International Journal of Biological Macromolecules 69 (2014) 344–352

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Diversified targets of FKBP25 and its complex with rapamycin

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 21 May 2014
Accepted 21 May 2014
Available online 29 May 2014

Keywords:
FKBP
Rapamycin
FKBP25
PPIase
Immunophilin

ABSTRACT

FKBP25 is a member of the super-family of peptidylprolyl *cis*/*trans* isomerases, which is a high affinity binder for the immunosuppressive antibiotic rapamycin (Rpm). FKBP25 isolated from natural sources, its recombinant murine homologue (mFKBP25) and their complexes with rapamycin bind to diverse DNAs, RNAs and heparin affinity beads. The recombinant mFKBP25/rapamycin complex binds to several proteins including the calcineurin–A/calcineurin–B/calmodulin complex and to elongation factor 1β. We solved the X-ray structure of the C-terminal domain of mFKBP25 bound to rapamycin that has a higher resolution than of its human counterpart, and which clearly illustrates that the positively charged 40s loop is an epitope of the FK506-like binding domain (FKBD) for interactions with various biopolymers.

1. Introduction

A 25-kDa protein belonging to FK506-binding family of proteins (FKBP25) was isolated from the bovine brain and shown to bind preferentially to the immunosuppressive macrocycle antibiotic rapamycin (sirolimus) [1]. FKBP25 binds to the macrocyclic immunosuppressive drug FK506 (tacrolimus) but with a weaker K_d than rapamycin. Although 18 FKBPs are expressed in mammalian tissues [2], but not all of them bind to these two antibiotics with equal affinity; FKBP25 and hFKBP12A are the best rapamycin binders [1–3]. FKBP25 has also been detected in centrosomes [4], RNA granules [5], cellular differentiation processes [6], and in developing neurons [7].

From its sequence, determined by Edman degradation, FKBP25 was predicted to be composed with two domains. Chow–Fasman analysis of the N-terminal portion predicted a positively charged helix–loop–helix (HLH) motif that has now been confirmed by its NMR structure [8,9]. The N-terminus is linked via a flexible and negatively charged segment to the C-terminal FK506-binding domain (FKBD) that has a moderate sequence similarity to hFKBP12A [10] and exhibits peptidylprolyl *cis–trans* isomerase (PPIase) activity, which is inhibited by diverse macrocyclic antibiotics [1–3]. The C-domain of FKBP25 also differs from the other FKBDs because of its unique lysines rich in highly charged 40s loop that can be an interaction site with negatively charged biopolymers including DNA [8].

To study certain aspect of interactions between FKBP25s and diverse cellular targets, we expressed in *Escherichia coli* the recombinant mouse FKBP25 (mFKBP25) fused to GST. The fusion protein is associated with the prokaryotic ribosomal proteins during its purification on glutathione beads. The purified whole protein was found to degrade at room temperature at a slow pace, probably due to a heterologous protease. The C-terminus of mFKBP25, resistant to degradation was the only component found in crystals of the mFKBP25/rapamycin complex. The structure of mFKBP25 is at a higher resolution than of its human counterpart [11], which clearly defines the lysine-rich nuclear translocation signal (NLS) region in 40s loop. In both hFKBP25 and mFKBP25 this region is the most flexible segment of the protein, probably because it protrudes from the main globular body of the FKBD. Pull-down experiments showed that the mFKBP25 itself and its complex with either rapamycin or FK506 bind to (calcineurin subunits A + B)/calmodulin ternary complex and elongation factor 1β. The functionally diversified targets of the mammalian FKBP25s [2], which are involved in chromatin remodeling machinery, may lend some support for its vital function in cancer development and as a molecular target for anti-cancer drugs.

2. Materials and methods

2.1. Materials

Acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), reduced glutathione, calcineurin, calmodulin, RNA type IV from calf liver, tRNA from *E. coli* strain W, single-stranded dodecamer $(dT)_{12}$,

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http://dx.doi.org/10.1016/j.ijbiomac.2014.05.060
0141-8130/

palindromic dodecamer deoxy-CGCGAATTCGCG, three short synthetic RNAs (heptamer, 5′-AGGGAUU; 15-mer, 5′-AGGGAUUUUUUCCCU; and 23-mer, 5′-CCUGGUAUUGCAGUACCACCAGG), rapamycin and FK506 were purchased from Sigma. Ampholites 3–10 and 8–10.5 were from Pharmacia (GE). Protein G Sepharose 4 FF was obtained from Pharmacia (GE). Endogenous FKBP25 was isolated from porcine brain as described [1]. Fusion of glutathione S transferase (GST) with hFKBP12A was a gift from Prof. SL Schreiber (Harvard University), whereas the expression vector of fusion GST-mFKBP25 was a gift from Prof. M. Simonneau (INSERM, Paris). Molecular mass markers were purchased from BioRad. GST-affinity gel, and Precision protease were purchased from Pharmacia (GE). Nitrocellulose and PVDF membranes were purchased from Whatman and Millipore, respectively.

2.2. Preparation of nuclear extracts

Two cell lineages were used for preparation of nuclear extracts, namely HeLa and U937 (each 10^9 cells) supplied by Cil Biotech S.A. Belgium. Cells were lysed in 50 mM Tris–HCl pH 7.4, 100 mM NaCl with 1 mM PMSF plus a cocktail of inhibitors (Roche) and were passed 10 times via 22G needles. The resulting cellular slurry was centrifuged at $1000 \times g$ during 15 min. The nuclear pellet was washed three times in the lyse buffer, divided into two parts and treated with the following buffers: (buffer-A) 20 mM Tris–HCl pH 7.4, 500 mM KCl, 500 mM NaCl, 2 mM $MgCl_2$, 0.2 M EDTA, 25% glycerol; and (buffer-B) 1 M potassium phosphate pH 7.5, 0.5 M KCl, 0.5 M NaCl, 2 mM $MgCl_2$, 10% glycerol. In each case the nuclear pellet was gently shaken at 7 °C during 2 h. Soluble proteins were centrifuged at $45{,}000 \times g$ during 2 h and dialyzed to 100 mM KCl, 20 mM potassium phosphate pH 7.5, 2 mM $MgCl_2$, 0.2 mM EDTA. Soluble nuclear aliquots were added to DNA-cellulose affinity gel slurry (Pharmacia Biotech), oligo-dT sepharose (Amersham) slurry or heparin-affinity beads (Sigma) and equilibrated during 14 h at 7 °C. The affinity gels were washed twice with the dialysis buffer containing 0.01% Tween-20 and treated with 8 M urea at 0 °C. The soluble aliquots were mixed with Laemmli buffer and resolved on 12% SDS-PAGE.

2.3. Size exclusion chromatography of nuclear extracts

Nuclear extracts from HeLa and U937 cells were passed via a chromatography on S200 sepharose column (1.5 cm × 1 m) that was equilibrated with 25 mM potassium phosphate at pH 7.4, 100 mM KCl, 2 mM $MgCl_2$ and 0.2 mM EDTA. The column was calibrated with protein markers supplied by BioRad. The endogenous FKBP25 was detected on Western blots using antiFKBP25 antibodies.

2.4. Immunoprecipitation of proteins

Soluble fractions of proteins isolated from U937 and HeLa cells were passed through 0.22 μM filter and treated with a filtered solution (0.22 μM) of antiFKBP25 for 20 h at 4 °C with gentle shaking. Protein-G affinity gel was added to the mixture and the slurry was gently shaken for another 4 h. The affinity gel was then washed twice with buffer containing 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl and 0.01% Triton X-100. The affinity beads were treated with 8 M urea at 0 °C and the eluted proteins were resolved either on a non-reducing 2D or SDS-PAGE gels.

2.5. Gel electrophoresis, blotting and sequencing of proteins

Immobiline gels (Pharmacia) 3–10 and 6–11 (18 cm) were used for 2D IEF/SDS-PAGE experiments. Protein samples were dissolved in the IEF buffer containing 8 M urea, 2% (v/v) Triton X100, 2% carrier ampholites 7–9 and 100 mM DTT. IEF/SDS-PAGE gels (12%, size 20 × 20 cm), blotting, Edman degradation (Applied Biosystems Precise 482HT protein sequencer) were carried as described [12].

2.6. Circular dichroism spectroscopy

CD spectra were measured in a rectangular double compartment cell (2 × 0.5 cm) using a JASCO J-815 CD spectrometer. In each case, the first CD spectrum of the unmixed components was followed with the CD spectrum after mixing the contents of both compartments. All CD spectra were made at room temperature in a low salt buffer at pH = 7.6 (25 mM Tris–HCl, 50 mM NaCl, 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$). CD spectra were expressed either in millidegrees for complexes of mFKBP25 with RNAs or DNAs, or in terms of mean residual molecular ellipticity θ (deg cm^2 $dmol^{-1}$) for each component of the sample. Concentrations of DNA and RNA samples were established using the ε's of deoxy- and ribo-nucleotides, respectively [13].

2.7. Crystallization

The mFKBP25 rapamycin complex was concentrated to 5 and 12 mg ml^{-1} in 50 mM sodium acetate, and screened for crystallization by sitting drop vapor diffusion using 24 selected conditions from the "Stura" screens (Molecular Dimensions). From the precipitation pattern it was decided to screen 2.4 M ammonium sulfate at acidic pH. Crystals were obtained at 20 °C in a cooled incubator at pH 4.5 and 5.0. The crystals obtained were large enough for synchrotron data collection. After a short soaking in cryo-protectant (2.4 M ammonium sulfate, 25% glycerol, 50 mM sodium citrate, pH 5.0), the crystals were cryo-cooled in liquid ethane (Table 1).

2.8. Data structure determination and refinement

Data for the mFKBP25-rapamycin complex were collected at the European Synchrotron Radiation Facility (ESRF) beamline ID14-1 (Grenoble, France) at 100° K from single crystals. The crystals diffracted to 1.8 Å resolution and processed using MOSFLM [14]. The crystals belong to the tetragonal space group $P4_12_12$ with cell parameters a = 85.65 Å, b = 85.65 Å, c = 53.0 Å. Molecular replacement was carried out with MOLREP [15] using the human counterpart (1PBK) as the starting model. After restrained refinement using REFMAC [16], the amino acid differences between the two FKBPs were corrected. Density fitting and refinement were carried out with the aid of electron density maps (omit σA-weighted 2Fo-Fc and Fo-Fc) calculated and displayed using the XtalView [17] suite of programs and COOT [18]. Stereochemical analysis of the final refined model was checked with the validation tools in COOT. The coordinates and structure factors of the FKBD of mFKBP25 bound to rapamycin have been deposited in the RCSB database with the access code 3KZ7.

2.9. Sequence information resources and analyses of X-ray crystallographic structures

The NCBI non-redundant database produced [19] was scanned with the BLAST program [20]. Coordinates of X-ray structures of several FKBPs were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB, http://www.rcsb.org) [21]. Multiple sequence alignments (MSA) were made with the ClustalW program [22]. Analyses of hydrophobicity profiles, HI indexes and multiple sequence alignments (MSA) were made as recently described [23]. The figures depicting the X-ray structures were made with PYMOL from DeLano Scientific LLC (www://pymol.sourceforge.net) [24]. Tandem 2D distance maps