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Complementary DNA for human T-cell cyclophilin

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Complementary DNA encoding human cyclophilin, a specific cyclosporin A-binding protein, has been isolated from the leukemic T-cell line Jurkat and sequenced. Comparison of the deduced amino acid sequence with the previously determined sequence of bovine thymus cyclophilin reveals only three differences: an additional amino acid at the carboxy terminus end and two internal changes. RNA transfer blot analysis indicates an mRNA size of ~1 kb for human T-cell cyclophilin. Phytohaemagglutinin and phorbol myristate acetate induction of T cells treated or not with cyclosporin A affects only marginally the level of cyclophilin mRNA. Southern blot analysis of human genomic DNA digested with different restriction enzymes strongly suggests the existence of a multigene family for cyclophilin.

Key words: cyclosporin A/cyclophilin/T-cell cDNA library

Introduction

In recent years cyclosporin A (CsA), a cyclic undecapeptide of fungal origin, has become recognized as one of the most useful therapeutic agents for the prevention of graft rejection. It is widely employed in kidney, heart, liver and bone marrow transplantation. In addition, its potential use in the treatment of certain autoimmune diseases is under clinical investigation (for a review see Beveridge, 1986). It appears to act primarily on the T-cell compartment (Shevach, 1985), but inhibition of B cells (Maraguchi *et al.*, 1983), hepatocellular toxicity, nephrotoxicity (Myers *et al.*, 1984) and disturbances in the central nervous system (Atkinson *et al.*, 1984) have also been documented. Its immunosuppressive action is thought to result at least in part from a specific and complete inhibition of lymphokine mRNA production in helper T cells (Elliott *et al.*, 1984; Krönke *et al.*, 1984). However, the induction of the interleukin-2 (IL-2) receptor mRNA is at the most only partially inhibited by CsA (Krönke *et al.*, 1984) and there appears to be little or no direct effect on T-cell proliferation in the presence of exogenously added IL-2 (Herold *et al.*, 1986).

A cytosolic protein with binding activity for CsA has recently been identified (Handschumacher *et al.*, 1984). This protein, termed cyclophilin, was first isolated from bovine thymus and later from human spleen. Two isoforms were identified in both cases. The complete amino acid sequence of the bovine protein has been established (Harding *et al.*, 1986) and it shows no significant homology to any other protein sequenced so far. The 72 amino-terminal amino acids of human spleen cyclophilin have also been determined and they are identical to the corresponding part in the bovine protein. This high evolutionary conservation together with a broad tissue and species distribution (Koletsky *et al.*, 1986) suggests that cyclophilin has an important functional role in the cell.

It has been proposed that cyclophilin is involved in the signal transduction from surface receptors to the nuclei and that binding to CsA interferes with this process, due to competition with a naturally occurring ligand (Harding *et al.*, 1986). However, binding of the drug to the prolactin receptor (Larson, 1986) and to calmodulin (Colombani *et al.*, 1985) has also been described and the primary role of cyclophilin in the mechanism of action of CsA remains to be clearly defined.

Further understanding of the function of cyclophilin in the cell would be facilitated by the availability of its complementary DNA clone. This would allow production of the large amounts of recombinant cyclophilin necessary for the detailed study of its binding properties to CsA. It would also be useful in the search for a putative natural ligand. We report here the molecular cloning and sequencing of the cDNA encoding human cyclophilin. A labelled probe transcribed from this cDNA detects one major mRNA species in uninduced, induced and CsA-treated cells. Finally, genomic DNA hybridization analysis shows the possible existence of multiple genes coding for cyclophilin.

Results

Isolation of cDNA clones for human T-cell cyclophilin

A cDNA library was constructed in the COS cell expression vector p91023B (Wong *et al.*, 1985) from poly(A)$^+$ mRNA isolated from induced Jurkat cells. The library was screened with two probes deduced from the published amino-terminal amino acid sequence of human cyclophilin (Harding *et al.*, 1986). The nucleotide sequence of the probes was based on the preferred codon usage in mammals, taking into account the relative infrequency of CG pairs in eukaryotic DNA (Lathe, 1985) and inverted repeats were avoided (Figure 1). Two positively hybridizing clones with inserts of similar length (700–800 bp) were detected by both probes. They were subcloned into the pBS-M13 sequencing vector.

Nucleotide sequence of human cyclophilin cDNA and deduced amino acid sequence

The restriction map and sequencing strategy for both cDNA clones (pBS-CYC21 and pBS-CYC5) are shown in Figure 2 (bottom). The nucleotide sequence of the insert in the pBS-CYC21 clone, as determined by sequencing the full length of both its strands, is indicated in Figure 2 (top). The sequence starts with a G which we believe to be part of the transcribed AUG initiation codon as in the pBS-CYC5 clone. This clone was found to contain an insert longer by 16 nucleotides at the 5′ end, with a complete transcribed AUG codon lying in a favourable position for translation initiation according to the rules defined by Kozak (1986). No difference was found further in the sequence except at position +6, where a deoxycytidine replaces a deoxythymidine. This single nucleotide difference does not alter the deduced amino acid sequence and may constitute an allelic difference. An open reading frame coding for 165 amino acids was determined from the nucleotide sequence. Positions 2–73 entirely match the published partial sequence of human cyclophilin (position 1 comes from the initiator Met codon). The region extending from residue 74

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                  58                          66
                 ProGlyPheMetCysGlnGlyGlyAsp
Probe A: 3' GGICCIAAATACACAGTICCICCICT 5'
                       G     G

                  7                                                    28
                 PhePheAspIleAlaValAspGlyGluProLeuGlyArgValSerPheGluLeuPheAlaAspLys
                     *         *  *  *  *     **     *  *  *                 *
Probe B: 5' TTCTTTGACATTGCTGTGGATGGTGAGCCTCTGGGCAGG 3'
                                                3' GGAGACCCGTCCCACAGGAAACTCGACAAACGACTGTTC 5'
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Fig. 1. Oligonucleotide probes. The two probes were based on the amino acid sequence shown. Mismatches between the predicted and observed nucleotide sequences are indicated with an asterisk. Probe A was a mixture of four 26-mers. Deoxyinosine residues or deoxyadenosine and deoxyguanosine residues were inserted at ambiguous positions as indicated. Probe B consisted of two overlapping 39-mers with 12 complementary nucleotides at their 3′ end. After annealing and filling in with the Klenow fragment of DNA polymerase I, two 66-mers corresponding to the coding or non-coding strands were obtained.

```
                1                15                30
GTGTACTATT AGCC AT   C
                  G GTT AAC CCC ACC GTG TTC TTC GAC ATT GCC GTC GAC GGC
                 MET Val Asn Pro Thr Val Phe Phe Asp Ile Ala Val Asp Gly

 45                60                75                90
GAG CCC TTG GGC CGC GTC TCC TTT GAG CTG TTT GCA GAC AAG GTC CCA AAG ACA
Glu Pro Leu Gly Arg Val Ser Phe Glu Leu Phe Ala Asp Lys Val Pro Lys Thr

      105               120               135               150
GCA GAA AAT TTT CGT GCT CTG AGC ACT GGA GAG AAA GGA TTT GGT TAT AAG GGT
Ala Glu Asn Phe Arg Ala Leu Ser Thr Gly Glu Lys Gly Phe Gly Tyr Lys Gly

                165               180               195
TCC TGC TTT CAC AGA ATT ATT CCA GGG TTT ATG TGT CAG GGT GGT GAC TTC ACA
Ser Cys Phe His Arg Ile Ile Pro Gly Phe MET Cys Gln Gly Gly Asp Phe Thr

    210               225               240               255
CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC TAT GGG GAG AAA TTT GAA GAT GAG
Arg His Asn Gly Thr Gly Gly Lys Ser Ile Tyr Gly Glu Lys Phe Glu Asp Glu

          270               285               300
AAC TTC ATC CTA AAG CAT ACG GGT CCT GGC ATC TTG TCC ATG GCA AAT GCT GGA
Asn Phe Ile Leu Lys His Thr Gly Pro Gly Ile Leu Ser MET Ala Asn Ala Gly

315               330               345               360
CCC AAC ACA AAT GGT TCC CAG TTT TTC ATC TGC ACT GCC AAG ACT GAG TGG TTG
Pro Asn Thr Asn Gly Ser Gln Phe Phe Ile Cys Thr Ala Lys Thr Glu Trp Leu

      375               390               405               420
GAT GGC AAG CAT GTG GTG TTT GGC AAA GTG AAA GAA GGC ATG AAT ATT GTG GAG
Asp Gly Lys His Val Val Phe Gly Lys Val Lys Glu Gly MET Asn Ile Val Glu

          435               450               465
GCC ATG GAG CGC TTT GGG TCC AGG AAT GGC AAG ACC AGC AAG AAG ATC ACC ATT
Ala MET Glu Arg Phe Gly Ser Arg Asn Gly Lys Thr Ser Lys Lys Ile Thr Ile

    480               495               508       518       528
GCT GAC TGT GGA CAA CTC GAA TAA  GTTTGACTTG TGTTTTATCT TAACCACCAG
Ala Asp Cys Gly Gln Leu Glu

      538       548       558       568       578       588
ATCATTCCTT CTGTAGCTCA GGAGAGCACC CCTCCACCCC ATTTGCTCGC AGTATCCTAG

      598       608       618       628       638       648
AATCTTTGTG CTCTCGCTGC AGTTCCCTTT GGGTTCCATG TTTTCCTTGT TCCCTCCCAT

      658       668       678       688       698       708
GCCTAGCTGG ATTGCAGAGT TAAGTTTATG ATTATGAAAT AAAAACTAAA TAACAATTGT C
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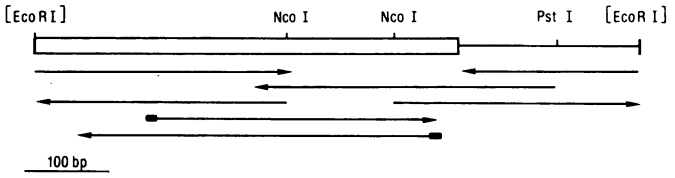

Fig. 2. (Top) Nucleotide and predicted amino acid sequences of human cyclophilin. The complete nucleotide sequence of the pBS-CYC21 insert is indicated. The additional nucleotides (position −14 to +2) and the difference (position +6) found in the pBS-CYC5 insert are also shown (higher line). The consensus signal for polyadenylation is underlined. **(Bottom)** Sequencing strategy and partial restriction map of the cDNA insert in pBS-CYC21. The open box represents the coding region. Arrows indicate the direction and extent of sequencing by the dideoxy chain termination method. Synthetic 17-mer primers complementary to the cDNA sequence are represented by closed boxes attached to some arrows. Only the restriction sites used for the subcloning are shown. The *Eco*RI sites in brackets were generated during the cloning procedure. Scale, 100 bp.

to the carboxy-terminal end is only known for the bovine protein (Harding *et al.*, 1986). Comparison with the sequence deduced from human cyclophilin cDNA shows three discrepancies: Asp-84 and Ile-164 are Glu and Leu respectively in the human protein which furthermore has an additional amino acid residue (Glu-165). The determined 3′ non-coding region contains 214 bp. A consensus polyadenylation signal AATAAA is located at positions 686–691.

RNA transfer blot analysis

Using a ^{32}P-labelled RNA probe transcribed from the pBS-CYC21 clone, we analysed Jurkat cell poly(A)$^+$ RNA by RNA transfer blotting under high-stringency conditions. One major band of 1000 nucleotides and two minor bands of ~1800 nucleotides were revealed (Figure 3). The size of the major band detected is compatible with that of the cyclophilin cDNA which lacks the poly(A)$^+$ tract and possibly part of the 5′ untranslated region. The minor bands may either correspond to less represented species or to a precursor form. Poly(A)$^+$ RNA isolated from uninduced or induced cells revealed the same bands. The amount of poly(A)$^+$ RNA hybridizing with the probe is only slightly higher in the case of lectin and phorbol ester-induced T cells. Treatment with CsA prior to the induction slightly diminished the level of cyclophilin mRNA (Figure 3).

Southern blot analysis

To investigate the genomic complexity of the cyclophilin genes, Southern blot analysis was performed. Multiple bands are detected in human DNA by high-stringency hybridization using the T3 RNA polymerase transcript of the pBS-CYC21 insert as a probe (Figure 4). Under the same conditions an IL-2 probe revealed one or two bands, as expected for a single copy gene (Fujita *et al.*, 1983). The finding that many discrete restriction fragments of DNA digested with four different enzymes hybridize to the cyclophilin probe is consistent with the existence of multiple functional genes coding for the different isoforms, but it is also possible that some of the hybridizing species represent pseudogenes. Further experiments using probes derived from the *Eco*RI–*Nco*I or the *Nco*I–*Pst*I fragment of the cyclophilin cDNA gave similar results (data not shown). Since the *Eco*RI–*Nco*I fragment represents part of the coding region only, the hybridization pattern truly represents cyclophilin sequences and is not an artifact caused by the possible existence of a repetitive sequence in the 3′ untranslated sequence.

Discussion

Despite extensive clinical use of CsA, the exact mode of action of this drug has so far not been fully established. It has been argued that it involves binding to calmodulin (Colombani *et al.*, 1985) or to the prolactin receptor (Larson, 1986). However, cyclophilin has been shown to constitute the largest pool for CsA-binding activity in the cell, but its cytosolic concentration (Koletsky *et al.*, 1986) is rather high for a protein with regulatory func-

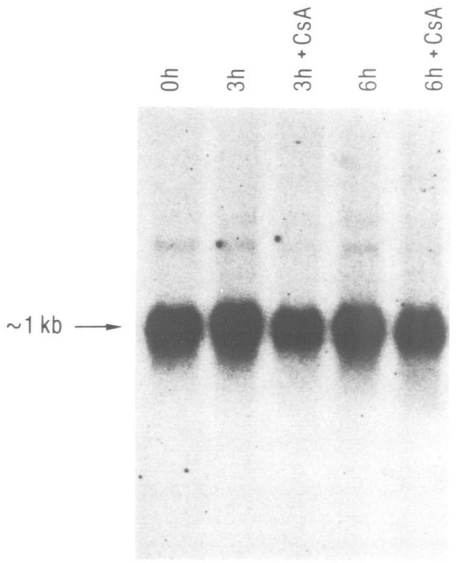

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA isolated from Jurkat cells. Induction with phytohaemagglutinin and phorbol myristate acetate was performed for the times indicated. In some cases CsA was added 1 h before induction. Five micrograms of RNA were analysed in each lane with the cyclophilin probe described in the text. Exposure time was 48 h at −70°C.

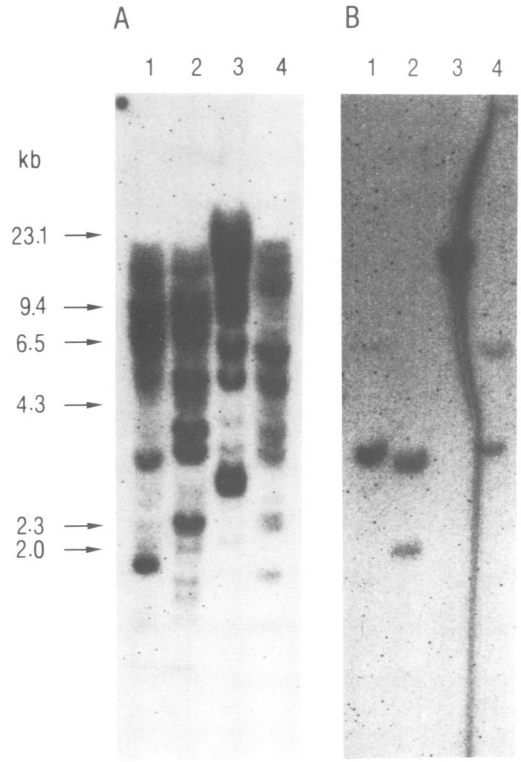

Fig. 4. Southern blot analysis of genomic DNA prepared from peripheral blood leukocytes. Twenty micrograms of high mol. wt DNA digested to completion by *Eco*RI **(1)**, *Hin*dIII **(2)**, *Bam*HI **(3)** or *Xba*I **(4)** were analysed with the cyclophilin probe described in the text **(panel A)**. The results obtained when using an RNA transcribed from an IL-2 cDNA, following the procedures used for the cyclophilin probe, are shown in **panel B**. Exposure time was 7 days at −70°C. Mol. wt markers are indicated in kb.

tions. Furthermore its wide tissue distribution cannot account for the specific action of CsA in helper T lymphocytes. In order to gain further knowledge about the precise role of cyclophilin in T cells, we undertook to clone its complementary DNA from the leukemic cell line Jurkat.

The deduced amino acid sequence shows ~98% homology to the sequence of the major bovine isoform which makes it likely that the clones we isolated correspond to the major form of human T-cell cyclophilin. This high evolutionary conservation also suggests that essentially all regions of the protein are functionally important and fosters the idea of a fundamental role in the cell. From the cDNAs we cloned we can predict that human cyclophilin has a higher content of glutamic acid and leucine than the bovine protein. This agrees with the amino acid analysis of the human protein performed by Harding *et al.* (1986).

One major band was revealed in Northern blot analysis of poly(A)$^+$ RNA from Jurkat cells. Neither induction of these cells by lectin and phorbol ester nor treatment with CsA prior to this induction dramatically alters the transcription level of cyclophilin. Only a slight increase of cyclophilin mRNA is visible after a 3- or 6-h induction whereas under the same conditions the level of lymphokine mRNAs is increased at least 100-fold. A similarly small effect was observed for cyclophilin at the protein level in concanavalin A-induced murine splenocytes (Koletsky *et al.*, 1986). Thus it appears that no reaction to induction comparable with that observed for lymphokines exists for cyclophilin. Neither is there a tightly regulated mechanism responding to the binding of CsA to cyclophilin in T cells for cyclophilin mRNA.

The genomic blots of human leukocyte DNA show multiple hybridizing fragments which make it likely that several related genes coding for cyclophilin exist, even though some of the bands may represent pseudogenes. Admitting a general function for cyclophilin in the signal transduction from surface receptors to specific genes, allows us to compare it with such proteins as protein kinase C and G proteins, both of which have been shown to be encoded by many genes (Coussens *et al.*, 1986; Bourne, 1986). The existence of a multigene family could be the necessary pre-requisite for a protein of general function which has to cope with slightly changing conditions in various cells or during different states of differentiation. The presence of several isoforms or combinations of isoforms in a cell type-restricted manner could also offer an explanation for the specific action of CsA in T cells.

Materials and methods

Cell culture

Jurkat cells (Gillis and Watson, 1980) were obtained from Dr M.Schreier, Sandoz Basel, and grown in RPMI medium (Gibco) supplemented with 10% fetal calf serum. Induction was performed 16 h before harvesting with phytohaemagglutinin (1 μg/ml) and 4β-phorbol 12β-myristate 13-acetate (20 ng/ml) in serum-free IMDM medium at a cell density of 2×10^6 cells/ml. CsA was added at a final concentration of 500 ng/ml 1 h before induction.

Construction of the cDNA library

RNA was isolated using the urea−LiCl method (Le Meur *et al.*, 1981) and polyadenylated RNA was selected by oligo(dT) cellulose chromatography (Maniatis *et al.*, 1982). It was then copied with avian myeloblastosis virus reverse transcriptase and double-stranded cDNA was made using RNase H and DNA polymerase I, following the protocol of Gubler and Hoffman (1983) with minor modifications. The cDNA was made blunt-ended with S1 nuclease and T4 DNA polymerase, methylated, ligated to kinase-treated synthetic *Eco*RI linkers and the products were digested with *Eco*RI (Maniatis *et al.*, 1982). cDNA fragments >500 bp were selected on a 1.0% agarose gel and inserted into the *Eco*RI site of the p91023B vector (Wong *et al.*, 1985). The ligation mixture was used to transform *Escherichia coli* 5K.

Screening of the library

A total of 20 000 transformants replica-plated on nylon filters (Pall) was screened using two probes. One was a mixture of four 26-mer oligonucleotides corresponding to a nine amino acid long portion of human cyclophilin (Figure 1). It was

labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham) as described by Maniatis *et al.* (1982). The other probe consisted of two 39-mer oligonucleotides with 12 complementary bases at their 3′ end which could therefore be filled in after annealing using [α-^{32}P]dGTP (Amersham) and the Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982). The filters were processed according to the manufacturer's manual. They were pre-hybridized for 5 h and then hybridized with the probes for 16 h at 42°C in 5 × NET (1 × NET = 150 mM NaCl, 1 mM EDTA, 20 mM Tris−HCl pH 7.5), 5 × Denhardt's solution (1 × Denhardt's = 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.2% SDS and 100 μg/ml yeast tRNA. The filters were washed at 42°C in 5 × NET, 0.2% SDS and then again at gradually increasing temperatures up to 65°C. The filters were exposed for at least 10 h on Kodak XAR-5 film after each step.

Subcloning and sequencing

Plasmids from the positive clones were extracted following the boiling method (Maniatis *et al.*, 1982). Their inserts were cleaved out by *Eco*RI and subcloned into the pBS-M13 vector (Stratagene Cloning Systems). The DNA sequencing was performed using the dideoxy chain termination method of Sanger *et al.* (1977). Using both T3 and T7 primers and cyclophilin-specific oligonucleotide primers, we determined the DNA sequence on the entire cloned insert or on the *Eco*RI−*Pst*I, *Eco*RI−*Nco*I and *Nco*I−*Eco*RI fragments.

Northern blot

Poly(A)$^+$ RNAs were fractionated on a 1.2% agarose gel containing 5 mM methylmercuryhydroxide (Maniatis *et al.*, 1982) and electrophoretically transferred to a nylon membrane (Pall). The filter was processed according to the manufacturer's manual and then pre-hybridized at 65°C for 5 h in 5 × NET, 5 × Denhardt's, 0.2% SDS, 100 μg/ml salmon sperm DNA. It was then hybridized for 16 h in the same solution containing 100 μg/ml yeast tRNA instead of salmon sperm DNA to a ^{32}P-labelled probe obtained by transcription of the *Bam*HI-restricted pBS-CYC21 clone with T7 RNA polymerase. The filter was subsequently washed at 65°C with decreasing concentrations of salt, the final washing being in 0.5 × NET, 0.2% SDS.

Southern blot

High mol. wt DNA isolated from human peripheral blood leukocytes (Maniatis *et al.*, 1982) was digested for 4 h with an excess amount of *Eco*RI, *Hin*dIII, *Bam*HI or *Xba*I, separated on a 0.8% agarose gel and analysed by Southern blot hybridization (Southern, 1975). The same procedure as in the RNA transfer blot analysis was followed except that T3 RNA polymerase was used to transcribe the *Pvu*I-restricted pBS-CYC21 clone.

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Note added in proof

These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00052.