Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 298-303

ISOLATION OF A HUMAN cDNA ENCODING A 25 kDa FK-506 AND RAPAMYCIN BINDING PROTEIN

Greg Wiederrecht*, Mary M. Martin, Nolan H. Sigal, and John J. Siekierka

Department of Immunology Research
Merck Research Labs
P.O. Box 2000, Rahway, New Jersey 07065

Received April 16, 1992

Summary: Recently, the nearly complete peptide sequence of a 25 kDa rapamycin and FK-506 binding protein that had been isolated from calf thymus, brain, and spleen was reported (1). Based upon the amino acid sequence of this bovine protein, bFKBP25, we have isolated from a JURKAT cDNA library the cDNA encoding the human homolog, hFKBP25. Translation of the open reading frame contained within this cDNA clone yields a sequence that, in its C-terminal half, is 41% identical to the major human FK-506 binding protein, hFKBP12, and 43% identical to hFKBP13. The N-terminal half of hFKBP25 is unrelated to any known protein.

FK-506 is a powerful immunosuppressive drug used clinically to enhance allograft survival in organ transplant patients. FK-506 exerts its effects on calcium-associated events that take place early during T-lymphocyte activation and prevents transcription of a set of early-phase lymphokine genes (2). However, the precise mechanism by which FK-506 inhibits transcription of these genes is not yet understood. Since the first report of an FK-506 receptor in the cytoplasm of JURKAT cells (3), a number of human FK-506 binding proteins, termed hFKBPs, have been purified and cloned (4-8) indicating that there is a family of FK-506 binding proteins in cells. Rather than being lymphocyte-specific, at least two of the FKBPs, the major 12 kDa FK-506 binding protein, FKBP12, as well as FKBP13 have a broad phylogenic distribution and are found in organisms ranging from humans to the yeast, *S. cerevisiae* (9-14). The relative abundance of FKBP12 (0.1-0.4% of total cellular protein) and the conservation of both FKBP12 and FKBP13 suggests that these proteins have important, and as yet unidentified, functions within cells. In addition to being well-conserved at the amino acid level, the FKBPs share at least two other characteristics. First, the known FKBPs all bind another immunosuppressant and a reciprocal antagonist of FK-506, the macrolide antibiotic rapamycin (4,5, 10-14). Although related in structure to FK-506, rapamycin exerts its effects in a manner

*To whom correspondence should be addressed.

0006-291X/92 $4.00

mechanistically distinct from that of FK-506 by inhibiting lymphocyte proliferation in response to growth-promoting lymphokines (15). Second, the known FKBPs all catalyze the *cis*-to-*trans* isomerization of peptidyl-prolyl bonds in peptides and proteins (3,4, 10-14). This PPIase activity is inhibited by both FK-506 and rapamycin. No physiological substrate for the PPIase activities of the FKBPs has yet been identified. Furthermore, there is no evidence to suggest that the PPIase activity of the FKBPs is required for T-lymphocyte activation (16,17).

In order to understand the mechanistic basis for the immunosuppresive effects of FK-506 and rapamycin in humans, we have been purifying, characterizing, and cloning the relevant receptors. Recently, a nearly complete amino acid sequence compiled from peptide sequencing of a 25 kDa FK-506 and rapamycin binding protein purified from bovine brain, spleen, and thymus was reported (1). Using the bovine sequence, we designed oligonucleotide probes and cloned the human homolog of the bovine protein from a JURKAT cDNA library. Translation of the open reading frame within this cDNA shows that the C-terminal half of the protein is 41% identical to hFKBP12 and 43% identical to hFKBP13. The N-terminal half of the protein is unrelated to any known protein.

MATERIALS AND METHODS

Generation of a DNA Probe: Restriction site-tailed degenerate oligonucleotides were used in PCR reactions to generate a DNA probe for screening a cDNA library. The sense oligonucleotide specific for the bovine sequence KKDIIK was:

5'-GGGAATTCAA(AG)AA(AG)GA(TC)AT(ATC)AT(ATC)AA-3'.

The antisense oligonucleotide specific for the bovine sequence IEPEWA was:

5'-GGGGATCCA(TC)TC(TCAG)GG(TC)TC(AGT)AT(TC)T-3'.

The PCR conditions for screening phage libraries directly have been described (18). To probe the products of the PCR reaction on a Southern blot, a degenerate, inosine-containing oligonucleotide specific for the bovine sequence IGKVIRGWDEA was used. The sequence of that oligonucleotide was:

5'-GC(TC)TC(AG)TCCCAICCIC(GT)IATIAC(TC)TTICCIAT-3'.

5' end-labeling of the oligonucleotide probe, prehybridization, hybridization, and washing of the Southern blot was performed exactly as described (19). PCR products of the appropriate size were subcloned into pUC19. Screening of bacterial transformants was performed essentially as described (20) except that prehybridization, hybridization, and washing of the filters were the same as those conditions used for the Southern blot.

Screening of the Phage Library: Plasmid was purified from one of the positive colonies and the insert, encoding part of the human FKBP25 cDNA, was excised from pUC19 with EcoRI and BamHI and gel purified. The insert was labeled with α-[^{32}P]-dATP by random priming (Stratagene) and used to screen a JURKAT cDNA library in λgt10.

Sequencing the Human FKBP25 cDNA: Inserts from positive plaques were subcloned into the EcoRI site of pUC19. Sequencing was performed by the dideoxy method using the Sequenase Kit from USB.

5' RACEing: The RACE (rapid amplification of cDNA ends) technique was performed essentially as described (21) using polyA purified JURKAT mRNA except for the modification described below. The primer used in the reverse transcription reaction was 5'-CAGCAGTGAACAACATC-3'. The amplification primer derived from sequencing of the hFKBP25 gene was 5'-GGTCTCTTCAGACTTGG-3' and the adapter and dT_{17} adapter primers were the same as described (21). To increase the specificity of the RACE technique, we used a small aliquot from the first PCR reaction and amplified again using a

primer specific for hFKBP25, 5'-GTTAGCTGTCTTGGCCA-3', and the adapter primer. A small aliquot from the products of the second PCR reaction were used in a third PCR reaction using another hFKBP25 specific primer, 5'-CTGTAACCAAGTGGTCC-3', and the adapter primer again. A single band was purified by gel electrophoresis, subcloned into pUC19, and sequenced. To enhance the fidelity of the PCR reactions, *Pyrococcus furiosus* DNA polymerase (Stratagene) was used in all PCR reactions.

Sequence Alignments: Multiple sequence alignments were constructed using the program CLUSTAL in the University of Wisconsin Genetics Computer Group package (22).

RESULTS AND DISCUSSION

Based upon the near-complete amino acid sequence of the bovine FKBP25 protein, we cloned the cDNA encoding the human FKBP25 equivalent. In order to clone the human homolog, we relied upon the very high amino acid sequence conservation between species of the known FKBPs and assumed that this conservation would hold for FKBP25. For example, bovine and human FKBP12 differ by only two amino acids. To clone the human gene, PCR primers specific for stretches of amino acids encoded by relatively non-degenerate codons were chosen. The sense primer covered the peptide KKDIIK while the anti-sense primer covered the peptide IEPEWA. The primers were used in a PCR reaction containing a JURKAT cDNA library in λgt10 as the template. One half of the PCR reaction was subjected to gel electrophoresis. A number of bands were detected and so the gel was subjected to Southern analysis with an oligonucleotide probe specific for a peptide sequence in the bovine protein between the two peptides described above. One band of about 500 bp hybridized to the probe. The other half of the PCR reaction was electrophoresed on a 1% agarose gel, the DNA of about 500 bp in length was purified, and subcloned into pUC19. Transformants were screened with the oligonucleotide encoding the internal peptide and plasmid from one of the positive colonies was purified. Sequencing of the pUC19 insert revealed an open reading frame highly homologous to the bovine protein.

The insert was gel purified, labeled by random priming, and used to screen 800,000 phage from a JURKAT cDNA library in λgt10. Six positive plaques were identified and four of the plaques were purified. Excision of the EcoRI inserts from the phage revealed that all had inserts of about 1 kB in length. These inserts were subcloned into pUC19 and sequenced. Based upon the bovine sequence, the cDNA clone contained almost all of the open reading frame. The RACE technique was used to obtain the remainder of the open reading frame as well as some of the 5' untranslated region. Only the codon specifying the initiator methione was missing from the phage cDNA clone and that was supplied by the RACE product.

The 964 bp cDNA encoding the human FKBP25 is shown in Figure 1. The open reading frame encodes a 224 amino acid protein of 25,178 daltons. The amino acid sequence of the human FKBP25 protein is 97% identical to that of the available bovine sequence, strongly suggesting that this cDNA encodes the human homolog. The translation product of the human cDNA also shares the potential nuclear targeting sequence,

```
   1  GAAAGCGGAGGCAGCGGGGGAAGATGGCGGCGGCCGTTCCACAGCGGGCGTGGACCGTGG   60
   1                          M  A  A  A  V  P  Q  R  A  W  T  V  E   13
  61  AGCAGCTGCGCAGTGAGCAGCTGCCCAAGAAGGATATTATCAAGTTTCTGCAGGAACACG   120
  14    Q  L  R  S  E  Q  L  P  K  K  D  I  I  K  F  L  Q  E  H  G   33
 121  GTTCAGATTCGTTTCTTGCAGAACATAAATTATTAGGAAACATTAAAAATGTGGCCAAGA   180
  34    S  D  S  F  L  A  E  H  K  L  L  G  N  I  K  N  V  A  K  T   53
 181  CAGCTAACAAGGACCACTTGGTTACAGCCTATAACCATCTTTTTGAAACTAAGCGTTTTA   240
  54    A  N  K  D  H  L  V  T  A  Y  N  H  L  F  E  T  K  R  F  K   73
 241  AGGGTACTGAAAGTATAAGTAAAGTGTCTGAGCAAGTAAAAAATGTGAAGCTTAATGAAG   300
  74    G  T  E  S  I  S  K  V  S  E  Q  V  K  N  V  K  L  N  E  D   93
 301  ATAAACCCAAAGAAACCAAGTCTGAAGAGACCCTGGATGAGGGTCCACCAAAATATACTA   360
  94    K  P  K  E  T  K  S  E  E  T  L  D  E  G  P  P  K  Y  T  K  113
 361  AATCTGTTCTGAAAAAGGGAGATAAAACCAACTTTCCCAAAAAGGGAGATGTTGTTCACT   420
 114    S  V  L  K  K  G  D  K  T  N  F  P  K  K  G  D  V  V  H  C  133
 421  GCTGGTATACAGGAACACTACAAGATGGGACTGTTTTTGATACTAATATTCAAACAAGTG   480
 134    W  Y  T  G  T  L  Q  D  G  T  V  F  D  T  N  I  Q  T  S  A  153
 481  CAAAGAAGAAGAAAAATGCCAAGCCTTTAAGTTTTAAGGTCGGAGTAGGCAAAGTTATCA   540
 154    K  K  K  K  N  A  K  P  L  S  F  K  V  G  V  G  K  V  I  R  173
 541  GAGGATGGGATGAAGCTCTCTTGACTATGAGTAAAGGAGAAAAGGCTCGACTGGAGATTG   600
 174    G  W  D  E  A  L  L  T  M  S  K  G  E  K  A  R  L  E  I  E  193
 601  AACCAGAATGGGCTTACGGAAAGAAAGGACAGCCTGATGCCAAAATTCCACCAAATGCAA   660
 194    P  E  W  A  Y  G  K  K  G  Q  P  D  A  K  I  P  P  N  A  K  213
 661  AACTCACTTTTGAAGTGGAATTAGTGGATATTGATTGAAATAGGCAGTGCTTCAGCTCTA   720
 214    L  T  F  E  V  E  L  V  D  I  D  *                         224
 721  AGGATATTAGCAACAATGATAAAACTTGGCCTTGAAGAAATTTACACAACTAGTTAGAAC   780
 781  TTGTTACTATTGTAAAGGAAGAGTCAACTGGAAAATTCAAGGAGTTAATAAAATTTGTTT   840
 841  ACTTGGTCCCAGCTTTTGAGAGATAAATCCCTTATGAATCCCTGGTCTAAAATACTTTCC   900
 901  TACAGCTGTGTAAAATACTGGTCAAGGAGAACTTTTTCCTTTTACCTCATGTTGTAAACT   960
 961  TAAG  964
```

Fig.1. Nucleotide sequence and predicted protein sequence of the human gene encoding FKBP25. The amino acid translation, beginning with the initiator methionine, is shown below the nucleotide sequence. The numbers flanking each row designate the amino acid and nucleotide positions.

$KK(X)_7KK(X)_{26}KKKK$, identified within the bovine protein (1). We believe that the methionine at amino acid position one is the initiator methione for two reasons. First, the initiator methione in the human cDNA clone is in an extremely good context for translation initiation (23). Second, there is very close agreement between the calculated molecular weight of the human protein, 25,161 daltons, and the weight of the bovine protein determined by mass spectroscopy, 25,235 daltons (1).

Shown in Figure 2 is a multiple sequence alignment, performed by the GCG program CLUSTAL, of all three human FK-506 binding proteins for which there is complete amino acid sequence. Most of the amino acids conserved between FKBP12 and FKBP25 or between FKBP13 and FKBP25 are in the C-terminal half of the FKBP25 protein. The conservation in the C-terminal half becomes even more apparent when the FKBP12 and FKBP13 amino acid sequences are aligned independently with the sequence of FKBP25 (independent Lipman-Pearson alignments not shown). The remainder of FKBP25 is unrelated to any protein presently in any protein sequence databases. Based upon independent sequence alignments between either FKBP12 or FKBP13 and FKBP25 the amino acid sequences of FKBP12 and FKBP13 are 41% and 43% identical, respectively, to that of FKBP25. Many of the residues conserved among all three of the proteins (denoted by asterisks) are those amino acids that, in FKBP12, have been shown by crystallographic analysis to form the hydrophobic pocket into which FK-506 binds (24).

```
FKBP12     1 MGV-------------------------------------------------------  3
FKBP13     1 MRLS-----W------------------------------------------------  5
FKBP25     1 MAAAVPQRAWTVEQLRSEQLPKKDIIKFLQEHGSDSFLAEHKLLGNIKNVAKTANKDHLV  60
               *

FKBP12     4 ----------------------QVETISPGDGR-----------TFPKRGQTCVV---  25
FKBP13     6 -----------FRVLTVLSICLSAVATATGAEGKRK-------LQIGVKKRVDHCPIKS-  46
FKBP25    61 TAYNHLFETKRFKGTESISKVSEQVKNVKLNEDKPKETKSEETLDEGPPKYTKSVLKKGD 120
                                  .* . . ....            .  *  .

FKBP12    26 -------------HYTGMLEDGKKFDSSRD-------RNKPFKFMLGKQEVIRGWEEGVA  65
FKBP13    47 -----RKGDVLHMHYTGKLEDGTEFDSSLP-------QNQPFVFSLGTGQVIKGWDQGLL  94
FKBP25   121 KTNFPKKGDVVHCWYTGTLQDGTVFDTNIQTSAKKKKNAKPLSFKVGVGKVIRGWDEALL 180
                          *** *.**. **..          ...*. * .*  .**.**....

FKBP12    66 QMSVGQRAKLTISPDYAYGATGHP-GIIPPHATLVFDVELLKLE 108
FKBP13    95 GMCEGEKRKLVIPSELGYGERGAP-PKIPGGATLVFEVELLKIERRTEL 142
FKBP25   181 TMSKGEKARLEIEPEWAYGKKGQPDAKIPPNAKLTFEVELVDID 224
               *. *.. .* * .. .**  * *   **  *.*.*.***....
```

Fig.2. Multiple sequence alignment of derived amino acid sequence of human FKBP25 with human FKBP12 and human FKBP13. Alignments were performed by the GCG program CLUSTAL. Asterisks (*) denote residues that are identical in all three sequences. Periods (.) denote residues that are conserved in all three sequences. The numbers flanking each row designate the amino acid positions.

It has been shown that the FKBP12-FK-506 complex, but not the FKBP12-rapamycin complex, binds to the phosphoprotein phosphatase, calcineurin and inhibits its phosphatase activity (25). FKBP25 has a marked preference for binding rapamycin over FK-506 and may be the relevant receptor by which rapamycin mediates its immunosuppressive effects. Expression of the cDNA clone for FKBP25 will allow further study of the mechanism of action of both FK-506 and rapamycin.

REFERENCES

1. Galat, A., Lane, W., Standaert, R., and Schreiber, S. (1992) *Biochemistry* **31**, 2427-2434.
2. Tocci, M., Matkovich, D., Collier, K., Kwok, P., Dumont, F., Lin, S., DeGudicibus, S., Siekierka, J., Chin, F., and Hutchinson, N. (1989) J. Immunol. **143**, 718-726
3. Siekierka, J., Staruch, M., Hung, S., and Sigal, N. (1989) *J. Immunol.* **143**, 1580-1583.
4. Siekierka, J., Hung, S., Poe, M., Lin, S., and Sigal, N. (1989) *Nature* **341**, 755-757.
5. Harding, M., Galat, A., Uehling, D., and Schreiber, S. (1989) *Nature* **341**, 758-760.
6. Maki, N., Sekiguchi, F., Nishimaki, J., Miwa, K., Hayano, T., Takahashi, N., and Suzuki, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5440-5443.
7. Standaert, R., Galat, A., Verdine, G., and Schreiber, S. (1990) *Nature* **346**, 671-674.
8. Jin, Y-J., Albers, M., Lane, W., Bierer, B., Schreiber, S., and Burakoff, S. (1991) Proc. Natl. Acad. Sci. USA **88**, 6677-6681.
9. Siekierka, J., Wiederrecht, G., Greulich, H., Boulton, D., Hung, S., Cryan, J., Hodges, P., and Sigal, N. (1990) *J. Biol. Chem.* **265**, 21011-21015.
10. Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N., and Siekierka, J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1029-1033.
11. Koltin, Y., Faucette, L., Bergsma, D., Levy, M., Cafferkey, R., Koser, P., Johnson, R., and Livi, G. (1991) *Mol. Cell. Biol.* **11**, 1718-1723.
12. Heitman, J., Movva, N., Hiestand, P., and Hall, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1948-1952.

13. Tropschug, M., Wachter, E., Mayer, S., Schonbrunner, E., and Schmid, F. (1990) *Nature* **346**, 674-677.
14. Nielsen, J., Siekierka, J., Bostian, K., Hsu, M., Dahl, A., Shafiee, A., Ramadan, N., Foor, F., Morin, N., Brizuela, L., Chrebet, G., and Parent, S. (1992) Proc. Natl. Acad. Sci. USA (submitted).
15. Dumont, F., Staruch, M., Koprak, S., Melino, M., and Sigal, N. (1990) *J. Immunol.* **144**, 251-258.
16. Bierer, B., Mattila, P., Standaert, R., Herzenberg, L., Burakoff, S., Crabtree, G., Schreiber, S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9231-9235.
17. Sigal, N., Lin, S., and Siekierka, J. (1991) *Transplant. Proc.* **23** (2), 1-5.
18. Friedman, K., Rosen, N., Newman, P., Montgomery, R. (1988) *Nucleic Acids Res.* **16**, 8718.
19. Itoh, N., Tanaka, N., Mihashi, S., and Yamashina, I. (1986) *J. Biol. Chem.* **262**, 3132-3135.
20. Grunstein, M. and Hogness, D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961-3965.
21. Frohman, M., Dush, M., and Martin, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002.
22. Deveraux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387-395.
23. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125-8148.
24. Van Duyne, G., Standaert, R., Karplus, P., Schreiber, S., and Clardy, J. (1991) *Science* **252**, 839-842.
25. Liu, J., Farmer, J., Lane, W., Friedman, J., Weissman, I., and Schreiber, S. (1991) *Cell* **66**, 807-815.