The Cytosolic-binding Protein for the Immunosuppressant FK-506 Is Both a Ubiquitous and Highly Conserved Peptidyl-Prolyl Cis-Trans Isomerase*

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We have recently isolated an abundant cytosolic protein from human T-cells which specifically binds the immunosuppressive agent, FK-506. The FK-506-binding protein (FKBP) is a member of a novel class of proteins possessing peptidyl-prolyl cis-trans isomerase activity. These proteins are believed to play an important role in accelerating the rate at which proteins fold into their native conformations. In the present study, we demonstrate that FKBP is not a lymphoid-specific protein, but is widely distributed and phylogenically conserved. FKBP, purified from three sources (a human T-lymphocyte cell line JURKAT, bovine calf thymus, and Saccharomyces cerevisiae) exhibit identical molecular weights, immunological cross-reactivities, and a high degree of NH2-terminal amino acid sequence homology. In addition, FKBP from all sources possesses peptidyl-prolyl cis-trans isomerase activity which can be specifically inhibited by FK-506. We conclude that FKBP may serve an important biological function in all eukaryotic cells.

The potent immunosuppressive agents, cyclosporin A (CsA)1 and FK-506, selectively inhibit early T-cell activation events required for lymphokine gene expression (1–3). In particular, FK-506 and CsA appear to inhibit those activation pathways that induce a measurable rise in intracellular Ca2+ (4–6). This selective action on T-cell activation has been exploited clinically for the prevention of allograft rejection.

Despite the highly selective action of CsA on lymphocytes, the distribution of its intracellular receptor, cyclophilin, is not restricted to lymphoid cells. Cyclophilin has been found in a wide variety of cell types and organisms (7). Cyclophilin isolated from various sources is a highly conserved protein which exhibits peptidyl-prolyl cis-trans isomerase activity (PPIase activity) (8, 9). This activity is believed to play an important role in the folding of proteins and peptides into their native conformations (for a recent review, see Ref. 10). CsA specifically inhibits cyclophilin PPIase activity at concentrations relevant to immunosuppression (8, 9). This observation has led to the hypothesis that critical T-cell signal transduction events (transcription factors, protein kinase activation, ion channel function, etc.) may be regulated via peptidyl-prolyl isomerization. In view of the ubiquitous and highly conserved nature of cyclophilin, the question arises as to its function in non-lymphoid cells.

Recently, a cytosolic-binding protein for the immunosuppressant agent, FK-506, has been identified (11) and purified to homogeneity from human T cells (12, 13). FKBP, like cyclophilin, is an abundant, cytosolic protein (0.2–0.4% of total cytosolic protein) which exhibits peptidyl-prolyl cis-trans isomerase activity (12, 13). FKBP, however, is clearly distinct from cyclophilin by a number of criteria including immunosuppressant binding specificity, molecular weight, and amino acid sequence (12, 13). We now report that FKBP, like cyclophilin, is a highly conserved protein with a wide phylogenetic distribution. These properties are suggestive of a fundamental biochemical role for FKBP and cyclophilin in all cells.

MATERIALS AND METHODS

Preparation of Crude Cytosol—All cells, except Saccharomyces cerevisiae, were washed two times with phosphate-buffered saline and disrupted by Dounce homogenization in four packed cell volumes of buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The resulting extracts were centrifuged at 1,000 X g for 20 min. The supernatants were further centrifuged for 20 min at 150,000 X g in a Beckman TL-100 ultracentrifuge (Beckman Instruments, Inc.). Protein was determined by the method of Bradford (14). Crude bovine thymus and S. cerevisiae homogenates were prepared as described in the next section. Crude homogenates from these sources were centrifuged as previously described.

Purification of FKBP—Human JURKAT FKBP was purified as previously described (12). Bovine calf thymus FKBP was purified by homogenizing 1 kg of bovine calf thymus (Roth Products Inc., Lantsdale, PA) in 4 liters of buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 0.02% sodium azide. The homogenate was centrifuged at 8,000 X g and the supernatant clarified by filtration through two 0.2 μm Pellicon cassettes (GVLP 000 05, Millipore Corp., Bedford, MA) in parallel. The filtrate was filtered through a 100,000 M, exclusion membrane (Pellicon cassette PTKH 000 05). The filtrate was concentrated using a similar membrane with a 10,000 M, exclusion. The remaining steps were as previously described for human JURKAT FKBP (12).

Yeast FKBP was purified from the protease-deficient S. cerevisiae strain E6226. Yeast were grown at 30 °C to A600 = 4.5 in YPD media, harvested by centrifugation, and the paste resuspended 1:2 (w/v) in a buffer solution containing 50 mM Tris-HCl, 7.8% glycerol, 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 400 μM diisopropyl fluorophosphate, 70 μg of leupeptin/g of yeast and 50 μg of pepstatin A/g of yeast. The suspension was frozen by dripping into liquid
nitrogen and a yeast extract prepared by grinding the frozen pellets under liquid nitrogen in a mortar and pestle. The yeast powder was thawed and insoluble debris removed by centrifugation. The yeast extract was subjected to heat-treatment, Cibacron Blue chromatography, and HPLC gel filtration as previously described for human JURKAT FKBP. Unlike the human and bovine FKBP, yeast FKBP failed to precipitate on calcium extraction. Final purification was therefore achieved by affinity purification using an affinity matrix synthesized as described below. FKBP purified through the HPLC gel filtration step was applied to the affinity column and nonspecifically bound protein eluted with a buffer containing 10 mM potassium phosphate, 100 mM NaCl, and 0.02% Tween-20. Yeast FKBP was eluted with 4 M guanidine hydrochloride and renatured by dialysis against buffer containing 10 mM potassium phosphate, pH 7.5, and 1 mM EDTA. A yield of 1.4 mg of yeast FKBP was obtained from 6.9 g of crude extract. Overall yields varied between 10–20%. Protein was determined by the method of Bradford (14).

Synthesis of an FK-520 Affinity Chromatography Resin—The FK-506 analog, FK-520, which differs from FK-506 by the presence of an ethyl substituent at position 21 (15) was used for the synthesis of an affinity resin. All reagents were obtained from Aldrich Chemical Co. To a stirred solution of FK-520 (1.58 g, 2.0 mmol) (isolated by Dr. Robert Borris of Merck from Streptomyces hygroscopicus) in dichloromethane (6 ml) and pyridine (0.5 ml) at 0 °C was added 4-nitrophenyl isocyanate (0.3 g, 2.0 mmol), portionwise. The mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. Processing of the organic phase gave a residue which was purified by flash column chromatography (elution with 30% ethyl acetate-hexane) to give the 4-nitrophenyl derivative of FK-520 as a pale yellow foam (1.39 g, 72%). To a stirred solution of this derivative (470 mg, 0.5 mmol) in dimethylformamide (1.5 ml) at room temperature was added β-alanine t-butyl ester hydrochloride (454 mg, 2.5 mmol) in dichloromethane (0.1 ml) at 0 °C was added dropwise trifluoroacetic acid (4.25 ml). The mixture was stirred at -10 °C for 3 h and then diluted with water (200 ml) and methanol (5 ml). The mixture was filtered, and the residue was purified by flash column chromatography (elution with 30:40:30 ethyl acetate-hexane) to give the β-alanine t-butyl ester hydrochloride-peptide portion. The mixture was stirred at 0 °C for 1 h and then at room temperature for 24 h. Processing of the organic phase gave a residue which was purified by flash column chromatography (elution with 30:40:30 ethyl acetate-hexane) to give the β-alanine t-butyl ester hydrochloride (434 mg, 0.87%). To a stirred solution of this (301 mg, 0.31 mmol) in dichloromethane (0.9 ml) at -10 °C was added dropwise trifluoroacetic acid (2.1 ml). The mixture was stirred at -10 °C for 3 h and then diluted with water (200 ml) and methanol (5 ml). The mixture was lyophilized, and the residue was purified by flash column chromatography (elution with 50:50:20 ethyl acetate-hexane-acetonitrile) to give a pure product (172 mg, 61%). FABMS, MLi + Li = 919. RF = 0.06 by flash column chromatography (elution with 80:20:2 chloroform/methanol/ammonia) on silica gel.

Western Blot Analysis—Following electrophoresis, proteins were transferred to Millipore Immobilon membranes (Millipore Corporation, Bedford, MA) using a Bio-Rad Mini Tran-Blot apparatus. Transfers were run at 100 V constant voltage for 2 h. Nonspecific sites were blocked for 2 h at room temperature with a solution containing 10 mM Tris-HCl, pH 7.5, 10% non-fat dry milk (Carnation), 0.09% NaCl, and 0.04% sodium azide. Membranes were rinsed twice in a solution containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 20% heat-inactivated fetal bovine serum (Hyclone), and 0.04% sodium azide (fetal bovine serum blocking solution). Membranes were incubated with biotinylated goat anti-guinea pig IgG (O.E.M. Concepts Inc., Toms River, NJ) at 1:10,000 in Tris-buffered saline for 1 h at room temperature. After washing in Western wash buffer as above, membranes were incubated with streptavidin/alkaline phosphatase (O.E.M. Concepts Inc., Toms River, NJ) at 1 μg/ml in Tris-buffered saline for 30 min at room temperature. Membranes were washed with Western wash buffer as above and briefly rinsed in 0.05 M carbonate buffer, pH 9.6, containing 0.02% sodium azide. Blots were developed with the BCIP/NBT color development solution as described by the manufacturer (Bio-Rad).

**RESULTS**

Crude cytoplasmic extracts (S-100) were prepared from a variety of cell types and from bovine calf thymus and assayed for FKBP activity with the [3H]FK-506 binding assay previously described (11, 12). The results of this survey are shown in Table I. FKBP activity was found in a wide variety of cell types and organisms. Wide variations in FKBP activity were not observed, with lower organisms (e.g. insects and yeast) containing levels of FK-506 binding activity comparable to those found in human cells. These results clearly indicate that FKBP is not a lymphoid-cell-specific protein. In a similar survey of cyclophilin, it was found that cyclophilin was present in all eukaryotic extracts examined (7).

In order to further compare the biochemical and molecular properties of FKBP from different organisms, we purified FKBP from human, mouse, bovine, thymus, and yeast. Western analysis of these proteins exhibited identical molecular weights as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10,000–11,000, Fig. 1A) and HPLC gel filtration (data not shown). The isoelectric point of human and bovine FKBP was 8.8, while that of yeast was 4.8 (Fig. 1B). The reason for this charge discrepancy is not immediately apparent but may be related to covalent modification of the...
The FK-506-binding Protein Is Ubiquitous and Highly Conserved

TABLE I

<table>
<thead>
<tr>
<th>Source</th>
<th>FK-506 binding activity* ng of [3H]FK-506 bound/mg protein (“Material and Methods”)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cells</td>
<td></td>
</tr>
<tr>
<td>JURKAT</td>
<td>Human, T-lymphoblastic leukemia 41</td>
</tr>
<tr>
<td>Hut-73</td>
<td>Human, T-cell lymphoma 36</td>
</tr>
<tr>
<td>CTLL-2</td>
<td>Murine, T-cell 21</td>
</tr>
<tr>
<td>EL-4</td>
<td>Murine, lymphoma 41</td>
</tr>
<tr>
<td>B-cells</td>
<td></td>
</tr>
<tr>
<td>DAUDI</td>
<td>Human, Burkitt lymphoma 20</td>
</tr>
<tr>
<td>Murine</td>
<td>Normal, purified 11</td>
</tr>
<tr>
<td>Other cells</td>
<td></td>
</tr>
<tr>
<td>MRC-5</td>
<td>Human, lung 46</td>
</tr>
<tr>
<td>PC-12</td>
<td>Rat, adrenal chromaffin 32</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Murine, embryo 21</td>
</tr>
<tr>
<td>BHK</td>
<td>Hamster, kidney 44</td>
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<tr>
<td>PMN</td>
<td>Human, polymorphonuclear cells 36</td>
</tr>
<tr>
<td>Endothelial</td>
<td>Human, umbilical cord 84</td>
</tr>
<tr>
<td>Sf9</td>
<td>Insect, Spodoptera frugiperda 29</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Yeast 15</td>
</tr>
</tbody>
</table>

| Tissue                        |                                                                  |
| Calf thymus                   | 26                                                               |

* ng of [3H]FK-506 bound/mg protein (“Material and Methods”).

DISCUSSION

The results presented here demonstrate that FKBP, like cyclophilin, is a ubiquitous and highly conserved protein. The wide distribution of these two proteins suggests that they serve a general function in cellular metabolism in addition to their immunomodulatory role(s). FKBP and cyclophilin almost certainly function in a critical signaling pathway in T- and B-lymphocytes since the immunosuppressive ligands which bind to these proteins (FK-506 and CsA, respectively) are potent inhibitors of T- and B-cell activation. FK-506 and CsA appear to selectively inhibit the accumulation of a set of early phase T-cell activation gene products such as interleukin 2 (3, 22, 23). Both FK-506 and CsA are without effect on events subsequent to early lymphokine gene expression such as the expression of intermediate/late phase T-cell activation genes or interleukin 2-dependent T-cell growth (2, 3).

FKBP and cyclophilin are members of a class of proteins possessing peptidyl-prolyl cis-trans isomerase activity (12, 13, 17, 18). These enzymes catalyze the highly efficient isomerization of Xaa-Pro peptide bonds in proteins, greatly accelerating the folding of proteins and peptides into their physiologically active conformations (10). In view of this, one can appreciate the ubiquitous nature of these proteins since a role in catalysis of protein refolding would be expected to be of fundamental importance to all organisms. However, in addition to a general role in accelerating protein folding, FKBP and cyclophilin appear to have important regulatory functions in cells. In view of the striking observation that both FKBP and cyclophilin are PPIases, we have proposed that these PPIases, either directly or as part of a yet undefined macromolecular complex, “conformationally” regulate some component(s) of the signal transduction pathway (e.g. protein kinases, ion channels, transcriptional regulatory factors, etc.) responsible for the activation of early T-cell genes (12). FK-506 and CsA, as selective inhibitors of FKBP and cyclophilin PPIase activity, would block events catalyzed by these proteins. The selective action of FK-506 and CsA on T-cells may be due to the presence of lymphocyte-specific PPIase substrates or cofactors. Indeed, recent kinetic analysis of FKBP and cyclophilin using synthetic peptides has revealed very different substrate requirements for these two PPIases (24). FK-506 and CsA may also function through the interaction with other FKBP or cyclophilin isoforms or perhaps through
interaction with an as yet undefined, unique receptor(s) which may be cell-specific. For example, the Drosophila ninaA gene encodes a photoreceptor-specific cyclophilin which contains a putative signal sequence and transmembrane domain (25, 26) and the fungus, N. crassa, has been shown to contain a mitochondrial form of cyclophilin (19). Similar forms (e.g. Aplysia) have been used as model systems to study the mechanism of action of CsA (19, 20). The potent antiproliferative effects of CsA in these two organisms have been shown to be mediated by cyclophilin. Generation of CsA-resistant mutants should allow the functional characterization of cyclophilin through genetic means. The ubiquitous nature of both FKBP and cyclophilin should allow a variety of molecular and biochemical approaches to be applied to the question of the role of FKBP and cyclophilin in general cellular metabolism. In turn, this information should provide new insights into the immunomodulatory role(s) of these proteins.

**REFERENCES**

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