Volume 315, number 3, 247–251 FEBS 11982 January 1993

On the localization of FKBP25 in T-lymphocytes

Sylvie Rivière, André Ménez and Andrzej Galat

Département d'Ingénierie et d'Etudes des Protéines (DIEP), Centre d'Etudes de Saclay, Bat. 152, F-91191 Gif-sur-Yvette Cedex, France

Received 18 November 1992

Using polyclonal rabbit antibodies against bovine FKBP25, NEPHGE/SDS-PAGE and Western blotting we demonstrate that the rapamycin-specific immunophilin FKBP25 is present in T-lymphoma Jurkat cells. Subsequent fractionations of the soluble Jurkat cell proteins have revealed that FKBP25 predominantly occurs in the nuclear fraction. FKBP25 has the ability to bind to DNA. The FKBP25/DNA complex can be dissociated in the presence of a high salt concentration. FKBP12, which shares high amino acid sequence homology to the C-terminal domain of FKBP25, has no tendency to bind to DNA. CD-constrained predictions of the secondary structures in FKBP25 suggest that an amphipathic helix–loop–helix occurs in the N-terminal part of the protein and may account for its binding to DNA.

PPIase; FKBP25; FKBP12; DNA binding

1. INTRODUCTION

Immunophilins form a subgroup of peptidyl-prolyl *cis-trans* isomerases (PPIases) which catalyze the interconversion of *cis-* and *trans-* rotamers of the peptidyl–prolyl amide bond of peptide and protein substrates [1]. They also bind the immunosuppressive drugs FK506 [1–3], rapamycin [2–4] and cyclosporin A [5]. At present 27 different amino acid sequences of cyclophilins, referred to as the cyclosporin-A binding proteins and 17 different sequences of FKBPs known as the FK506 or rapamycin binding proteins, have been deposited to the MIPSX protein data-bank. However, only few immunophilins have been isolated from organs and cell lines and partially characterized [1–7]. Cyclophilin was the first PPIase whose isolation from porcine kidney cortex and bovine thymocytes was described [8,9]. Subsequently FKBP12 was isolated from bovine thymocytes and human spleen [2] and T-lymphoma Jurkat cells [3]. Various cellular targets for the complexes of cyclophilin with CsA, and FKBP12 with FK506 or rapamycin were identified and correlated with the immunosuppressive actions exerted by the drugs [6,7,10]. However, no molecular targets for FKBP25 [4] have yet been identified.

Analyses of the amino acid sequence of FKBP25 revealed putative nuclear translocation signals [4]. Moreover, the primary extraction of FKBP25 [4] from bovine organs required a high concentration of salt [4]. These properties prompted us to further investigate FKBP25 and to examine the possiblity that the protein is located in the nucleus of T-cells. In this paper we report on the localization of FKBP25 in T-lymphoma Jurkat cells and on its ability to bind to DNA under in vitro conditions.

Correspondence address: A. Galat, Département d'Ingénierie et d'Etudes des Protéines (DIEP), Centre d'Etudes de Saclay, Bat. 152, F-91191 Gif-sur-Yvette Cedex, France. Fax: (33) (1) 6908 7190.

2. MATERIALS AND METHODS

2.1. *Tissue source, chemicals and chromatography materials*

Calf brain was supplied fresh on ice by the Henri Meunier slaughter house (Meaux, 77, France). The fresh tissue was shredded and frozen in liquid nitrogen. SDS-PAGE and NEPHGE chemicals were purchased from Serva and Pharmacia/LKB. All other chemicals were of analytical grade and purchased from either Bio-Rad or Sigma.

2.2. *NEPHGE and SDS-PAGE electrophoresis*

NEPHGE was performed in glass tubes (18 cm × 2.4 mm). Mixtures of proteins were treated with the lysis buffer (9.8 M urea, 2% w/v NP-40, 2% carrier ampholytes and 100 mM DTT) and loaded immediately onto the tubes. NEPHGE was carried out for 4 h at a constant voltage of 400 V. The following protein markers were used to establish an approximate pI scale: FKBP12 from bovine thymus, two isoforms of cyclophilin from bovine spleen, FKBP25 from bovine brain, two isoforms of aspartate aminotransferase from bovine brain and cyclophilin from *E. coli*. The gels were excised from the tubes, soaked in SDS-PAGE buffer for 15 min and reloaded onto a 12% SDS-PAGE (20 cm × 20 cm) - Protean II apparatus (Bio-Rad). Proteins were visualized by either silver staining [11] or immunostaining.

2.3. *Preparation of FKBP25 antibodies*

FKBP25 was isolated as recently described [4] with a small modification, namely preparative IEF was replaced by chromatofocusing on PBE118. Male de Bouscat White rabbits were injected subscapulary with approximately 100 mg of protein in complete Freund's adjuvant. It was followed by two other injections with incomplete Freund's adjuvant. Antibody titers were monitored by immunoblotting with purified bovine FKBP25.

2.4. *Cell culture and cell fractionation*

Jurkat cells (10^8) were grown in a RPMI 1640 medium supple-

mented with 10% fetal calf serum, L-glutamine (2 mM), HEPES (10 mM), gentamycine 100 mg/ml, sodium pyruvate (2 mM) at 37°C in a humidified 7% carbon dioxide atmosphere. The cells were harvested by centrifugation at $300 \times g$ and washed twice with PBS (10 mM phosphate, pH 7.4, 3 mM KCl, 150 mM NaCl). The cells were fractionated for cytosolic and nuclear fractions according to the procedure of Challberg and Kelly [12]. Briefly, the cells were washed once in cold hypotonic buffer (20 mM HEPES pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT and 0.2 M sucrose), resuspended in 1 ml of the hypotonic buffer without sucrose and let to swell for 10 min on ice. The cells were lysed by passing the mixture through a 25-G needle. The mixture was centrifuged for 5 min at $2000 \times g$. The resulting supernatant was centrifuged at $15\,000 \times g$ for 20 min which yielded clear cytoplasmic fraction. The nuclear pellet was resuspended in 500 ml of 50 mM HEPES pH 7.5, 10% sucrose, frozen in liquid nitrogen and thawed. After addition of NaCl to reach 100 mM salt concentration the suspension was incubated 1 h on ice and then centrifuged at $15\,000 \times g$ for 20 min. The supernatant which contained soluble nuclear proteins was concentrated. Each fraction was mixed with the NEPHGE lysis buffer and subjected to NEPHGE. Protein concentrations were estimated using the Bradford reagent (Bio-Rad).

2.5. *Western blotting*

Samples (ca. 40 mg of Jurkat proteins) isolated by fractionation of 10^8 cells were successively subjected to NEPHGE and SDS-PAGE electrophoresis (2D). Proteins from SDS-PAGE gels were electrotransferred (400 mA for 2 h) to nitrocellulose membranes (Bio-Rad) in 25 mM Tris pH 8.35, 192 mM glycine and 10% methanol. Membranes were regenerated in PBS, 1% Triton X-100, for 0.5 h, blocked for 1 h with a solution of 1% bovine serum albumin and 1% bovine caseine in PBS and incubated overnight with a 1/500 dilution of anti-FKBP25 antiserum in the blocking buffer. Normal rabbit serum was used as a negative control. The membranes were incubated for 1 h with biotinylated monoclonal anti-rabbit Ig (Sigma) diluted 1/5000 which was followed by incubation with a streptavidine–peroxidase complex 0.1 units/ml (Sigma). Immunoreactive bands were visualized with 0.04% DAB (Sigma), 0.3 mg/ml $CoCl_2$ (Sigma) and H_2O_2 (30%) 1 ml/ml in PBS. Between each step the membranes were washed (4×5 min) with PBS/Tween 20 (0.05%) buffer.

2.6. *FKBP25/(calf thymus-DNA) complexes*

50 mg of a double-stranded calf thymus-DNA-cellulose affinity matrix (4 mg of double-stranded DNA per 1 g of solid phase, Sigma – lot 101H7165) was mixed with 1 ml of 20 mM HEPES pH 7.3, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, 0.5% Triton X-100 (binding buffer) and let to swell for 24 h. A series of the complexes FKBP25/DNA-affinity matrix was prepared by mixing 75 ml of DNA-affinity matrix suspension in the binding buffer which contained 0.204 mg of the double-stranded DNA from calf thymus per 1 ml with 3.3 mg of bovine FKBP25. DNA-affinity matrices were sparingly vortexed and let to equilibrate for at least 3 h and aliquots with unbound FKBP25 were collected by centrifugation. Each DNA-affinity matrix was washed three times by vigorous vortexing in the buffer. The retained FKBP25 on DNA-affinity matrix was eluted with an increasing concentration of NaCl in the binding buffer as given in the caption to Fig. 2. The salt-eluted FKBP25 was precipitated with an equal volume of 20% trichloroacetic acid, treated with SDS lysis buffer and loaded on 12% SDS-PAGE gels. Similar steps were performed for binding of bovine FKBP12 to the same amount of DNA-affinity matrix as above.

2.7. *CD spectroscopy and analyses of protein sequences and protein database*

Circular dichroism spectra of FKBP25 and a mixture of FKBP25 with a double-stranded DNA from calf thymus (27 kb, Sigma) were recorded with a Jobin Yvon Mark VI dichrometer. The CD spectra were measured at room temperature in 20 mM HEPES, pH 7.2, 1 mM EDTA. The secondary structure of FKBP25 was predicted with the SEQPRO program using the CD-constrained procedure [4]. The predicted α-helical segments were analysed for potential amphipathic character using the Fourier transform procedure [13]. The hydrophobicity index (H_i) was calculated as a ratio of the amino acid residues being in the hydrophobic segments to their total. The hydrophobic segments were calculated with SEQPRO using a nine-residue window and the Kyte–Doolittle hydrophobicity scale [14]. The current edition of the MIPSX protein data base (release 33 with 61811 sequences) was searched with SEQPRO.

3. RESULTS

Soluble proteins from the nuclear and cytosolic compartments of T-lymphoma Jurkat cells were separated from each other. Subsequently, they were subjected to NEPHGE/SDS-PAGE and Western blotting. Only one protein of the nuclear fraction cross-reacted with a rabbit antiserum raised against bovine FKBP25 (see Fig. 1A). The coordinates of this immunospot are identical to those of bovine FKBP25 on a 2D map of the standard proteins (see Fig. 1B and section 2). Control experiments revealed that no protein was immunostained using a normal rabbit serum. The presence of FKBP25 in the nuclear fraction of T-lymphoma Jurkat cells was thus firmly established. However, a proportion of FKBP25 was also found in the cytosolic fraction (data not shown).

In the presence of a low salt concentration FKBP25 was retained on DNA covalently bound to cellulose. The protein was gradually released from the affinity matrices with an increasing concentration of NaCl (see Fig. 2). In contrast, FKBP12 was not retained by DNA-affinity matrix (data not shown).

4. DISCUSSION

Our experiments revealed that FKBP25 is present in the nucleus of T-lymphoma Jurkat cells. This observation is in agreement with the previous proposal [4] that the amino acid sequence of FKBP25 contains nuclear translocation signals. A proportion of FKBP25 was found, however, in the cytosolic fraction which might be due to its extraction from the nucleus during cell fractionation under hypotonic conditions, like in the case of the Ets-1 transcription factors and retinoblastoma [15,16]. Alternatively it might come from an association of FKBP25 with a cytosolic component.

At a low salt concentration FKBP25 was retained on DNA-affinity matrices. The range of NaCl concentration which was needed to elute FKBP25 from DNA-affinity matrices suggests that the protein has the ability to bind to DNA. Similar concentrations of NaCl were required for the initial extraction of FKBP25 from various animal organs [4]. Likewise high salt concentration is frequently used to isolate various DNA-binding proteins [17]. Hence, the ability of FKBP25 to bind to DNA is compatible with its nuclear localization.

Association between FKBP25 and DNA could be rationalized in terms of structural elements present in the former. The secondary structures of FKBP25 pre-

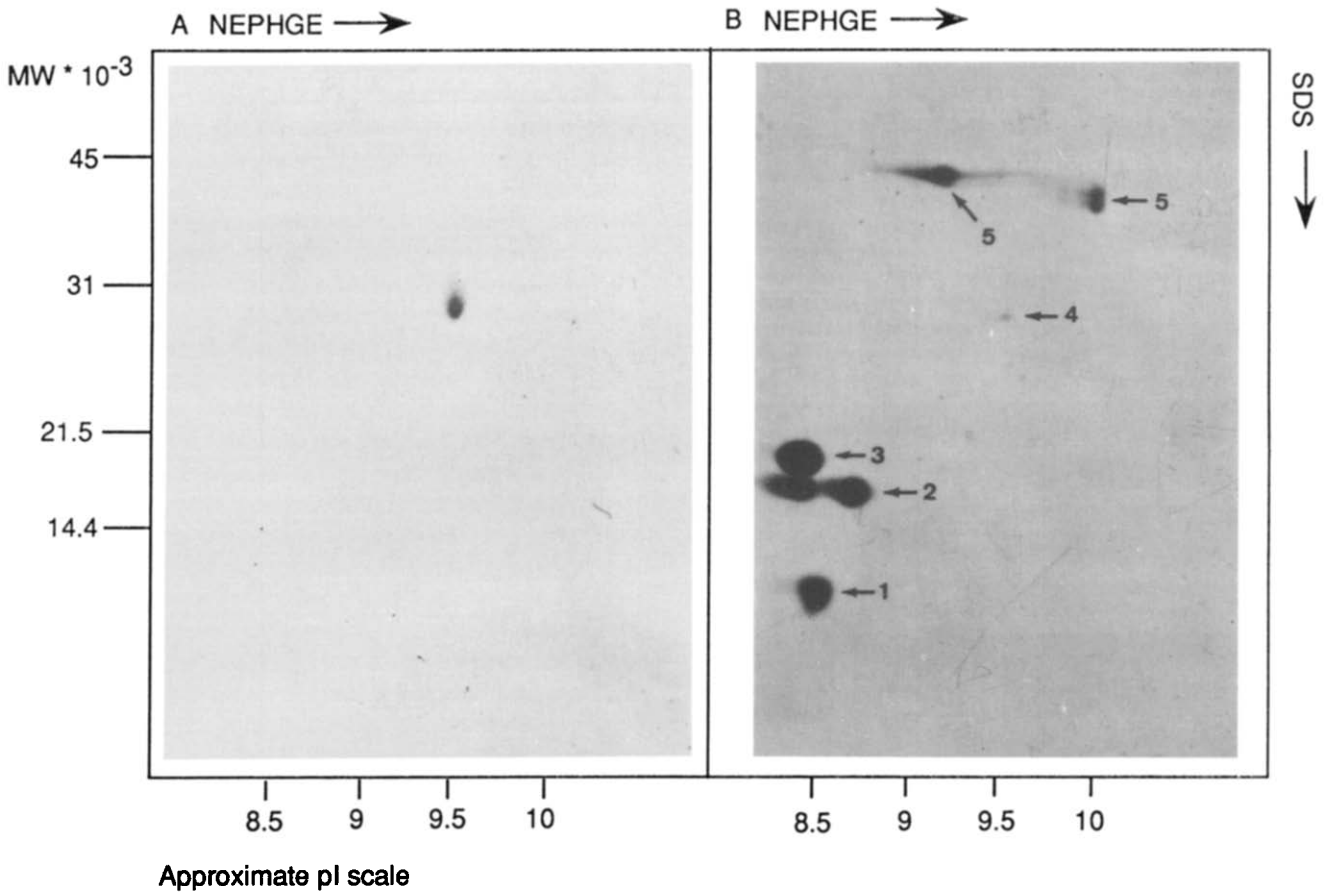

Fig. 1. (A) A specifically immunostained Western blot of nuclear proteins from the T-lymphoma Jurkat cells. Non-immune rabbit serum was used as a negative control. The positions of prestained molecular weight markers are indicated on the side of the gel (Bio-Rad). (B) NEPHGE/SDS-PAGE of the protein markers which are described in section 2: 1, FKBP12; 2, bovine cyclophilin; 3, *E. coli* cyclophilin; 4, FKBP25; 5, two isoforms of aspartate aminotransferase. The positions of molecular weight markers (Bio-Rad) and approximate pI scale are indicated on the sides of the gel.

dicted with the CD-constrained algorithm have revealed (see Fig. 3) that the protein could be divided into two domains [4]. The N-terminal domain (1 to 104) was predicted to comprise a number of α-helical segments, whereas the C-terminal domain (from 122 to 223) should have a chain fold similar to that of FKBP12 [18,19]. The predicted α-helices (7–19) and (33–50) are connected to each other by an extended loop-β-turn segment (21–32) and form an amphipathic helix–loop–helix (HLH). This characteristic DNA binding motif [20] occurs in the sequences of various transcription factors [21] and other DNA-binding proteins [20]. Furthermore, FKBP25 is a very hydrophilic protein (H_1 = 17.9% for FKBP25, H_i = 0% for histones whereas it is over 60% for membrane-associated proteins) and contains a number of Lys residues which may further stabilize the FKBP25/DNA complex. However, the CD spectra of a mixture of FKBP25 and double-stranded DNA have shown no significant change in protein conformation. Thus if the putative HLH motif exists in the N-terminal part of FKBP25 its binding to DNA is not accompanied by any significant change of the secondary structures of the immunophilin. However, the complex also could be due to charge–charge interaction between DNA and the multiple repeats of K and R residues separated by six or seven residues (see Fig. 3). The latter could also be aided by such elements as the NTS specific -KKKKNAK- sequence.

Different targets for FKBP12 and cyclophilin bound to their respective ligands have been identified [6,7,10]. These findings have led to several models accounting for the immunosuppressive actions exerted on T-cells by CsA, FK506 and rapamycin [6,7].

FK506 and CsA act early on T-cell activation cascade and stop the antigen induced transcription of the IL2 gene [6,7]. The drugs block cell transition from G_o to G_1 while they do not arrest cell proliferation. In contrast, rapamycin acts later in the T-cell activation cycle and arrests an IL2-receptor initiated T-cell progression from G_1 to the S phase. The latter process involves a large number of gene products essential for the cell division cycle. It has been established that FKBP25 has a higher affinity for rapamycin than for FK506 [4], yet the reason for this difference remains unclear. It could be of interest to explore the possibility of a linkage between highly specific binding of rapamycin to FKBP25 [4], the

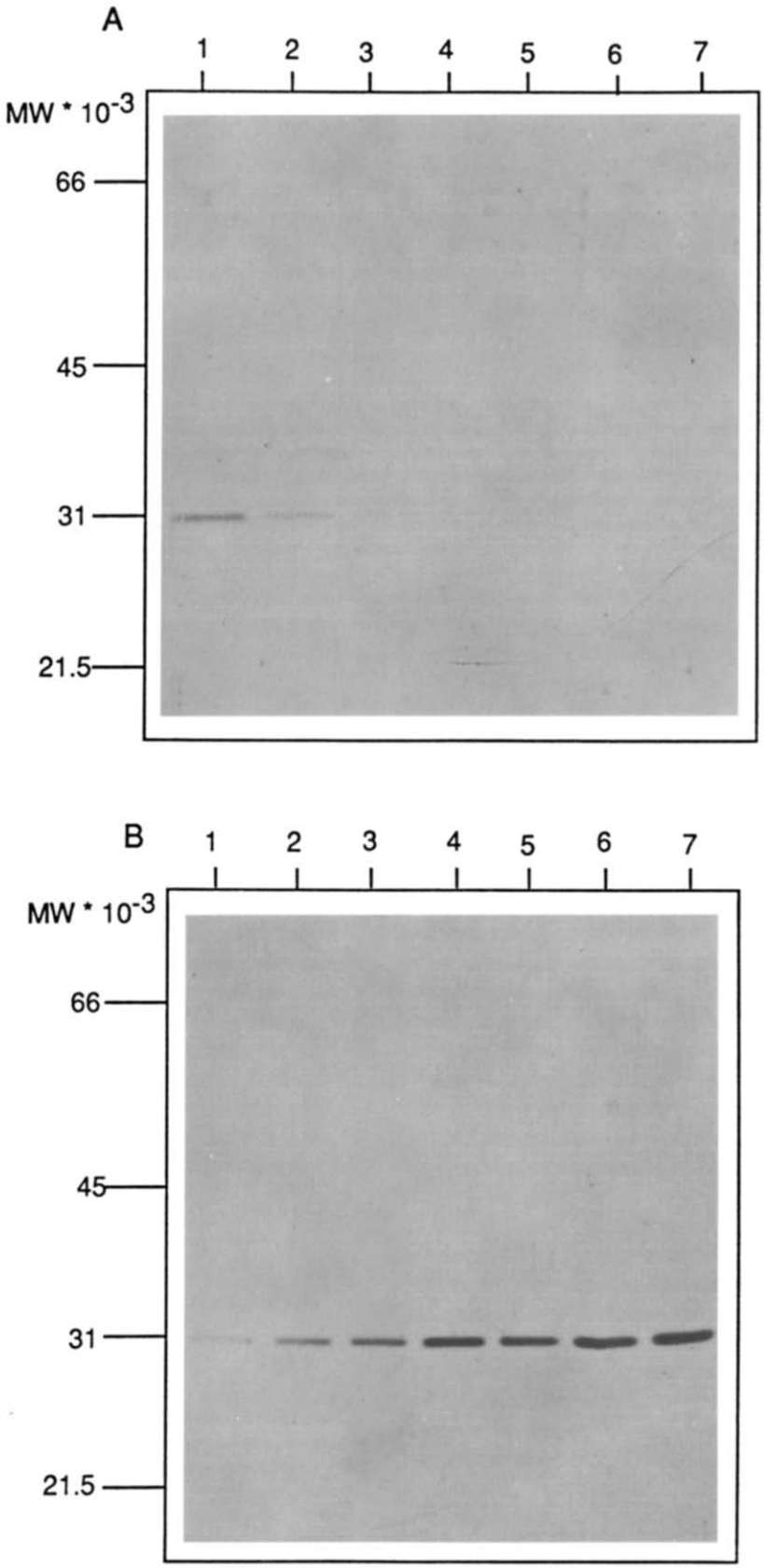

Fig. 2. (A) Lanes 1–7 show FKBP25 that was eluted from 7 DNA-affinity matrices after their treatment with the following concentrations of NaCl: 1 (500 mM), 2 (300 mM), 3 (250 mM), 4 (200 mM), 5 (150 mM), 6 (100 mM), 7 (50 mM). (B) FKBP25 retained on the respective 7 DNA-affinity matrices after their treatment with NaCl.

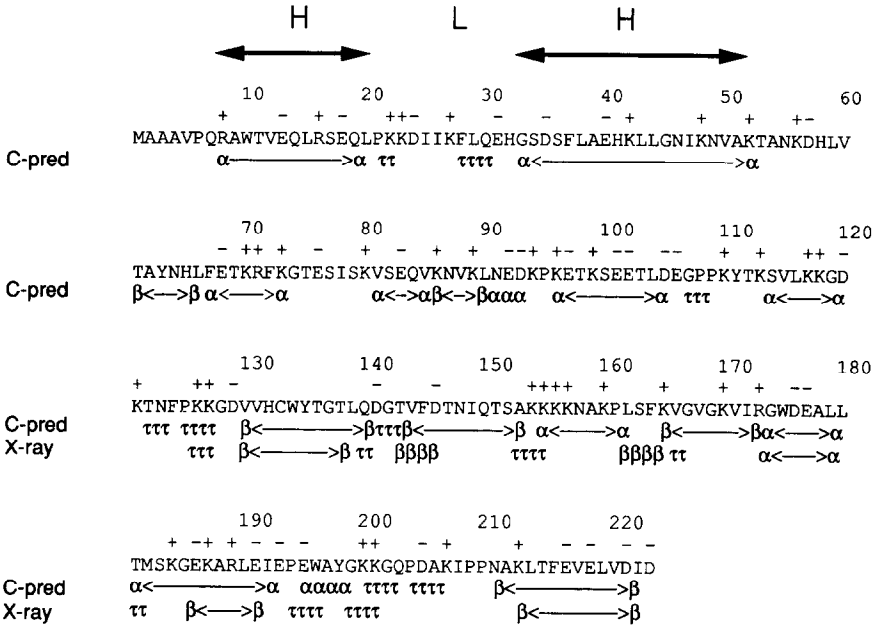

Fig. 3. CD-constrained prediction of the secondary structures (C-pred) in human FKBP25 [22]. Since the C-terminal part of FKBP25 shares considerable sequence homology to FKBP12 the X-ray assignment of its secondary structure (X-ray) is also given [19] ; τ, β-turn; α, α-helix; β, β-structure; $\leftrightarrow$ an extension of the structure.

ability of FKBP25 to bind to DNA, and the action of rapamycin on the cell division process.

Acknowledgements: We are indebted to Francoise Bouet for sequencing several proteins used as the NEPHGE markers, Patrick Héry for help to inject FKBP25 immunization mixtures to rabbits and B. Maillère, M. Léonetti and J. Grassi for advise on the preparation of the immunization mixtures.

REFERENCES

[1] Schreiber, S.L. (1991) Science 251, 283–287.
[2] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) Nature 341, 761–763.
[3] Siekerka, J.J., Hung, S.H.Y., Poe, M., Lin, S.C. and Sigal, N.H. (1989) Nature 341, 755–757.
[4] Galat, A., Lane, W.S., Standaert, R.F. and Schreiber, S.L. (1992) Biochemistry 31, 2427–2434.
[5] Fischer, G., Wittman-Liebold, B., Kang, K., Kiefhaber, T. and Schmid, F.X. (1989) Nature 337, 476–478.
[6] Sigal, N.H. and Dumont, F.J. (1992) Annu. Rev. Immunol. 10, 519–560.
[7] Schreiber, S.L. and Crabtree, G.E. (1992) Trends Biochem. Sci. 13, 136–142.
[8] Fisher, G., Bang, H. and Mech, C. (1984) Biomed. Biochem. Acta 43, 1101–1112.
[9] Handschumaker, R.E., Harding, M.W., Rice, J., Drugge, R.J. and Speicher, D.W. (1984) Science 226, 544–546.
[10] Schreiber, S.L. (1992) Cell 70, 365–368.
[11] Blum, H., Beier, H. and Gross, H.J. (1987) Electrophoresis 8, 93–99.
[12] Challberg, M.D. and Kelly Jr., T.S. (1979) Proc. Natl. Acad. Sci. USA 76, 655–659.
[13] Cornette, J.L., Cease, K.B., Margalit, H., Spouge, J.L., Berzofsky, J.A. and DeLisi, C. (1987) J. Mol. Biol. 195, 659–685.
[14] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[15] Mittnacht, S. and Weinberg, R.A. (1991) Cell 65, 381–393.
[16] Pognanec, P., Boulukas, K.E. and Ghysdael, J. (1989) Oncogene 4, 691–697.
[17] Kadonaga, J.T. (1991) in: Methods of Enzymology (R.T. Sauer, Ed.), Academic Press, New York, Vol. 208, pp. 10–23.
[18] Standaert, R.F., Galat, A., Verdine, G.L. and Schreiber, S.L. (1990) Nature 346, 671–674.
[19] Van Duyne, G.D., Standaert, R.F., Karplus, P.M., Schreiber, S.L. and Clardy, J. (1991) Science 252, 839–842.
[20] Steitz, T.A. (1990) Quart. Rev. Biophys. 23, 205–280.
[21] Murre, C., McCaw, P.S. and Baltimore, D. (1989) Cell 56, 777–783.
[22] Hung, D.T. and Schreiber, S.L. (1992) Biochem. Biophys. Res. Commun. 184, 733–738.