully active, and we have calculated the $k_{cat}/K_m$ of rFKBP65 (described under "Experimental Procedures") for the cis-trans isomerization of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide of $0.64 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, which is comparable to the PPIase activity of the other FKBP5s (21, 22). Additionally, the PPIase activity of rFKBP65 is specifically inhibited by the addition of FK506 in a dose-dependent manner (Fig. 3B, curves b–d). Since the assay is not under pure Michaelis-Menten conditions, only an $IC_{50}$ rather than a $K_i$ can be obtained. An $IC_{50}$ value of 45 nM was...
FKBP65 is also potently inhibited in a dose-dependent manner by the addition of 27 (curve a) or without (curve c) purified rFKBP65 (16 nM). The PPIase activity of purified rFKBP65 is inhibited in a dose-dependent manner by the addition of 27 (curve b), 54 (curve d), or 200 nM (curve d) FK506.

To detect FKBP65 protein, two polyclonal antisera (Pep 2 and Pep 4) were raised in rabbits against synthetic peptides derived from the predicted sequence of FKBP65, as described under "Experimental Procedures." Although both peptide antisera generated similar results, we used Pep 4 antiserum, raised against the carboxyl terminus of FKBP65 (Fig. 1A, underlined sequence) because it reacted more strongly in immune precipitations and Western blots. Pep 4 antiserum was used to immunoprecipitate total cell lysates of [35S]methionine-labeled NIH3T3 cells. Despite the calculated molecular mass of 64,683 or 60,576 for the NH2-terminal cleaved protein, two prominent bands migrating at approximately 72 and 68 kDa were observed (Fig. 4, lane 2) that were specifically competed when peptide was added during immune precipitation (Fig. 4, lane 3). The discrepancy between apparent and calculated molecular masses is observed with other FKBP family members (9, 10, 12, 14, 16, 33).

The increase in apparent molecular weight may be due to the presence of post-translational modifications such as glycosylation and/or phosphorylation. The predicted amino acid sequence contains seven potential N-glycosylation sites and 14 potential casein kinase II phosphorylation sites. Additionally, FKBP65 contains one cAMP-dependent protein kinase phosphorylation site, one protein kinase C phosphorylation site, and two tyrosine kinase phosphorylation sites. Furthermore, FKBP65 contains nine proline-proline sequences that may alter the structure of the protein. FKBP65 contains one cAMP-dependent protein kinase phosphorylation site, one protein kinase C phosphorylation site, and two tyrosine kinase phosphorylation sites. Furthermore, FKBP65 contains nine proline-proline sequences that may alter the structure of the protein.

To determine if FKBP65 was glycosylated, NIH3T3 cells were labeled with [35S]methionine and immunoprecipitated with recombinant FKBP65. Western blot analysis of Endo-F-treated lysates revealed a single specific band that migrates at an apparent molecular mass of 58 kDa (lane 4) of preimmune serum coupled to either preimmune (lane 1) or with anti-peptide 4 antiserum in the absence (lanes 2 and 3) or presence of competing peptide (lanes 3 and 5) as described under "Experimental Procedures." The discrepancy between apparent and calculated molecular masses is observed with other FKBP family members.

DISCUSSION

We have identified a mouse gene encoding a 65-kDa protein (FKBP65) that shares homology with members of the FK506-binding protein class of immunophilins. In this study, we report the cloning, sequencing, and biochemical characterization of a unique 2636-bp clone isolated from a NIH3T3 fibroblast λ Zap cDNA library. The 2.6-kb FKBP65 clone contains an ORF encoding a protein with a predicted molecular mass of 64,683 daltons; however, the presence of a hydrophobic leader sequence also was observed. These results demonstrate that FKBP65 is a glycoprotein.

The observance of two precipitated bands may reflect the difference between phosphorylated and unphosphorylated forms of FKBP65 or alternatively may represent the product from a different translational start site. There is a second methionine residue 60 amino acids downstream from the first ATG sequence. This second ATG contains a nearly perfect Kozak consensus sequence (26) and may encode a protein with a molecular weight of approximately 57,000; however, typically only a single 2.6-kb transcript is seen (Fig. 2). To determine if FKBP65 also is phosphorylated, NIH3T3 cells were labeled with [32P]orthophosphate followed by immune precipitation with Pep 4 antiserum as described under "Experimental Procedures." The immune precipitates were visualized by autoradiography after SDS-PAGE and electroblotting onto Immobilon™. A specific band at a relative molecular mass of 72,000 (Fig. 5A, lane 2) was observed. The [32P]-labeled blot was allowed to decay prior to Western blot analysis (Fig. 5B), and alignment of the two autoradiographs confirms that the [32P]-labeled protein is FKBP65 and represents the top 72-kDa band observed during immune precipitation.
quence and a predicted cleavage site would result in a mature protein with a molecular mass of 60,576. Amino acid sequence comparison of FKBP65 to other FKBP family members revealed from 26% (human FKBP52) to 46% (hFKBP12) overall sequence homology. Additionally, FKBP65 contains four FK506-binding protein PPIase signature domains. The regions flanking the four PPIase domains retain 30% similarity and appear to be unique as they are not homologous to other FKBP52 or any sequences reported in available data bases. The first three domains of FKBP65 conserve six of the seven amino acid residues reported to be involved with FK506-binding proteinase. Historically, FKBP12-like PPIase domains have been shown to associate with the ryanodine receptor (40), and, recently, the inositol 1,4,5-trisphosphate receptor (41), both of which mediate calcium release processes. The predicted amino acid sequence of FKBP65 reveals a potential ER retention sequence and a possible membrane-spanning sequence, suggesting localization to the ER. Using various cellular fractionation protocols, we have localized FKBP65 to the membrane and cytosolic fractions; however, due to the presence of N-linked oligosaccharides, the appearance of FKBP65 in the cytosolic fraction may be a result of inadequate fractionation and/or contamination from the ER fractions.

The presence of PPIase domains of FKBP65 suggest that they may function to interact with more than one molecule within a complex of proteins or act stoichiometrically with the same molecule. We are currently investigating proteins that associate with FKBP65, using affinity-purified protein and NIH3T3 lysates with or without FK506 or rapamycin treatment. To identify the possible functions of FKBP65 and associated proteins, we are currently generating FKBP65 mutants to assess the functional roles of the four PPIase domains. Using the Pep 4 polyclonal antibody, immunoreactive FKBP65 also has been detected by immunoprecipitation and Western blotting techniques in human cell lines, and we are currently in the process of isolating the human homologue of FKBP65. This will allow precise comparisons of the various human FK506-binding proteins and possible detection of additional genes related to FKBP65.

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Fig. 5. **FKBP65 is a phosphoprotein.** A, total cellular lysates of NIH3T3 cells labeled with [32P]orthophosphate were precipitated with either preimmune antiserum (lane 1) coupled to protein A-Sepharose or with Pep 4 antiserum in the absence (lane 2) or presence of competing peptide (lane 3), separated on SDS-PAGE, and electrophoretically onto Immobilon-P™ as described under “Experimental Procedures.” B, Western blot analysis of the filter in A.
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