FEBS Letters 347 (1994) 31–36

FEBS 14115

Cyclophilin-B is an abundant protein whose conformation is similar to cyclophilin-A

Andrzej Galat*, Françoise Bouet

Département d'Ingénierie et d'Etudes des Protéines, D.S.V., C.E.A., C.E. Saclay, F-91191 Gif-sur-Yvette Cedex, France

Received 13 April 1994

Abstract

Cyclophilin-B (bCyP-20) was isolated in a relatively high quantity from calf brain and spleen tissues consecutively applying weak cation exchange, chromatofocusing and strong cation exchange chromatographies. Edman degradation yielded the N-terminal sequence NH_2-DEKKKGPKVTVK-VYFDLRIGDEDIGRVVIGLFGKTVPKTVDNFVAL. Bovine cyclophilin-B possesses the peptidylproline cis-trans isomerase activity which is inhibited by nM concentrations of CsA. bCyP-20 has a strong tendency to bind to cation exchangers including DNA and heparin. It could be released from DNA affinity column at concentrations of NaCl higher than 200 mM. Circular dichroism spectroscopy revealed that bovine cyclophilin-A (bCyP-18) and bCyP-20 in aqueous solution have similar conformations.

Key words: PPIase; Cyclophilin; Cyclosporin-A; Circular dichroism

1. Introduction

The mammalian group of cyclophilins consists of at least eight different proteins which show high sequence similarity of their peptidylproline cis-trans isomerase (PPIase) domains [1]. The PPIase activity of the majority of cyclophilins can be inhibited by nanomolar concentration of the clinically useful immunosuppressant cyclosporin-A (CsA) [1]. The apo forms of hCyP-18 and hCyP-20 bind to various proteins including the Gag protein of the HIV class II virus [2] whereas their complexes with CsA bind and inhibit the phosphatase activity of calcineurin [3–4]. The latter effect is believed to be a crucial step which effectively blocks early T-cell activation cascade and constitutes the base of the immunosuppressive activity of CsA and its derivatives [5]. CyP-18 was localized in the cytosol [6] in contrast to CyP-20 which was found in the endoplasmic reticulum associated with the highly acidic calcium-storage protein calreticulin [7]. The calmodulin-dependent phosphatase calcineurin which binds to CyP-18/CsA and CyP-20/CsA complexes with high affinities occurs both in the cytosol and membrane fraction [8]. It is thus possible that the CyP-18/CsA complex inhibits in vivo the activity of the cytosolic fraction of calcineurin which in turn prohibits translocation of the cytosolic form of nuclear factor of activated T-cells (NFATc) to the nucleus [5]. The membrane-associated fraction of calcineurin may thus bind in vivo the CyP-20/CsA complex which in turn may affect other cellular processes and lead to suppression/toxicity of various lymphoid and non-lymphoid cells.

In this communication we show that cyclophilin-B is an abundant protein in bovine brain tissues and that in aqueous solution it has a conformation similar to that of bCyP-18 and hCyP-18. This latter finding may explain strong in vitro inhibiton of the phosphatase activity of calcineurin by the CyP-20/CsA or CyP-18/CsA complexes [3,4] and may imply that both proteins have other common intracellular targets.

Materials and methods

2.1. Tissue sources, chemicals, chromatography materials and gel electrophoresis

Calf brain and spleen were supplied fresh on ice by the Henri Meunier slaughter house (Meaux, 77, France). The fresh tissue was shredded and frozen in liquid nitrogen. Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, *N,N,N',N'*-tetramethyle nediamine were purchased from Serva. Pharmalyte 8–10.5 and Ampholite 9–11 were from Pharmacia/LKB. Molecular mass markers were purchased from Bio-Rad. PBE118 and CM-sepharose were from Pharmacia/LKB. All other chemicals were analytical grade and purchased from either Serva or Sigma. SDS-PAGE, 2D (NEPHGE/SDS-PAGE) gels (12%, size 20 × 20 cm) and binding of bCyP-20 or bCyP-18 to calf thymus DNA and heparin affinity gels (Sigma) were performed according to the procedures described previously [9]. The peptidylproline cis-trans isomerase (PPIase) assay was performed at 10°C using the succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide tetrapeptide as a substrate [10].

2.2. Preparation of tissue samples and primary processing of soluble proteins

Frozen brain tissues were dry-homogenized with a Waring blender and dispersed in buffer containing 0.250 M NaCl, 10% glycerol, 50 mM phosphate, pH 7.2, 5 mM β-ME, 0.5% Triton X-100, 0.02% NaN_3 and 1 mM PMSF. The mixture was cleared from insoluble debris by centrifugation and the resulting supernatant was dialyzed against 50 mM phosphate buffer (pH 7.2) containing 10% (v/v) of ethanol, 2 mM β-ME

*Corresponding author. Fax: (33) (1) 6908 9137.

The N-terminal sequence of bCyP-20 has been deposited to the EMBL Sequence Data Bank with the following accession number EMBL:P80311. The prefixes (h,b,m) in front of cyclophilin (CyP) correspond to: h, human; b, bovine; m, murine; the numbers correspond to approximate molecular mass (kDa) of the protein.

SSDI 0014-5793(94)00501-L

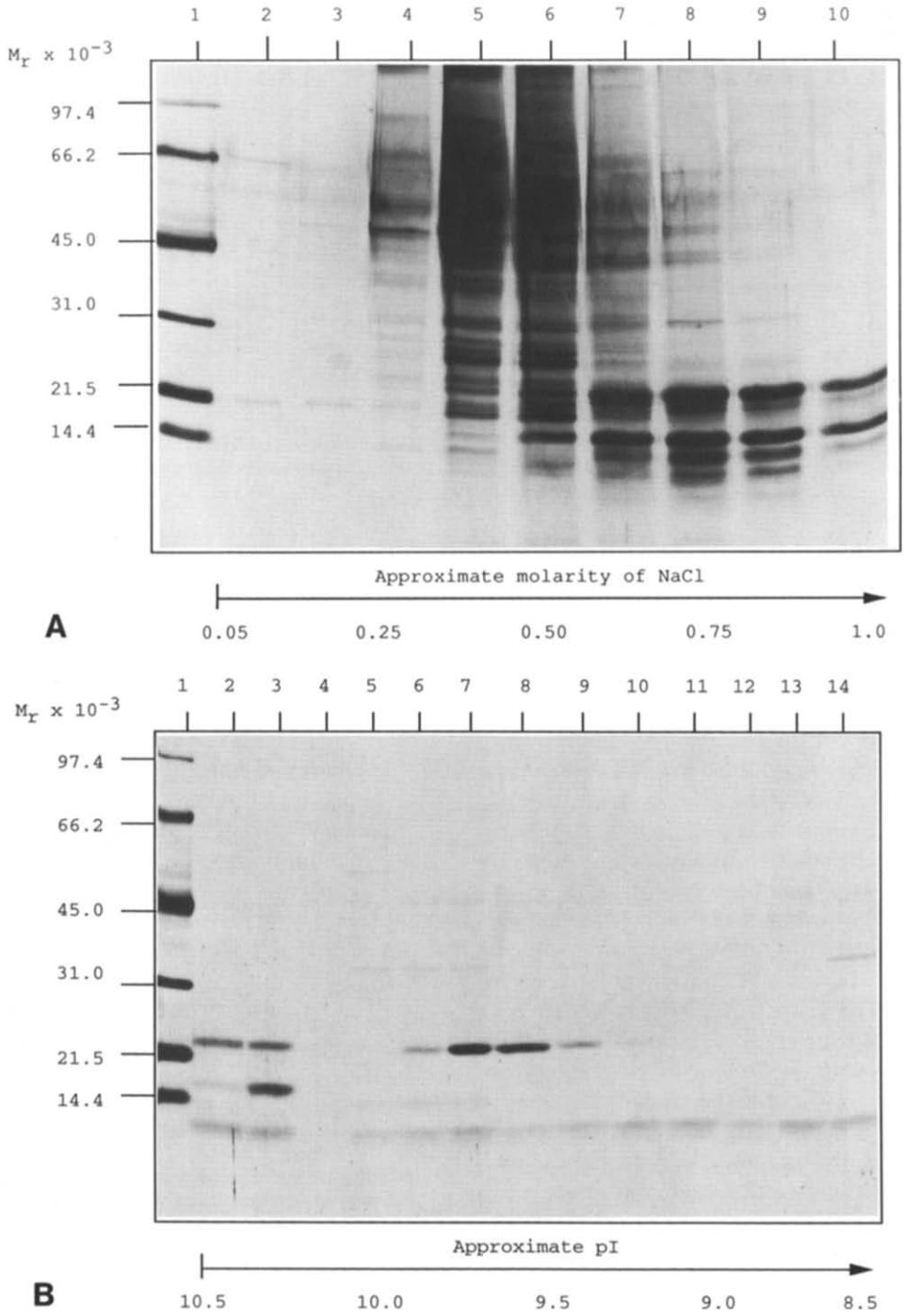

Fig. 1. SDS-PAGE analyses of the elution profiles of bCyP-20 from CM-sepharose and PBE118. (A) Aliquots of 9 different fractions from a CM-sepharose column were fractionated on a 12% SDS-PAGE and visualized by silver staining: lane 1, molecular mass markers; lanes 2–10, proteins which were eluted with increasing concentration of NaCl from 0.05 M to 1.0 M. (B) Aliquots from a PBE118 chromatofocusing column were resolved on a 12% SDS-PAGE gel: lane 1, molecular mass markers; lanes 2–14, proteins in fractions whose pHs decrease from 10.5 to 8.5; the majority of bCyP-20 was found in fractions 7 and 8.

and 1 mM PMSF. Proteins were loaded onto a CM-separose column (5 cm × 40 cm) and eluted with increasing concentration of NaCl in phosphate buffer.

2.3. Fractionation of basic proteins and final purification of bCyP-20

The mixture of proteins enriched with bCyP-20 was concentrated, followed by extensive dialyzis against 0.025 M triethylamine · HCl, pH 11 (PBE118 buffer). The dialized mixture of proteins was applied onto a PBE118 column (1.6 cm × 20 cm) which was equilibrated with 100 void volumes of the PBE118 buffer. Proteins were eluted with the Ampholite 8–10.5 (1:45 v/v in water, pH 8.0). The fractions (8 ml each) were collected at a speed of about 80 ml per hour. The pH of each fraction was measured with a pH electrode. The fractions containing bCyP-20 were dialized against 25 mM phosphate buffer and applied

directly onto a MonoS (Pharmacia) column equilibrated with 25 mM phosphate, pH 7.2. bCyP-20 was eluted in a gradient of salt from 25 mM to 1 M NaCl.

2.4. Sequencing of proteins and computer analyses of protein sequences

The N-terminal sequences of bCyP-20 and bCyP-18 were established by Edman degradation using an Applied Biosystems Model 477A protein sequencer. Amino acid compositions were established with an Applied Biosystem 420A derivatizer. rmsd is the root-mean-square-difference of amino acid compositions of proteins A and B calculated according to equation (1) for $n = 16$ (see Table 1),

$$\text{rmsd} = \{1/n \sum_{i=1}^{n} [P_{Ai}(\%) - P_{Bi}(\%)]^2\}\ 1/2 \quad (1)$$

where P_A and P_B are the percentages of each amino acid in proteins A and B. The sequences of cyclophilins were extracted from the MIPSX, PIR and EMBL protein data-banks (rel. 36, 37 and 28, containing 73,891, 56,849 and 36,000 protein sequences, respectively) using the SEQPRO program [10]. Total hydrophobicity index (H_i) was previously defined [1].

2.5. Circular dichroism spectroscopy

Circular dichroism spectra (CD) were recorded with a Jobin-Yvon Mark VI dichrograph. The spectra were measured with quartz squared cuvettes at room temperature in 20 mM phosphate, pH 7.3. Data were averaged over 10 repetitive scans. Protein concentrations were estimated by means of molar extinction coefficients at λ 278 nm, ε = 8,400 and ε = 15,000 $M^{-1} \cdot cm^{-1}$ for bCyP-18 and bCyP-20, respectively. The mean molecular ellipticity θ was calculated using a mean residual mass of 111 and expressed in deg · cm^2 · $dmol^{-1}$ units.

Table 1
Amino acid compositions of hCyP-20, bCyP-20 and bCyP-18

No	Amino acid*	hCyP-20	bCyP-20	bCyP-18
1	Ala	4.4 (8)	5.7 (10)	5.7 (9)
2	Asx	11.1 (20)	10.8 (20)	10.7 (17)
3	Arg	3.9 (7)	3.7 (7)	3.8 (6)
4	Glx	7.2 (13)	7.7 (14)	8.2 (13)
5	Gly	12.8 (23)	12.7 (23)	14.6 (24)
6	His	1.7 (3)	1.4 (3)	2.5 (4)
7	Ile	5.6 (10)	5.5 (10)	7.0 (11)
8	Leu	3.9 (7)	5.0 (9)	3.8 (6)
9	Lys	13.9 (25)	12.7 (23)	8.7 (14)
10	Met	1.7 (3)	1.4 (3)	2.5 (4)
11	Phe	7.8 (14)	7.9 (14)	9.5 (15)
12	Pro	3.3 (6)	4.2 (8)	3.8 (6)
13	Ser	3.3 (6)	2.9 (5)	5.0 (8)
14	Thr	6.7 (12)	7.6 (14)	7.0 (11)
15	Tyr	2.2 (4)	2.3 (4)	1.3 (2)
16	Val	10.6 (19)	8.4 (15)	5.7 (9)
17	Total**	183 (180)		163 (158)

*The amino acid compositions (expressed in %) were calculated without taking into account Cys and Trp residues.
**Total is the number of amino acid residues in the processed proteins while the number of residues used to calculate % is given in parantheses.

3. Results and discussion

Cyclophilin-B was isolated from bovine brain tissues using the following three consecutive steps: (i) trapping of proteins on a weak cation exchanger; (ii) sorting of proteins according to their pIs on a PB118 chromatofocusing column; (iii) final purification on a strong cation exchanger (see Fig. 1A,B). From 1 kg of dry brain matter we purified nearly 5 mg of bCyP-20. Only one isoform of bCyP-20 with pI ca. 9.8 (see Fig. 2A) was found in calf brain tissues. On a 12% SDS-PAGE gel bCyP-20 migrates as a protein with M_r of 22 kDa (see Fig. 2B). bCyP-20 has the highest pI in the cyclophilin family of proteins and is flanked on NEPHGE/SDS gels only by the high pI ribosomal proteins [12]. bCyP-20 has relatively strong affinity to cation exchangers including DNA and heparin. Both the apo form of bCyP-20 and the bCyP-20/CsA complex bind to DNA (see Fig. 3) and heparin whereas bCyP-18 does not bind well to cation exchangers (data not shown).

Edman degradation yielded the N-terminal sequence NH_2-DEKKKGPKVTVKVYFDLRIGDEDIGRVVI-GLFGKTVPKTVDNFVAL- which is very similar to those of human cyclophilin-S [13], murine cyclophilin-S

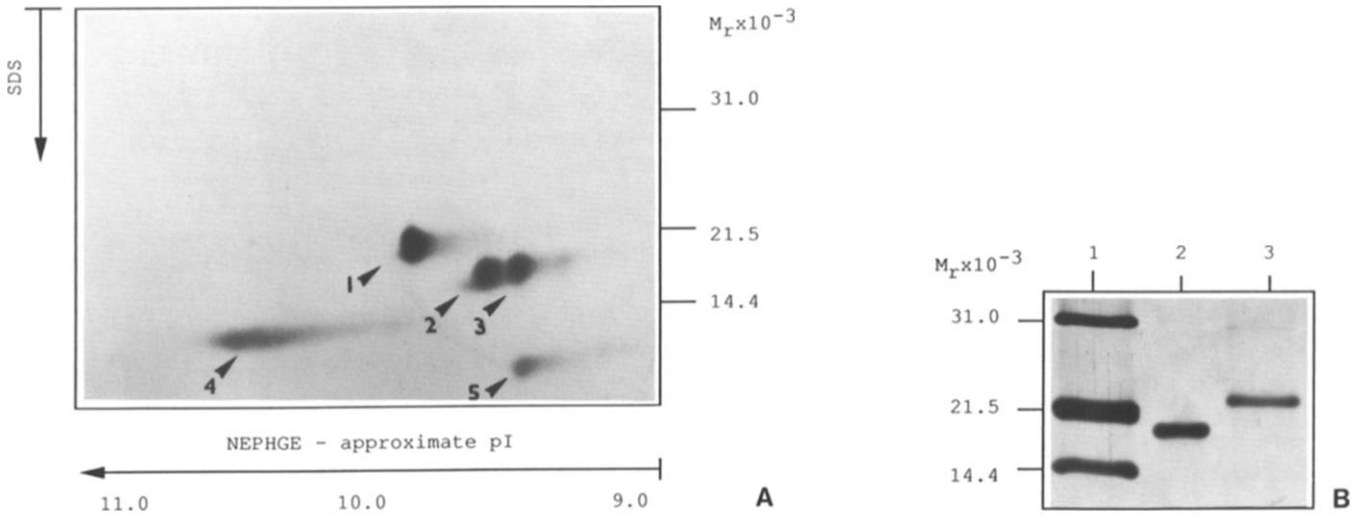

Fig. 2. (A) NEPHGE/SDS-PAGE of the following proteins: 1, bCyP-20; 2 and 3, two isoforms of bCyP-18; 4, lysozyme; 5, bovine macrophage migration inhibitory factor [11]. The positions of molecular mass markers and approximate pI scale are indicated on the sides of the gel; (B) Relative migration on a 12% SDS-PAGE gel of bCyP-18 (lane 2) vs. bCyP-20 (lane 3); lane 1, molecular mass markers.

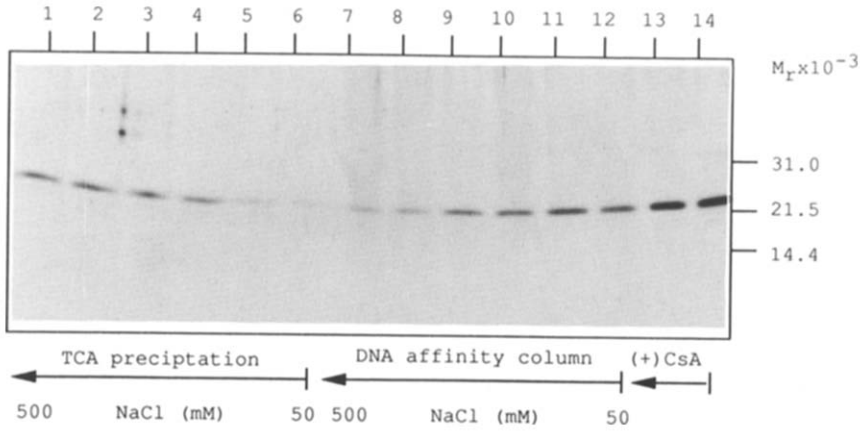

Fig. 3. SDS-PAGE gel of bCyP-20 eluted/retained by DNA affinity gels. Lanes 1–6, bCyP-20 eluted from DNA affinity gels with decreasing concentration of NaCl; lanes 7–12, bCyP-20 retained by DNA affinity gels treated with increasing concentration of NaCl: 7 (500 mM), 8 (300 mM), 9 (200 mM), 10 (150 mM), 11 (100 mM), 12 (50 mM); lane 13, the bCyP-20/CsA complex bound to DNA affinity gel; lane 14, CsA binding to the preformed bCyP-20/DNA complex.

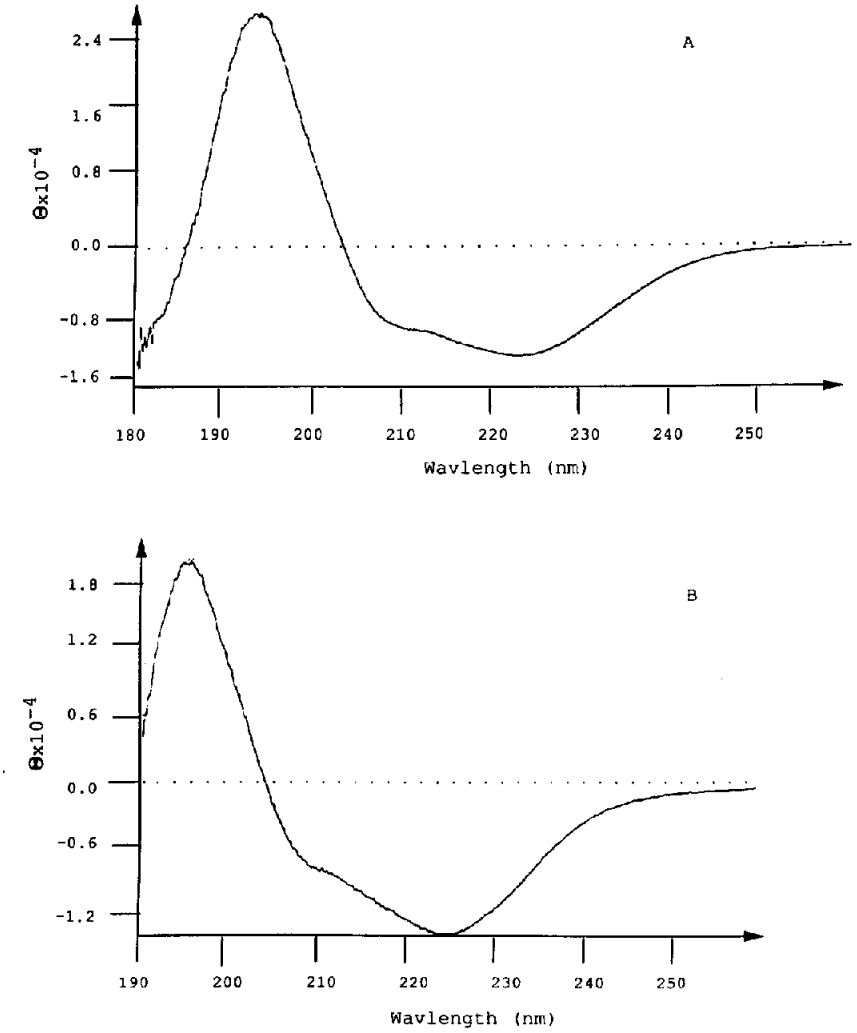

Fig. 4. Circular dichroism spectra: (A) bCyP-18; (B) bCyP-20.

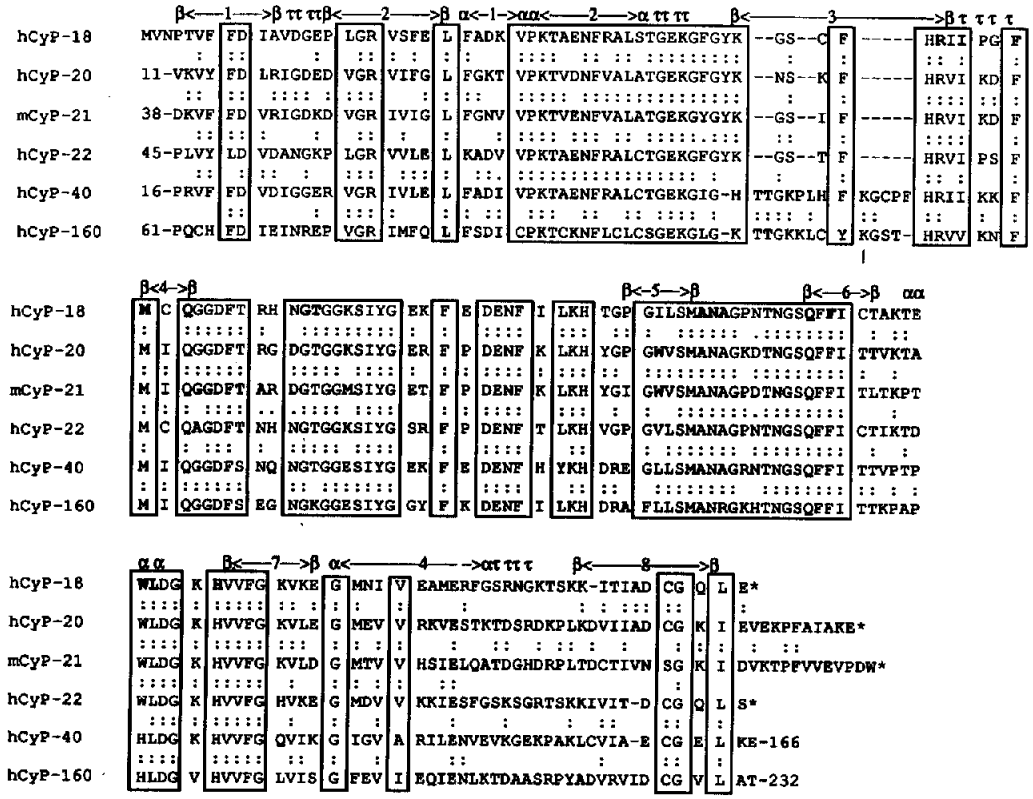

Fig. 5. Alignment of several sequences of the cyclophilin family of proteins; the stars indicate the C-terminal amino acid. The sequences come from the following sources: (1) hCyP-18, GB:X52851 [20]; (2) hCyP-20, GB:M63573 [21]; (3) mCyP-23, GB:M74227 [22]; (4) hCyP-22, GB:M80254 [23]; (5) hCyP-40, GB:L11667 [24]; (6) hCyP-150, GB:LH04888 [25]. The consecutively numbered secondary structures were taken from the X-ray structure of hCyP-18 elucidated by Ke [19]; α–α-helix, β–β-structure, τ–β-turn, $\leftrightarrow$ an extension of the structure. The side chains of hCyP-18 residues (bold face) contact CsA [27–28].

[14] and chicken cyclophilin-S [15]. There are only two conservative substitutions in the N-terminal part of bCyP-20 vs. hCyP-20, namely 24V/24I and 28I/28V. Likewise, the amino acid composition of bCyP-20 is very similar to that of hCyP-20, rmsd = 0.8355 (see Table 1). In contrast, the amino acid composition of bCyP-18 differs significantly from that of bCyP-20 (rmsd = 1.6263). bCyP-20 has PPIase activity which is inhibited by CsA at nM concentrations. Circular dichroism spectra of bCyP-18 and bCyP-20 show very similar patterns (see Fig. 4) which may suggest that in aqueous solution both proteins have nearly identical three-dimensional structures. Furthermore, the CD spectra of periplasmic CyP-18 from *Escherichia coli* [16] is similar to those of bCyP-18, hCyP-18 and bCyP-20. A recent NMR study on the periplasmic CyP-18 from *E. coli* [17] and the X-ray structure of the mCyP-23/CsA complex [18] revealed that both proteins have structures very similar to that of hCyP-18 [19].

Sequence alignment of peptidylproline cis-trans isomerase domains of mammalian cyclophilins indicate for remarkable sequence conservancy (see Fig. 5) which could result from a universal requirement imopsed on this domain to catalyze in vivo protein folding and stabilize nascent polypeptide chains. The cDNA of hCyP-20 contains a signal sequence [21] which should translocate the protein to the endoplasmic reticulum. The processed protein does not possess the signal sequence and begins at the amino acid similar to that established by Edman degradation for mCyP-20(s) [26]. It is likely however, that both due to the high hydrophobicity index of the cyclophilin family of proteins, H_i = 36–55 [1], and the tendency to bind to weak cation exchangers very basic mammalian CyP-20's could be associated with the hydrophobic/polar interfaces of various membranes. Moreover, the hCyP-20/CsA complex has even higher affinity for calcineurin than the hCyP-18/CsA complex itself [4] which may suggest that these complexes can effectively compete with each other under in vivo conditions. Relatively high content of CyP-20 in bovine tissues is concomitant with an equally high content of calcineurin. Since CyP-20 is one of the major components of the PPIase family of proteins in the brain, its complex with CsA may cause serious neurotoxicity. Morover, mammalian CyP-20s may play a variety of functions in cellular milieu including its involvement in the signaling pathways which comprise both the cytosolic and membrane-bound calcineurins.

Acknowledgements: We are indebted to Prof. B. Ryffel for cyclosporin-A and Dr. André Ménez for encouragement.

References

[1] Galat, A. (1993) Eur. J. Biochem. 216, 689–707.
[2] Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V. and Goff, S.P. (1993) Cell 73, 1067–1078.
[3] Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[4] Swanson, S.K.-H., Born, T., Zydowsky, L.D., Cho, H., Chang, H.Y., Walsh, C.T. and Rusnak, F. (1992) Proc. Natl. Acad. Sci. USA 89, 3741–3745.
[5] MacCaffrey, P.G., Luo, C., Kerppola, T.K., Jain, J., Badalian, T.M., Ho, A.M., Burgeon, E., Lane, W.S., Lambert, J.N., Curran, T., Verdine, G.L., Rao, A. and Hogan, P.G. (1993) Science 262, 750–754.
[6] Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J. and Speicher, D.W. (1984) Science 226, 544–546.
[7] Arber, S., Krause, K.-H. and Caroni, P. (1992) J. Cell. Biol. 116, 113–125.
[8] Alexander, D.R., Hexham, J.M. and Crumpton, M.J. (1988) Biochem. J. 256, 885–892.
[9] Rivière, S., Ménez, A. and Galat, A. (1993) FEBS Lett. 315, 247–251.
[10] Galat, A., Lane, W.S., Standaert, R.F. and Schreiber, S.L. (1992) Biochemistry 31, 2427–2434.
[11] Galat, A., Rivière, S. and Bouet, F. (1993) FEBS Lett. 319, 233–236.
[12] Celis, J.E., Rasmussen, H.H., Madsen, P., Leffers, H., Honoré, B., Dejgaard, K., Gesser, B., Olsen, E., Gromov, P., Hoffman,, H.J., Nielsen, M., Celis, A., Basse, B., Lauridsen, J.B., Ratz, G.P., Nielsen, H., Andersen, A.H., Walbum, E., Kjaergaard, I., Puype, M., van Damme, J. and Vandekerckhove, J. (1992) Electrophoresis 13, 893–959.
[13] Spik, G., Haendler, B., Delmas, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H.P., Keller, R., Hiestand, P.C. and Movva, N.R. (1991) J. Biol. Chem. 266, 10735–10738.
[14] Hasel, K.W., Glass, J.R., Godbout, M. and Sutcliffe, J.G. (1991) Mol. Cell. Biol. 11, 3484–3491.
[15] Caroni, P., Rothenfluh, A., McGlynn, E. and Schneider, C. (1991) J. Biol. Chem. 266, 10739–10742.
[16] Compton, L.A., Davis, J.M., MacDonald, J.R. and Bachinger, H.P. (1992) Eur. J. Biochem. 206, 927–934.
[17] Clubb, R.T., Thanabal, V., Fejzo, J., Ferguson, S.B., Zydowsky, L., Baker, C.H., Walsh, C.T. and Wagner, G. (1993) Biochemistry 32, 6391–6401.
[18] Ke, H., Zhao, Y., Luo, F., Weissman, I. and Friedman, J. (1993) Proc. Natl. Acad. Sci. USA 90, 11850–11854.
[19] Ke, H. (1992) J. Mol. Biol. 228, 539–550.
[20] Haendler, B., Hofer-Warbinek, R. and Hofer, E. (1987) EMBO J. 6, 947–950.
[21] Price, E.R., Zydowsky, L.D., Jin, M.J., Baker, C.H., McKeon, F.D. and Walsh, C.T. (1991) Proc. Natl. Acad. Sci. USA 88, 1903–1907.
[22] Friedman, J. and Weissman, I. (1991) Cell 66, 799–806.
[23] Bergsma, D.J., Eder, C., Gross, M., Kersten, H., Sylvester, D., Appelbaum, E., Cusimano, D., Livi, G.P., McLaughlin, M.M., Kasyan, K., Porter, T.G., Silverman, C., Dunnington, D., Hand, A., Prichett, W.P., Bossard, M.J., Brandt, M. and Levy, M.A. (1991) J. Biol. Chem. 266, 23204–23214.
[24] Kieffer, L.J., Seng, T.W., Li, W., Osterman, D.G., Handschumacher, R.E. and Bayney, R.M. (1993) J. Biol. Chem. 268, 12303–12310.
[25] Anderson, S.K., Gallinger, S., Roder, J., Frey, J., Young, H.A. and Ortaldo, J.R. (1993) Proc. Natl. Acad. Sci. USA 90, 542–546.
[26] Schumacher, A., Schroter, H., Multhaup, G.V. and Nordheim, A. (1991) Biophys. Biochem. Acta 1129, 13–22.
[27] Pflugl, G., Kallen, J., Schirmer, T., Jansonius, J.N., Zurini, M.G.M. and Walkinshaw, M.D. (1993) Nature 361, 91–94.
[28] Theriault, Y., Logan, T.M., Meadows, R., Yu, L., Olejniczak, E.T., Holzman, T.F., Simmer, R.L. and Fesik, S.W. (1993) Nature 361, 88–91.