The 13-kD FK506 Binding Protein, FKBP13, Interacts with a Novel Homologue of the Erythrocyte Membrane Cytoskeletal Protein 4.1

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Abstract. We have identified a novel generally expressed homologue of the erythrocyte membrane cytoskeletal protein 4.1, named 4.1G, based on the interaction of its COOH-terminal domain (CTD) with the immunophilin FKBP13. The 129-amino acid peptide, designated 4.1G–CTD, is the first known physiologic binding target of FKBP13. FKBP13 is a 13-kD protein originally identified by its high affinity binding to the immunosuppressant drugs FK506 and rapamycin (Jin, Y., M.W. Albers, W.S. Lane, B.E. Bierer, and S.J. Burakoff. 1991. Proc. Natl. Acad. Sci. USA. 88:6677–6681); it is a membrane-associated protein thought to function as an ER chaperone (Bush, K.T., B.A. Henrickson, and S.K. Nigam. 1994. Biochem. J. [Tokyo]. 303:705–708). We report the specific association of FKBP13 with 4.1G–CTD based on yeast two-hybrid, in vitro binding and coimmunoprecipitation experiments. The histidyl-proline moiety of 4.1G–CTD is required for FKBP13 binding, as indicated by yeast experiments with truncated and mutated 4.1G–CTD constructs. In situ hybridization studies reveal cellular colocalizations for FKBP13 and 4.1G–CTD throughout the body during development, supporting a physiologic role for the interaction. Interestingly, FKBP13 cofractionates with the red blood cell homologue of 4.1 (4.1R) in ghosts, inside-out vesicles, and Triton shell preparations. The identification of FKBP13 in erythrocytes, which lack ER, suggests that FKBP13 may additionally function as a component of membrane cytoskeletal scaffolds.

The immunophilins comprise two classes of protein receptors for the immunosuppressive drugs cyclosporin A, FK506, and rapamycin (28). Whereas cyclosporin A interacts with a family of proteins designated cyclophilins (31), FK506 and rapamycin bind to a growing family of FK506 binding proteins (FKBPs). A common feature of immunophilins is their peptidyl–prolyl isomerase activity (33). FKBP12 was the first member of the FKBP family to be characterized (32). In the presence of FK506, FKBP12 binds to calcineurin and inhibits its phosphatase activity, ultimately leading to T-cell immunosuppression (26, 42, 43). FKBP12 associates physiologically with two intracellular calcium channels, the ryanodine receptor (36) and the inositol 1,4,5-trisphosphate (IP_3) receptor (36) and the inositol 1,4,5-trisphosphate (IP_3) receptor (11), and regulates their ability to flux calcium. FKBP12 also interacts with type I TGFβ receptors and can inhibit the signaling pathways of TGFβ ligands (57, 58).

FKBP13 was the second member of the FKBP family to be cloned and shares 43% amino acid identity with FKBP12 (37). Whereas FKBP12 is cytosolic, FKBP13 is a membrane-associated protein, possessing an NH_2-terminal signal sequence and COOH-terminal RTEL motif, which are believed to play roles in protein targeting and ER retention, respectively (37). FKBP13 has been identified in rough microsomal subcellular fractions and is enriched in samples containing ER luminal proteins (46). Expression of FKBP13 is upregulated in response to heat shock...
and the accumulation of unfolded proteins in the ER (8, 48). Taken together, these data suggested that FKBP13 may function as an ER molecular chaperone, which binds to and catalyzes the folding and/or assembly of proteins in the ER.

To further elucidate the physiologic role(s) of FKBP13, we sought to identify potential FKBP13 binding targets using the yeast two-hybrid method. We report that FKBP13 associates with the COOH-terminal domain (CTD) of a novel homologue of the red blood cell (RBC) protein 4.1 (4.1R). We have designated the new gene product 4.1G because of its general, widespread distribution compared to 4.1R, which is enriched in hemapoietic tissues and discrete neuronal populations (Walensky, L.D., Z.T. Shi, S. Blackshaw, A.C. DeVries, G.E. Demas, P. Gascard, R.J. Nelson, J.G. Conboy, E.M. Rubin, S.H. Snyder, and N. Mohandas. 1997. Mol. Biol. Cell. (Suppl.) 8:275a). 4.1R is a critical structural component of the erythrocyte membrane cytoskeleton (15). The 135- and 80-kD isoforms of 4.1R are generated by the use of alternate start codons in the coding sequence; many additional isoforms are produced by complex alternative exon splicing (16, 17, 35). The spectrin–actin binding domain (SABD) of 4.1R potentiates the interactions of spectrin tetramers with F-actin in the RBC cytoskeleton (20). 4.1R also provides a linkage between the cytoskeletal scaffold and the plasma membrane through interactions of its membrane-binding domain (MBD) with band 3 and glycophorin C (2, 49). The role of the CTD of 4.1R is unknown. 4.1G is the closest homologue to 4.1R within the 4.1 superfamily whose members contain homologous MBDDs. In addition to alternative splicing of 4.1R (4, 17), 4.1G contributes to the protein 4.1 diversity observed by immunologic methods in a wide range of tissues (19, 24, 30, 54).

**Materials and Methods**

**FKBP13 Yeast Two-hybrid Screen**

A two-hybrid screening was conducted using the Y190 yeast strain containing the HIS3 and β-galactosidase (β-gal) reporter genes and the pPC86 and pPC97 expression vectors (12). The cDNA encoding rat FKBP13 was subcloned into the pPC97 vector (encoding the GAL4 DNA–binding domain [GAL4 DB]) and then used as bait to screen a rat hippocampal library using the lithium acetate/PEG method, restreaked onto leu2 plates, and then DNA sequenced. The plasmid encoded a 129-amino acid (aa) peptide with sequence homology to the CTD of 4.1R, as determined by searching the National Center for Biotechnology Information sequence databases (Bethesda, MD) using the BLAST network service. The sequence alignment was generated using the Geneworks program (Oxford Molecular Group, Oxford, UK).

pPC97 containing the FKBP13 target (rat 4.1G[r4.1G–CTD], fos), pPC12, and pPC86 containing jun, were generated for control experiments. Vectors lacking inserts were likewise used to assess nonspecific lacZ activation by r4.1G–CTD with the GAL4 binding or activating domains alone. The yeast were cotransformed with pPC97 and pPC86 using the lithium acetate/PEG method, restreaked onto leu2 tryp2 plates, and then assayed for β-gal activity using the nitrocellulose lift assay. To determine whether the interaction between FKBP13 and its target was sensitive to FK506, the double transformants were plated on leu2 tryp2 plates containing vehicle (ethanol), 1-, 10-, and 50-μM FK506. Fos-jun double transformants were used as a positive control in these experiments.

**Southern Analysis of 4.1G–CTD**

Genomic DNA prepared from rat tail was digested with 20 U BamH1/µg DNA. The DNA was subjected to 0.7% agarose gel electrophoresis and then stained with ethidium bromide. The gel was then depurinated with 0.2 N HCl for 10 min, denatured with 0.5 N NaOH/1.5 M NaCl for 45 min, neutralized with 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, 0.1% SDS, and then subjected to a short (5 min) and subsequent transferred and cross-linked to Hybond nylon membrane (Amer sham Corp., Arlington Heights, IL). 32P-labeled probes corresponding to r4.1G–CTD and the human 4.1R (h4.1R–CTD) were generated using the Multikite primer (Amersham Corp.) according to the manufacturer's protocol. After prehybridizing for 4 h at 65°C in nylon wash buffer (14% SDS, 12% Dextran sulfate, 2× SSC, 0.1% SDS, and 0.2% Triton X-100), the blots were incubated overnight at 65°C with 5.0 × 106 cpm/mL probe in 0.75× wash buffer. The membranes were subsequently washed at 65°C in 0.5× wash buffer for 2 min, 0.3× wash buffer for 30 min, and 0.2× wash buffer for 20 min and then exposed to film for 2 d at −70°C.

**Identification of the Mouse 4.1G cDNA**

To identify the full-length cDNA containing the 387-bp 4.1G COOH-terminal sequence obtained from the yeast screen, degenerate primers were designed against 5’ 4.1R sequences conserved across species and 3’ r4.1G sequence and used in reverse transcriptase (RT)-PCR experiments using mouse brain cDNA as template. PCR was conducted with high-fidelity pfu polymerase (Stratagene, La Jolla, CA). The degenerate primers made against the CVEEEHHT (5’-TGGTTG[A/G]GA[A/G]CATCACACGTT) motif of the 4.1R MBD and the QHPDM (3’-CAT-[A/G]CTT[A/G]GG[G/ A]TGCTG) motif of the r4.1G CTD yielded a product of 965 bp. To obtain 5’ mouse 4.1G (m4.1G) sequence, the dbest database was searched using human 4.1G (h4.1G) as the query sequence (47). Mouse expressed sequence tag (EST) clone with EMBL/GenBank/DDBJ accession number AA218250 was identified and sequenced; the 5’ overlapping with the 3’ end of the 1652-bp clone contained the CVEEEHHTYRVLSSPPKTFKLTSGLK motif that overlapped with the 5’ end of the original PCR product. Sequencing of additional mouse EST clones (Genome Systems Inc., St. Louis, MO) identified exact match sequence to the PCR product and EST clone AA218250. A full-length cDNA was assembled using the following sequence data: AA218250 (bp 1–1525), WSS304 (1001–1533), W17544 (1162–1526), PCR product (1441–2907), AA220495 (1807–2173), AA030412 (2554–2964), and AA009193 (2575–2964). To confirm the full-length mouse 4.1G sequence, two pairs of nondegenerate primers were used in PCR experiments to identify overlapping products that covered the full-length cDNA. The primers were as follows: (Pair 1) 5’-5MTTEVEG: ATGACTAAGTAATGGGC and 3’-RVTPLP: AGGCCAGGGTGTACCG; (Pair 2) 5’-CVEEHHHT: GTGTTGGAACATCACACT and 3’-AEEGGG: GCGGAGGGAGGAGGAAGGAAGA. Primers to the extreme 5’ and 3’ sequence preferentially generated shorter PCR products that represented 4.1G splice forms, and for this reason the above PCR strategy was used instead. Products of 1977 and 1287 bp were generated (in addition to smaller products determined to be splice forms) from primer pairs 1 and 2, respectively, using both mouse brain cDNA and a mouse brain cDNA library (Stratagene) as templates. All EST clones and PCR products were double-strand sequenced using the fluorescent terminator method of cycle sequencing on an automated DNA sequencer (model 373a; Applied Biosystems, Inc., Foster City, CA) at the DNA Analysis Facility of the Johns Hopkins University (45, 55). Oligonucleotides used for sequencing were generated by a synthesizer.
FKBP13 extract was preincubated with 50 ng of the NH2-terminal signal sequence of FKBP13 was omitted in each case. Preincubation with 50 ng of the GST protein extract diluted in the same buffer up to 500 μL. After rotating the tubes for 20 min at room temperature, the glutathione beads were washed three times in 1 mL of buffer B and then resuspended in 480 μL of the same buffer. 20 μL of the T7 his fusion protein lysate was added and the tubes were rotated for 1 h at 4°C. In FK506 inhibition experiments, the T7 his-FKBP13 extract was preincubated with 50 μM FK506 for 15 min at room temperature and control T7 his-FKBP13 extract was incubated with vehicle (1% ethanol). The beads were then washed five times with 500 μL of 50 mM Hepes, pH 7.4, 400 mM NaCl, 0.1% Triton X-100 (Tx-100), resuspended in PBS/protein load buffer, and subjected to electrophoresis using 18% Tris-glycine minigels (Novex). Proteins were wet transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA), blocked for 2 h in 5% nonfat dry milk, and incubated with 1:5,000 rabbit anti-FKBP (Santa Cruz Biotechnolog, Inc., Santa Cruz, CA) and 1:5,000 mouse anti-T7 (Novagen Inc.) primary antibodies for 1 h at room temperature. Blots were washed in 5% milk one time for 15 min and two times for 5 min, followed by a 1-h incubation at room temperature. 100 μL of a 50% protein A–agarose slurry (Oncogene Science Inc., Cambridge, MA) was then added followed by a 1-h incubation at 4°C. The matrix was washed by incubation at 4°C for 1 h. The matrix was washed by incubation for 10 min at 4°C. Then, FKBP13 was eluted with 100 mM glycine, pH 2.5, and 100 mM triethylamine, pH 11.5, and dialyzed against PBS and PBS/40% glycerol for storage. Antibody specificity was evaluated by Western analysis using brain extracts prepared by homogenizing whole rat brain in ice-cold lysis buffer C containing 1:5,000 anti-rabbit and 1:5,000 anti-mouse antibodies for 1 h at 4°C. The extract was protein assayed using DC reagents (Bio-Rad Laboratories) and 5 μg of protein per lane was electrophoresed on an 18% Tris-glycine polyacryl- amide gel. A silver-stained lane containing FKBP1s purified from whole brain on an FK506 column (see below) served as FKBP molecular weight markers. Western analysis was conducted as described above. Anti-FKBP12 and -FKBP13 antibodies were diluted 1:250 in 3% BSA/PBS. Blocking experiments were conducted by preadsorbing the antibodies with purified FKBP fusion protein overnight at 4°C.

FK506 Column Synthesis
FK506 was chemically derivatized and coupled to affigel-10 (Bio-Rad Laboratories) as previously described (25). FK506 was a gift of S. Hase- moto (Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Tsukuba, Japan).

Red Blood Cell Preparations
Sprague Dawley rat RBCs and ghosts were isolated according to established procedures (5). The cytoblast was obtained by hypotonic lysis of purified RBCs and subsequently treated with chloroform and water extraction to remove the hemoglobin (38). Ghosts were solubilized in lysis buffer C (see above). The RBC fractions were protein assayed using DC reagents (Bio-Rad Laboratories), and 20 μg of each were analyzed by gel electrophoresis on 18% Tris-glycine polyacrylamide gels, with transferred to PVDF, and then probed with anti-FKBP12 and -FKBP13 antibodies as described above. 10 μg of brain extract (prepared as above) served as positive controls. The solubilized ghosts were also incubated with the FK506 matrix to concentrate ghost-associated FKBP. 200 μL of solubilized rat ghosts were incubated with 30 μL of a 50% FK506 matrix slurry preincu- bated with 50 mM Trizma, pH 7.4, 100 mM NaCl, 0.1% TX-100 and brought to a final volume of 500 μL with this buffer. Specific binding to the matrix was assessed by adding 100 μM of free FK506 to control samples. The final vehicle (ethanol) concentration in each tube was 2.0%. After rotating the samples for 2 h at 4°C, the matrix was washed by incubating for 10 min at 4°C. The matrix was washed by incubating for 10 min at 4°C. Then, FK506 was centrifuged at 39,000 g for 20 min at 4°C. A silver-stained lane containing FKBP1s purified from whole brain on an FK506 column (see below) served as FKBP molecular weight markers. Western analysis was conducted as described above. Anti-FKBP12 and -FKBP13 antibodies were diluted 1:250 in 3% BSA/PBS. Blocking experiments were conducted by preadsorbing the antibodies with purified FKBP fusion protein overnight at 4°C.

Mutational Analysis
Mutation analysis was performed by PCR and subcloned into pUC18 using SalI and NotI restriction sites. Pr(108) to alanine (ala), his(107) to leu, and his(107) to arginine (arg) point mutations were constructed by the overlap extension method (34). GAL4(TA)–r4.1G–CTD constructs were cotransformed into Y190 yeast with GAL4(DB)- FKBP13 as described above. Double transformants were restreaked onto leu” trp” plates and assayed for β-gal activity using the nitrocellulose lift filter assay.

FKBP Antibodies
cDNAs encoding FKBP13 (without the NH2-terminal signal sequence) and FKBP12 were subcloned into the pet22b expression vector (Novagen Inc.). E. coli BL21 (DE3) bacteria (Novagen Inc.) were transformed and the fusion proteins expressed and purified over nickel columns (Novagen Inc.) according to the manufacturer’s protocol. New Zealand white rabbits were immunized with the FKBP antigens according to established protocols (Hazleton Labs, Denver, PA) except that alternating injections consisted of FKBP/45-nm colloidal gold (E.Y. Laboratories, Inc., San Mateo, CA) conjugates to increase the immunologic response (50). Production bleeds were affinity purified by first passing the serum over affigel-10 (Bio-Rad Laboratories, Hercules, CA) columns containing pet22b fusion protein lacking the FKBP inserts. Flowthroughs were then passed over the respective FKBP affigel-10 columns. After extensive washing with 10 mM Tris, pH 7.5, and 10 mM Tris, pH 7.5, 500 mM NaCl, the antibodies were eluted with 100 mM glycine, pH 2.5, and 100 mM triethylamine, pH 11.5, and dialyzed against PBS and PBS/40% glycerol for storage. Antibody specificity was evaluated by Western analysis using brain extracts prepared by homogenizing whole rat brain in ice-cold lysis buffer C containing 100 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, protease inhibitors (as above), followed by centrifugation at 39,000 g for 20 min at 4°C. The extract was protein assayed using DC reagents (Bio-Rad Laboratories) and 5 μg of protein per lane was electrophoresed on an 18% Tris-glycine polyacrylamid-
onto a column of equal volume containing 3:1 alpha-cellulose (Sigma Chemical Co.) to Siggmacell cellulose type 30 (Sigma Chemical Co.) pre-equilibrated in PBS. Blood was collected by gravity flow and washed with PBS three times. Ghosts were obtained by lysing purified RBCs in 30 vol of hypotonic lysis buffer (ice-cold 10 mM sodium phosphate, pH 7.4) for 5 min on ice. After centrifugation for 10 min at 4°C (SS34 rotor, 16,000 rpm; Sorvall Instruments Division, Dubport, Co., Newton, CT), the pelleted ghosts were washed in 30 vol of hypotonic lysis buffer until they became white. Inside-out vesicles (IOVs) and stripped IOVs were prepared as previously described (29). Briefly, ghosts were incubated in 30 vol 0.1 mM EGTA, pH 8.5, at 37°C for 30 min. The resulting IOVs were centrifuged for 20 min at 4°C, washed once with hypotonic lysis buffer, and then resuspended in the same buffer. Stripped IOVs were prepared by incubating IOVs in 30 vol of 0.1 mM EGTA, pH 11, at 25°C for 20 min, followed by centrifugation and washing as described for IOVs. Triton shells were generated by incubating ghosts in 20 vol of 625 mM NaCl/6.25 mM sodium phosphate, pH 7.0, 0.625 mM EGTA, 0.625 mM DTT, 2.0% Tx-100 for 30 min at 4°C (23). The resulting Triton shells were centrifuged and washed as described for IOVs. 20 μg of each fraction was electrophoresed on both 12 and 18% tris-glycine gels and then subjected to Western analysis using the FKBP13 antibody and anti-24-1, an affinity-purified anti-4.1R peptide antibody raised against the TRDYPIVHTETKITYEAQQ motif of the CTD (18).

In Situ Hybridization

In situ hybridization using digoxigenin-labeled probes was conducted using FKBP13 and r4.1G-CTD coding sequence. Frozen 20-mm cryostat sections of 18-d-old rat embryos and 4-d-old mouse newborns were cut onto Superfrost Plus slides (Fisher Scientific Co., Pittsburgh, PA), allowed to air dry for 1–3 h, and then postfixed for 2 h at room temperature (80°C). Sections were prehybridized for 2 h at 65°C in 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0, washed three times for 3 min in TBS, and then prehybridized for 2 h at room temperature in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt’s solution, 500 mg/ml sonicated herring sperm DNA, 250 mg/ml yeast tRNA). The tissue was incubated with 0.1 mL of buffer D containing 40 ng of cRNA probe under a siliconized coverslip at 65°C overnight. After removal of the coverslips in 5× SSC at 65°C, sections were washed as follows: two times for 1 h in 0.2× SSC, one time for 5 min in 0.2× SSC, and one time for 5 min in TBS. After a 1-h block at room temperature in 4% normal goat serum (NGS)/TBS, the slides were incubated overnight at 4°C in 1:5,000 dilution of anti-digoxigenin Fab fragment (Boehringer Mannheim Corp.) in 4% NGS/TBS. Sections were then washed three times for 5 min in TBS and one time for 5 min in buffer D (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 50 mM MgCl2). The color signal was developed in buffer D containing 3.375 mg/ml nitro blue tetrazolium (Boehringer Mannheim Corp.), 3.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Corp.), and 0.24 mg/ml levamisole. The color reaction was carried out in the dark for 24–72 h at room temperature and terminated with a TE wash. The developed slides were coverslipped with Aquapolymount (Polysciences, Inc., Warrington, PA). Control sections were hybridized with identical quantities of sense cRNA and no signal was observed. The in situ hybridization protocol has been used to distinguish the localizations of transcripts sharing as much as 85–90% nucleic acid identity (7, 56).

Results

Identification of a Novel 4.1 Homologue Whose CTD Interacts with FKBP13

Using the yeast two-hybrid method, we screened 2,000,000 rat hippocampal cDNA library transformants with FKBP13 as bait. Whereas 27 colonies were his positive, a single colony was both his and β-gal positive (Fig. 1A). The deduced aa sequence of the plasmid derived from the β-gal positive colony is 129 aa in length and is 71% identical to the corresponding portion of the 4.1R-CTD encoded by exons 18–21 of the 4.1R gene (14, 35) (Fig. 1 B). No β-gal activity was observed in control experiments in which yeast were doubly transformed with combinations of FKBP13 or 4.1G-CTD and the GAL4(BD) alone, the GAL4(TA) alone, and fos and jun constructs (Fig. 1 C). Despite the robust interaction of FKBP13 with 4.1G-CTD by β-gal filter assay, no activity was observed in parallel

Figure 1. FKBP13 yeast two–hybrid screen. (A) Y190 yeast were transformed with a rat hippocampal library and 2 × 10⁶ transformants were screened for his and β-gal activity. Whereas 27 colonies were his positive, a single colony was both his and β-gal positive. (B) The deduced aa sequence of the plasmid derived from the β-gal–positive colony is 129 aa in length and is 71% identical to the corresponding region of the 4.1R–CTD encoded by exons 18–21 of the 4.1R gene. (The domains of 4.1R were originally defined by chymotryptic digestion [40], and thus, the CTD of 4.1R technically begins 11 aa upstream of exon 18; in this study, 4.1G–CTD refers to the 129-aa COOH-terminal peptide identified by yeast two-hybrid experiments.) Amino acid identities shared by r4.1G, m4.1R, and h4.1R are boxed and shaded, whereas conservative aa substitutions are shaded. (C) Control experiments were performed using yeast doubly transformed with combinations of FKBP13 or 4.1G–CTD and the GAL4(BD) alone, the GAL4(TA) alone, and fos and jun constructs. A potential interaction between 4.1G–CTD and FKBP12 was also evaluated. Time (t) to positive β-gal signal in hours: ++ + ++ = t < 0.25; + + + = 0.25 < t ≤ 1; + + = 1 < t ≤ 6; + = 6 < t ≤ 12; = negative assay.
two-hybrid experiments performed with 4.1G–CTD and FKBP12.

To determine if 4.1G–CTD is encoded by a distinct gene from 4.1R, Southern analysis was performed using rat genomic DNA digested with BamH1 and then incubated with 4.1R– and 4.1G–CTD probes (Fig. 2). Although the 4.1R–CTD probe hybridized with two bands of 9.0 and 6.5 kb, the 4.1G–CTD probe detected two bands of 9.8 and 5.6 kb. This finding indicates that 4.1G–CTD represents a distinct gene and thus, is a novel member of the 4.1R superfamily.

The complete m4.1G cDNA was obtained by sequencing overlapping EST clones and RT-PCR products (Fig. 3A). The sequence was confirmed by high fidelity RT-PCR using two different cDNA sources and two nondegenerate primer pairs that generated overlapping products covering the complete cDNA (Fig. 3A). The starting methionine was assigned on the following basis: (a) it is preceded by an in frame stop codon, (b) it is located in the context of a Kozak translation initiation sequence (−6 to +4 = GTAACCATGA), and (c) it encodes the MTTE motif that initiates the 135-kD isoform of 4.1R (14, 35). m4.1G is a 988-aa protein with a predicted mol wt of 110 kDa, and is 53% identical to m4.1R (Fig. 3B). m4.1G sequences corresponding to the MBD, SABD, and CTD of m4.1R are

Figure 2. CTDs of 4.1G and 4.1R are encoded by distinct genes. Southern analysis of BamH1-digested rat genomic DNA yielded bands of 9.0 and 6.5 kb when hybridized with the 4.1R–CTD probe, and bands of 9.8 and 5.6 kb when incubated with the 4.1G–CTD probe.

Figure 3. Identification and aa sequence of m4.1G. (A) The m4.1G cDNA was identified by double-strand sequencing of overlapping EST clones and RT-PCR products. The schematic demonstrates how the m4.1G cDNA was assembled: (from top left) AA218250 (1–1521), W83204 (1030–1533), W17544 (1162–1528), PCR 1 (1441–2907), AA220495 (1807–2173), AA030412 (2554–2964), AA009193 (2575–2964). Whereas EST clone AA218250 extends into the 5′ untranslated region (left gray arrowhead), clones AA030412 and AA009193 extend into the 3′ untranslated region (right gray arrowhead). The sequence was confirmed by high-fidelity RT-PCR using two different cDNA sources and two nondegenerate primer pairs that generated overlapping products covering the complete cDNA (PCR 2.3: 1–1977 and PCR 4.5: 1441–2964). The corresponding location of the rat peptide identified in the yeast two-hybrid screen (4.1G–CTD) is also indicated. (B) The m4.1G cDNA encodes a protein of 988 aa with a predicted molecular weight of 110 kD. An aa-alignment of m4.1G and m4.1R highlights the identical sequence shared by the two proteins (boxed and shaded portions) and residues with conservative aa changes (shaded portions). m4.1G is 53% identical to m4.1R at the aa level, with 70, 68, and 64% aa identities in the MBD (black arrows), SABD (gray arrows), and CTD (striped arrows), respectively. The location of 4.1G–CTD is marked by an asterisk and occurs 30 aa beyond the start of the 4.1R–CTD, whose boundaries were originally defined by chymotryptic digests of 4.1R (40). m4.1G and m4.1R sequences diverge at the NH2 termini and in regions separating the defined domains. Whereas there is increased m4.1R sequence between the MBD and SABD, the region between the SABD and CTD is expanded in m4.1G. m4.1G sequence data is available from EMBL/GenBank/DDBJ accession number AF044312.
highly conserved, with aa identities of 70, 68, and 64%, respectively. Discrete spans of divergence are located at the NH₂ termini (16% identity) and in the regions separating the defined domains. In addition, m4.1G exhibits 80% aa identity to the human 4.1G (h4.1G) isolated concurrently in our laboratories in a study of nonerythroid 4.1 species (47). Sequencing of PCR products and EST clones further revealed that, like 4.1R, 4.1G has multiple splice forms (data not shown).

Specificity of the FKBP13/4.1G–CTD Interaction
Whereas FK506 stimulates the binding of FKBP12 to calcineurin (42), stoichiometric quantities of FK506 dissociate FKBP12 from the ryanodine and IP₃ receptors (11, 36). Sequence motifs in the calcium channels are believed to mimic the structure of FK506, and thus compete with FK506 at the FKBP12 binding pocket (9). If FK506 and FKBP12 are peptidyl-prolyl isomerases that bind FK506 and rapamycin with differing affinities (27). Because 4.1G–CTD binds to FKBP13 but not to FKBP12, we were interested in identifying the aa sequence that interacts with FKBP13 to shed light on the differing specificities of FKBP12 and FKBP13. Leu-pro and val-pro residues are optimal substrates for FKBP12 rotamase activity.

Figure 4. Specificity of the FKBP13/4.1G–CTD interaction. (A) FKBP13 and 4.1G–CTD double transformants were grown on plates containing 0, 1, 10, and 50 μM FK506. Fos and jun double transformants served as a positive control. The FKBP13/4.1G–CTD interaction, as assayed by β-gal filter assay, was inhibited by FK506 in a dose-dependent manner with complete blockage at 50 μM; the fos–jun interaction was unaffected by FK506. Time (t) to positive β-gal signal in hours: ++++ = t < 0.25; +++ = 0.25 < t ≤ 1; ++ = 1 < t ≤ 6; + = 6 < t ≤ 12; − = negative assay. (B) In vitro binding experiments were conducted by incubating T7 his–FKBP13 lysate with a GST–4.1G–CTD matrix (left), T7 his–FKBP12 lysate with a GST–4.1G–CTD matrix (middle), and T7 his–4.1G–CTD lysate with a GST–FKBP13 matrix (right). Whereas T7 his–FKBP13 bound to the GST–4.1G–CTD matrix, T7 his–FKBP12 did not. The T7 his–FKBP13 binding was blocked by 50 μM FK506. FKBP13/4.1G–CTD binding also occurred when the fusions were switched (right). (C) (Top) HEK293 cells were transfected with NH₂-terminal c-myc–tagged 4.1G–CTD with or without COOH-terminal HA-tagged FKBP13, and then lysates were subjected to Western analysis using a mixture of anti-HA and anti-c-myc monoclonal antibodies (top). (Bottom) Incubation of the lysates with mouse monoclonal HA antibody resulted in coimmunoprecipitation of c-myc–4.1G–CTD (arrow) with HA–FKBP13. c-myc–4.1G–CTD is not detected when anti-HA immunoprecipitation is conducted using lysates containing c-myc–4.1G–CTD alone. The arrowhead indicates the band corresponding to light chain IgG (l-IgG). Western blot exposures were kept short (5–20 s) so that the immunoprecipitated band could be resolved from the robust l-IgG band.
in vitro (1); FK506 binds with high affinity to FKBP12 and inhibits its rotamase activity by mimicking the transition state of peptidyl-prolyl isomerization. Leu-pro 1400–1401 of the IP3 receptor has recently been shown to mediate the constitutive binding of FKBP12 to the calcium channel and presumably has structural characteristics similar to FK506 (9).

4.1G–CTD contains three peptidyl-prolyl bonds that could potentially participate in FKBP13 binding, including val-pro at aa 18–19, ser-pro at 32–33, and his-pro at 107–108. We used yeast two–hybrid assays to determine which residues are required for the FKBP13 interaction. Yeast were doubly transformed with FKBP13 and truncations of 4.1G–CTD corresponding to aa 1–67 and 35–129. Only the transformants containing the 35–129 aa-construct exhibited β-gal activity, which occurred with the same time course as yeast transformed with the complete 4.1G–CTD (Fig. 5). The inability of the 1–67 aa construct to produce β-gal activity, eliminated val-pro and ser-pro as FKBP13 targets. To determine if the his-pro was responsible for FKBP13 binding, a 4.1G–CTD construct containing aa 1–106 that lacks this moiety and two constructs containing pro 108 to ala mutations (1–129:P108A and 35–129:P108A) were evaluated. These modifications completely abolished the FKBP13 interaction measured in yeast two-hybrid assays, suggesting that pro 108 of 4.1G–CTD is important for FKBP13 binding. The finding that a pro preceded by his is involved in the interaction (rather than a leu- or val-pro), prompted us to assess whether his 107 is required for binding. 4.1G–CTD constructs containing his 107 to leu (1–129: H107L) and his 107 to arg (1–129:H107R) mutations both failed to promote the FKBP13/4.1G–CTD interaction in yeast two–hybrid assays. His-pro 107–108 may confer binding specificity through direct interaction with FKBP13 or by generating/stabilizing a requisite secondary structure.

**Colocalization of FKBP13 and 4.1G–CTD In Vivo**

Whereas protein 4.1 has been identified in a wide range of tissues by immunologic methods (19, 24, 30, 54), the discovery of a new homologue indicates that protein 4.1 localizations in the body should be redefined in an isotype-specific manner. In situ hybridization studies of E18 rat embryos and 4-d-old newborn mice demonstrated 4.1G–CTD–containing mRNAs in a wide variety of tissues including the central nervous system, olfactory epithelium, toothbuds, skeletal muscle, salivary glands, thymus, thy-

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**Figure 5.** Identification of the FKBP13 binding site. Truncations of 4.1G–CTD were evaluated for interaction with FKBP13 in yeast by β-gal filter assay. A positive interaction using the 35–129-aa construct implicated pro 108 in FKBP13 binding. The requirement of pro 108 was confirmed using a construct truncated immediately upstream of pro 108 (1–106) and two constructs containing pro 108 to ala mutations (1–129:P108A and 35–129:P108A) were evaluated. These modifications completely abolished the FKBP13 interaction measured in yeast two-hybrid assays, suggesting that pro 108 of 4.1G–CTD is important for FKBP13 binding. The finding that a pro preceded by his is involved in the interaction (rather than a leu- or val-pro), prompted us to assess whether his 107 is required for binding. 4.1G–CTD constructs containing his 107 to leu (1–129: H107L) and his 107 to arg (1–129:H107R) mutations both failed to produce a positive β-gal signal. Time (t) to positive β-gal signal in hours: ++++ = t < 0.25; +++ = 0.25 ≤ t < 1; ++ = 1 ≤ t < 6; + = 6 ≤ t < 12; − = negative assay.

**Figure 6.** Colocalization of FKBP13 with 4.1G–CTD in E18 rat embryos. In situ hybridization identified FKBP13 and 4.1G–CTD–containing mRNAs in identical distributions during embryonic development (A and C). Colocalizations identifiable at low power include the brain (Br), spinal cord (Sc), olfactory epithelium (OE), submandibular gland (SG), thymus (Ts), thyroid (Td), skeletal muscle (Sm), lung (Lu), liver (Li), intestines (In), and rectum (Re). No signal is detected, for example, in heart (He) or bladder (Bl). The specific in situ hybridization signals are absent in control sections incubated with FKBP13 and 4.1G–CTD sense cRNAs (B and D). Bar, 2.1 mm.

Walensky et al. **FKBP13 Interacts with a Novel Homologue of Protein 4.1**

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Identification of FKBP13 in RBC Ghost Preparations

We investigated whether FKBP13 occurs in RBCs for the following reasons: (a) the CTDs of 4.1G and 4.1R are highly conserved in the defined FKBP13 binding region; (b) RBCs are one of the most abundant sources of 80-kD 4.1R in the body (16); (c) in situ hybridization studies revealed that 4.1G mRNAs also localize to hematopoietic tissues; and (d) because RBCs lack ER, we could determine if FKBP13 is found outside of the ER in association with the membrane cytoskeleton. Rat RBCs were purified from whole blood and then hemoglobin-free cytosol and ghost fractions were isolated. Whereas 4.1R is not present in the cytosol of RBCs, it is highly enriched in the ghost fraction (5). RBC ghosts are produced by hypotonic lysis of RBCs followed by extensive washing, and represent the intact membrane and cytoskeletal structure of RBCs without cytosolic contents. Affinity-purified polyclonal antibodies that specifically recognize FKBP12 or FKBP13 (Fig. 8A) identified FKBP12 exclusively in the RBC cytosol fraction, and FKBP13 solely in the ghost fraction (Fig. 8B). Brain extracts served as positive controls for Western analysis. To confirm that the protein identified in RBC ghosts has FK506 binding activity, ghosts were solubilized and then incubated with an FK506 matrix in the presence or absence of soluble FK506, which should block binding. Bound proteins were eluted from the matrix with SDS sample buffer, electrophoresed, and then subjected to FKBP12 and FKBP13 Western analysis. The FKBP13 antibody recognized a 13-kD protein from solubilized ghosts that bound to the FK506 matrix and was specifically displaced by free FK506 (Fig. 8C). In contrast, the FKBP12 antibody did not recognize any proteins extracted from the ghost preparation.

We subfractionated RBC ghosts to determine if FKBP13 segregates with 4.1R (Fig. 8D). Western analysis of the preparations was conducted using the FKBP13 antibody (Fig. 8D, bottom panel) and an affinity-purified anti-4.1R peptide antibody (18) (top panel). IOVs that are depleted of spectrin and actin retained 4.1R (80-kD doublet [17]) and FKBP13. IOVs that are then depleted of peripheral membrane proteins by a stripping procedure lose 4.1R and FKBP13. Finally, Triton shells were prepared from RBC ghosts to further enrich for 4.1R and FKBP13. Western analysis of this preparation demonstrated increased amounts of 4.1R. The quantity of FKBP13 in Triton shells was likewise enriched compared to ghosts and IOVs.

It should be noted that these experiments do not completely rule out the possibility that the 13-kD protein identified in RBC ghosts is a distinct immunophilin that is recognized by the FKBP13 antibody and comigrates with brain FKBP13.

Discussion

In the present study we have identified 4.1G–CTD as the first known binding target of FKBP13. 4.1G is a novel, widely expressed homologue of the erythrocyte membrane cytoskeletal protein (4.1R). The FKBP13/4.1G–

Figure 7. Examples of the FKBP13/4.1G–CTD colocalization within specific tissues during development. (A and B) FKBP13 and 4.1G–CTD-containing mRNAs colocalize in the olfactory epithelium (OE), hippocampus (H), and cerebellum (Cb) of 4-d-old mice. High-power images of E18 rat embryos demonstrate colocalizations in skeletal muscle bundles (C and D), crypts of Lieberkuhn in the rectum (E and F), secretory epithelium of the submandibular gland (G and H), and olfactory epithelium (I and J). Bars: (A and B) 1.30 mm; (C, D, G, and H–J) 0.32 mm; (E and F) 0.16 mm.
CTD interaction was discovered using the yeast two-hybrid system and then confirmed by in vitro binding and coimmunoprecipitation studies. Specificity of the association was established by blocking in vitro binding and the yeast interaction with FK506.

The cyclophilin and FKBP immunophilins possess peptidyl-prolyl isomerase activity, which interconverts the cis and trans isomers of peptidyl-prolyl bonds (33, 51). FK506 mimics the transition state of peptidyl-prolyl isomerization, and thus effectively competes with natural substrates for FKBP binding. Interestingly, the binding domains of FKBP12 and FKBP13 for FK506 are virtually superimposable, as determined by X-ray crystallographic studies (53). However, the respective $K_d$ of FK506 and rapamycin are 0.6 and 0.26 nM for FKBP12, and 74 and 3 nM for FKBP13 (27), suggesting that the two proteins may have different substrate specificities in vivo. The differences between the affinities of FKBP12 and FKBP13 for FK506 and rapamycin have been explained by the cumulative effect of multiple small distortions in their protein structures (53). Another distinction between the binding properties of FKBP12 and FKBP13 is exemplified by the ability of FKBP12, but not FKBP13, to form a complex with calcineurin in the presence of FK506. The inability of FKBP13–FK506 to interact with calcineurin is attributed to the distinct gln-50, ala-95, and lys-98 surface residues of FKBP13 (27).

The binding of 4.1G–CTD to FKBP13 but not to FKBP12 underscores the selective nature of immunophilin interactions in vivo. Protein motifs that mimic the transition state of peptidyl-prolyl isomerization are believed to produce high affinity, steady-state interactions with FKBP (9). In this study, we have determined that his-pro 107–108 of 4.1G–CTD is required for FKBP13 binding. The crystallographic structure of the FKBP12–FK506 complex indicates that the leu-like moiety of FK506, the pyranose methyl group region, shares a complementary surface with the side chains of his-87 and ile-91 of FKBP12. The corresponding aa in FKBP13 are ala-95 and lys-98. There is predicted complementarity between the IP3R-leu 1401 and FKBP12–his 87 in the FKBP12–IP3R complex (9); the analogous aliphatic and his residues are reversed in FKBP13 and 4.1G–CTD, suggesting that the 4.1G–CTD–his 107 may interface with FKBP13–ala 95. We speculate that an aliphatic–his complementarity may participate in FKBP-substrate interactions in vivo, conferring FKBP specificity based on the residues preceding pro in the physiologic targets.

A physiologic role for the FKBP13/4.1G–CTD interaction is supported by the striking tissue colocalizations demonstrated throughout the body during development by in situ hybridization. FKBP13 and 4.1G–CTD-containing mRNAs are widely expressed with enrichment in skeletal muscle, salivary glands, and the gastrointestinal, hematopoietic, respiratory, and nervous systems. Within these tissues, the proteins colocalize, for example, in intestinal crypt, salivary gland, and olfactory epithelium. Areas
where expression of both transcripts is negligible include
the heart and smooth muscle tissue of the gastrointestinal
tract, urinary bladder, and blood vessels. Whereas 4.1R
transcripts predominantly localize to hematopoietic tissues
and select neuronal populations (Walensky, L.D., Z.T. Shi,
S. Blackshaw, A.C. DeVries, G.E. Demas, P. Gascard,
R.J. Nelson, J.G. Conboy, E.M. Rubin, S.H. Snyder, and
N. Mohandes. 1997. Mol. Biol. Cell [Suppl.] 8:275a), 4.1G
mRNAs are broadly distributed, indicating a more gener-
alized role for this 4.1 homologue.

The strong homology between 4.1G and 4.1R suggests
that the novel 4.1G gene encodes a membrane cytoskeletal
protein. The primary structure of FKBP13, however, con-
tains a putative signal sequence and a variant of the de-
defined KDEL motif, which may serve to target and retain
FKBP13 in the ER, respectively (37). The identification of
a specific binding interaction between FKBP13 and 4.1G–
CTD, coupled with their mRNA tissue colocalizations,
suggested that FKBP13 may additionally be found outside
of the ER in association with 4.1G in the membrane cyto-
skeleton. Whereas FKBP13 has been identified in rough
microsomal fractions enriched with ER membrane and lu-
mina proteins (46), we were interested in determining if
the membrane cytoskeleton could be a further source of
FKBP13 protein. Thus, we conducted FKBP Western analysis
on purified RBCs because they lack ER and are en-
riched in 4.1R, which shares sequence identity with 4.1G
in the region implicated in FKBP13 binding. FKBP12 (38)
and a 12-kd inositol phosphate-binding protein (22), be-
lieved to be an immunophilin, have previously been identi-
fied in RBCs. Our affinity-purified FKBP antibodies iden-
tified FKBP12 in the cytosol of RBCs and FKBP13 in
RBC ghosts, where 4.1R is located. In addition, FKBP13
can be extracted from solubilized ghosts on the basis of its
affinity for an FK506 matrix. Ghosts stripped of actin and
spectrin retain both FKBP13 and 4.1R, whereas further
treatment to remove peripheral membrane proteins liber-
ates FKBP13 and 4.1R from the vesicles. The Triton shell
preparation that enriches for 4.1R likewise contains in-
creased FKBP13 compared to ghost and IOV samples.

The development of isoform-specific antibodies is re-
duced FKBP13 compared to ghost and IOV samples.
The development of isoform-specific antibodies is re-
quired to determine if 4.1G is found in RBCs and if the
FKBP13 of RBCs interacts with 4.1G and/or 4.1R. These
questions (all three from Johns Hopkins School of Medicine), E. Fung (Stanford Medical School,
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