A nuclear RNA-binding cyclophilin in human T cells

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Abstract Cyclophilins (CyPs) are binding proteins for the immunosuppressive drug cyclosporin A (CsA). CyPs are evolutionarily highly conserved proteins present in both pro- and eukaryotes as well as in different subcellular locations. CyPs possess enzymatic activity, namely peptidyl-prolyl *cis-trans* isomerase (PPIase) activity; CyPs are involved in cellular protein folding and protein interactions. To date, only cyclosporins and proteins are known to interact with CyPs. Here we describe a novel nuclear cyclophilin (hCyP33) from human T cells with an additional RNA-binding domain. This combines for the first time RNA binding and protein folding in one protein.

Key words: RNA binding; RNP motif; Prolyl isomerase; Cyclophilin; Protein folding; Cyclosporin A

1. Introduction

Cyclophilins (CyPs) are highly conserved proteins present in both pro- and eukaryotes and in different subcellular locations [1–3]. Besides their high-affinity binding of cyclosporin A (CsA), CyPs possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, which is inhibited by CsA. Due to the PPIase activity CyPs are able to accelerate protein folding in vitro (reviewed in [2,3]). There is increasing evidence that CyPs are involved in cellular protein folding after ribosomal synthesis and after passage of proteins across intracellular membranes [4–8]. CyPs are also involved in transient protein interactions, e.g. with steroid receptors [9], gag protein of HIV [10,11], or rhodopsin [12]. Abundant cytosolic CyPs (hCyP18 in human cells [13] and NcCyP20 [14] in the fungus *Neurospora crassa*) mediate the immunosuppressive [15] or antifungal effect [16], respectively, of CsA by inhibiting the protein phosphatase calcineurin.

In higher eukaryotic cells a variety of CyPs are present in different concentrations and cellular compartments. Abundant small forms (18–24 kDa) are present in the cytosol, mitochondria and the endoplasmic reticulum, perhaps being part of the protein folding machinery [4–8]. Larger CyPs (40–360 kDa) containing additional protein-binding domains are present in hormone receptor complexes [9], nuclear pores [17] or on the cell surface [18]. To date, two kinds of molecules are known to interact with CyPs: cyclic peptides (cyclosporins) and proteins.

Here we describe a novel nuclear cyclophilin (hCyP33) from human T cells. hCyP33 contains two functional domains: an RNA-binding domain at the amino-terminus and a PPIase domain at the carboxy-terminus of the protein. This combines for the first time RNA binding and protein folding in one protein.

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The amino acid sequence for hCyP33 has been deposited under MIPS accession number S66681.

2. Material and methods

2.1. Cloning of a full-length cDNA for hCyP33

PCR amplification of DNA-fragments from different human Jurkat T cell line cDNA libraries were performed with two degenerate primers, which correspond to amino acid sequences QGGDF and KHVVFG (boxed in Fig. 2b) in the highly conserved regions of known cyclophilins [3,13,17–20]. The resulting PCR products were subcloned into pUC18 and sequenced. Nested PCR was performed to extend the sequence using two libraries: a Marathon cDNA library of the human Jurkat T cell line (Clontech) and a cDNA library made of mRNA from human T cells. Both libraries yielded as longest cDNA one with a length of 1.6 kb. The cDNA was sequenced on both strands.

2.2. Detection of hCyp33 mRNA using Northern hybridization

Human Jurkat T cell poly(A)$^+$ RNA (2 μg per lane) was resolved on a formaldehyde gel, blotted onto a nylon membrane and hybridized as described [14].

Jurkat T cells were subfractionated according to [21]. A hCyP33-specific rabbit antiserum was obtained by immunization with a glutathione transferase-fusion protein [22] containing amino acids 67–164 of hCyP33 at the carboxy-terminus.

2.3. In vitro expression

A full-length cDNA coding for hCyp33 was subcloned into pGEM4. Coupled in vitro transcription/translation using ^{35}S-labeled methionine was performed in a TNT SP6 coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol.

Nucleic acid binding assays were performed using ^{35}S-labeled hCyP33 from the in vitro expression system (see Fig. 4a, lane 1) as described [23].

PPIase assays were performed in a standard peptide assay [24] using a GST-hCyP33 fusion protein and Suc-Ala-Ala-Pro-Phe-pNA as a substrate.

3. Results

A variety of different cyclophilins have been cloned from higher eukaryotic cells (reviewed [1–3]). Using a CsA affinity column, we recognized minor bands in addition to the known CyPs (unpublished data). We therefore used a PCR approach to identify new CyPs from the human T cell line Jurkat. Degenerate primers directed against regions which are highly conserved in known CyPs (QGGD and KHVVFG; see boxes in Fig. 2b) were used to amplify short DNA fragments from different Jurkat cDNA libraries. Ninety CyP-related cDNA fragments were cloned and sequenced. Sixty-nine clones were identified as part of the coding region of human CyP18 [13], 19 were identical to secretory hCyP23 [19], one was identical to mitochondrial hCyP22 [20] and one clone (M1) represented a new hCyP. Using additional PCR approaches extending the cDNA sequence (5′- and 3′-RACE) to identify a full-length cDNA, we finally isolated and sequenced a 1.6 kb cDNA. This cDNA codes for a protein of 33430 Da, which was named hCyP33.

PII S0014-5793(96)01248-3

a

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  1 MATTKRVLYVGGLAEEVDDKVLHAAFIPFGDITDIQIPLDYETEKHRGFA  50
 51 FVEFELAEDAAAAIDNMNESELFGRTIRVNLAKPMRIKEGSSRPVWSDDD 100
101 WLKKFSGKTLEENKEEEGSEPPKAETQEGEPIAKKARSNPQVYMDIKIGN 150
151 KPAGRIQMLLRSDVVPMTAENFRCLCTHEKGFGFKGSSFHRIIPQFMCQG 200
201 GDFTNHNGTGGKSIYGKKFDDENFILKHTGPGLLSMANSGPNTNGSQFFL 250
251 TCDKTDWLDGKHVVFGEVTEGLDVLRQIEAQGSKDGKPKQKVIIADCGEY 300
301 V
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b

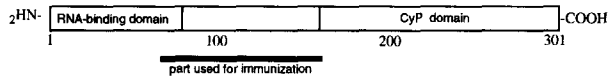

Fig. 1. hCyP33 contains an RNA-binding domain at the amino-terminus and a cyclophilin domain at the carboxy-terminus. a: Amino acid sequence of hCyP33 derived from the cDNA. b: Domain organization of hCyP33.

Fig. 1a shows the amino acid sequence of hCyP33 derived from the cDNA sequence. Sequence databank comparisons revealed that hCyp33 is a novel protein. Surprisingly, the protein exhibits *two* regions of high sequence identity to other proteins. The first region showed similarities to cyclophilins, as expected, whereas the second region was similar to RNA-binding proteins containing an RNA recognition motif (RRM, also known as RNA-binding domain or RNP consensus sequence; reviewed in [25–27]).

Separate sequence alignments of both domains are shown in Fig. 2. The putative amino-terminal RNA-binding domain of hCyP33 contains both RNP-1 and RNP-2 submotifs (Fig. 2a) located 33 amino acids apart. The RNP-1 octapeptide, Lys/Arg-Gly-Phe/Tyr-Gly/Ala-Phe-Val-X-Phe/Tyr (K/R-G-F/Y-G/A-F-V-X-F/Y), is the most highly conserved segment of the RNP motif (being RGFAFVEF in hCyP33); RNP-2 is a less well conserved hexapeptide that is rich in aromatic

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                    RNP-2                                                RNP-1
hCyP33        1 MATTKRVLYV GGLAEEVDDK VLHAAFIPFG DITDIQIPLD YETEKHRGFA FVEFELAEDA  60
RBP U2        8 ERNQDATVYV GGLDEKVSEP LLWELFLQAG PVVNTHMPLD RVTGQHQGYG FVEFLSEEDA  67
12RNP1            1 MSIYV GNLSYEVTEA DLTAVFTEYG AVKRVQLPID RETGRMRGFG FVEMSADAEE  55
RGP-1a        1 MAEVEYRCFV GGLAWATTDQ TLGEAFSQFG EILDSKIIND RETGRSRGFG FVTFKDEKAM  60
PABP 3          220 NLYV KNINSETTDE QFQELFAKFG PIVSASLEKD ADGKL-KGFG FVNYEKHEDA 272

hCyP33       61 AAAIDNMNES ELFGRTIRVN LAKP  84
RBP U2       68 DYAIKIMNMI KLYGKPIRVN KASA  91
12RNP1       56 DAAIAALGGA EWMGRGLRVN KAKP  79
RGP-1a       61 RDAIEGMNGQ DLDGRNITVN IAQS  84
PABP 3      273 VKAVEALNDS ELNGEKLYVG RAQK 296
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b

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hCyP33      137 RSNPQVYMDI KIGNKPAGRI QMLLRSDVVP MTAENFRCLC THEKGFG... ........FK 185
hCyP18cy      1 MVNPTVFFDI AVDGEPLGRV SFELFADKVE KTAENFRALS TGEKGFG... ........YK  49
hCyP23sec    33 KVTVKVYFDL RIGDEDVGRV IFGLFGKTVP KTVDNFVALA TGEKGFG... ........YK  81
hCyP22mito   43 SGNPLVYLDV DNAGKPLGLV VLELKADVVP KTAENFRALC TGEKGFG... ........YK  91
hCyP41cy     13 PSNPRVFFDV DIGGERVGRI VLELFADIVP KTAENFRALC TGEKGFG... HTTGKPLHFK  69
hCyP158mem   58 QDRPQCHFDI EINREPVGRI MFQRFSDICP KTCKNFLCLC SGEKGIGHTT KTTGKKLCYK 117
RanBP2     3064 ---PVVFFDV CADGEPLGRI TMELFSNIVP RTAENFRALC TGEKGFG... ........FK 3109

hCyP33      186 GSSFHRIIPQ FMCQGGDFTN HNGTGGKSIY GKKFDDENFI LKHTGPGLLS MANSGPNTNG 245
hCyP18cy     50 GSCFHRIIPG FMCQGGDFTR HNGTGGKSIY GEKFEDENFI LKHTGPGILS MANAGPNTNG 109
hCyP23sec    82 NSKFHRVIKD FMIQGGDFTR GDGTGGKSIY GERFPDENFK LKHYGPGVLS MANAGKDTNG 141
hCyP22mito   92 GSTFHRVIPS FMCQAGDFTR NHGTGGKSIY GSPFRDENFT LKHVGPGVLS MANAGPNTNG 151
hCyP41cy     70 GCPFHRIIKK FMIQGGDFSN QNGTGGKSIY GEKFEDENFH YKHDLEGLLS MANAGRNTNG 129
hCyP158mem  118 GSTFHRVVKN FMIQGGDFSE GNGKGGESIY GGYEKDENFI LKHDRAFLLS MANAGKHTNG 177
RanBP2     3110 NSIFHRVIPD FVCQGGDITK HDGTGGQSIY GDKFEDENFD VKHTGPGLLS MANQGQNTNN 3169

hCyP33      246 SQFFLTCDKT DWLDGKHVVF GEVTEGLDVL 275
hCyP18cy    110 SQFFICTAKT EWLDGKHVVF GKVKEGMNIV 139
hCyP23sec   142 SQFFITIVKT AWLDGKHVVF GEVLEGMEVV 161
hCyP22mito  152 SQFFICTIKT DWLDGKHVVF GHVKEGMDVV 171
hCyP41cy    130 SQFFITTVPT PHLDGKHVVF GQVIEGIGVA 159
hCyP158mem  178 SQFFITTKPA PHLDGVHVVF GLVISGFIVI 207
RanBP2     3170 SQFVITLKKA EHLDFKHVVF GFVKDGMDTV 3199
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Fig. 2. Sequence comparisons of the RNA-binding domain (a) and PPIase (cyclophilin) domain (b) of hCyP33. a: Alignment of amino acids 1–84 of hCyP33 with RNA-binding domains (RNP-2 and RNP-1) of the following proteins: RBP U2, human spliceosome-associated protein SAP 49 [29]; *Synechococcus* 12 RNP1 [23]; RGP-1a, an RNA-binding protein in wood tobacco [30] and PABP 3, a yeast poly(A)-binding protein [31]. The region of two conserved motifs of RNA-binding proteins, RNP-2 and RNP-1 are indicated. b: Alignment of amino acids 137–275 of hCyP33 with the PPIase domains of different cyclophilins: hCyp18cy, human cytosolic cyclophilin A [13]; hCyP23sec, human secreted cyclophilin B [19]; hCyP22mito, human mitochondrial cyclophilin [20]; hCyP41cy, a human cyclophilin present in hormone receptor complexes [32]; hCyP158mem, a plasma membrane cyclophilin of human natural killer cells [18]; RanBP2, a Ran/TC4-binding nucleopore protein [17]. The regions used for initial design of PCR primers to identify hCyP33 are boxed. Identical amino acids are printed in bold type in a and b.

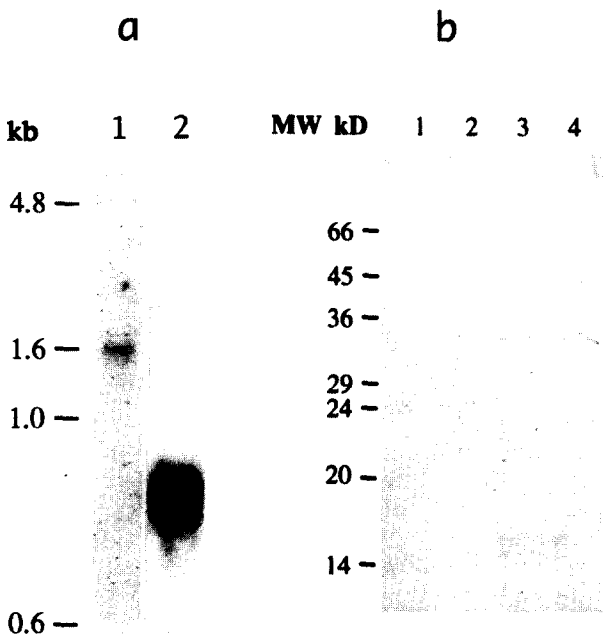

Fig. 3. hCyP33 is a low abundance nuclear protein in human T cells. a: Detection of hCyp33 mRNA using Northern hybridization. Human Jurkat T cell poly(A)$^+$ RNA (2 μg per lane) was hybridized to part of the cDNA of hCyP33 (corresponding to amino acid sequence 1–152; lane 1) or the cDNA coding for hCyP18 [13] (lane 2). Exposure times of the autoradiographic films were 2 h for hCyP18 mRNA (lane 2), and 7 days for hCyP33 mRNA (lane 1). b: Immunoblot of different cellular fractions from the human Jurkat T cell line using affinity-purified anti-hCyP33 rabbit antibodies. Jurkat T cells were subfractionated according to [21]; lane 1, 12000×g supernatant of nuclear free cell extract, 2, 12000×g pellet of nuclear free cell extract, 3, 12000×g supernant of nuclear extract, 4, 12000×g pellet of nuclear extract. The polyclonal rabbit antiserum was obtained by immunisation of a glutathione transferase fusion protein [22] containing amino acids 67–164 of hCyP33 at the carboxyterminus.

and aliphatic amino acids [27] (LYVGGL in hCyP33). In addition, several other amino acids throughout the RNP motif are conserved, when other RNA-binding proteins are compared (see Fig. 2a). The human protein whose RNA-binding domain is most closely related (43.8% identity in a 73 amino acid overlap) to the hCyP33 RNA-binding domain is the spliceosome-associated protein SAP49 (RBP U2 [29]). Other proteins in the identity range of 40% can be found in cyanobacteria (12 RNP1 [23]), an RNA-binding protein from tobacco (RGP-1 [30]) and a yeast poly(A)-binding protein (PABP3 [31]). Despite their different functions, these proteins all share this RNA-binding domain. This domain is typical for a large family of RNA-binding proteins like snRNP proteins, hnRNP proteins and other proteins regulating RNA processing or translation [25–27].

In addition to this putative RNA-binding domain, hCyP33 contains a typical cyclophilin domain (Fig. 2b), which is 70% identical to cytosolic hCyP18 [13], a protein probably involved in protein folding (like *Neurospora crassa* cytosolic and mitochondrial NcCyP20 [7,14,28], E. Zimmermann and M. Tropschug, unpublished data) and stabilization [10,11].

Cytosolic hCyP18 is an abundant protein. We used its cDNA [13] as a control probe in a Northern blot analysis (Fig. 3a, lane 2), which showed its abundant hCyP18 mRNA (0.8 kb); using hCyP33 cDNA as a probe, we recognized a rare mRNA of 1.6 kb (Fig. 3a, lane 1), in the range of approximately 1/1000 the amount of hCyP18 mRNA. Since the longest hCyP33 cDNA isolated was 1.6 kb long, we conclude that we have cloned a full-length cDNA (see also below).

For antibody production, a GST fusion protein [22] was constructed containing the interconnecting region (amino acids 67–164; see schematic representation in Fig. 1b) between the RNA-binding and the PPIase domain (to avoid cross-reactivity of the antiserum with other CyPs and/or RNA-binding proteins). Polyclonal antibodies were raised in rabbits against the fusion protein and affinity-purified. Jurkat cells were fractionated into nuclei and nuclear-free fractions [21]. These fractions were tested for the presence of hCyP33 in a Western blot analysis. Only nuclear soluble and nuclear insoluble (matrix) fractions showed the presence of a protein with an apparent molecular weight of 33 kDa (Fig. 3b, lanes 3 and 4 respectively). We conclude that hCyP33 is a nuclear protein in Jurkat cells and that we have indeed cloned a full-length cDNA coding for this protein (see above).

Using a coupled transcription/translation system (TNT lysate; Promega), the protein was expressed in vitro from the cDNA cloned into pGEM4. After translation using [^{35}S]methionine as label, total translation products were separated on SDS gels. Fig. 4a (lane 1) shows the result of an in vitro expression experiment. Two major bands with apparent molecular weights of 33 kDa and 30 kDa showed up, the upper band running exactly at the same position in SDS-PAGE as hCyP33 in Western blots (see Fig. 3b, lanes 3, 4). Since both proteins are coded by the same cDNA, we suggest that the 33/30 kDa proteins reflect different forms (e.g. post-translational modifications) of hCyP33. RNA-binding proteins are often phosphorylated in vivo [26,27]. Specific antiserum raised against hCyP33 (see Fig. 1b) precipitated both the 33 kDa and 30 kDa proteins from the reticulocyte lysate with equal efficiency (Fig. 4a, lane 2), showing that both proteins are hCyP33 related. The nature of the differences in both isoforms of hCyP33 are unknown to date.

We used in vitro expressed hCyP33 to test whether the putative RNA-binding domain (Fig. 1bFig. 2a) is functional. Reticulocyte lysate containing [^{35}S]methionine-labeled hCyP33 was incubated with agarose-coupled nucleic acids. Very weak binding was observed using single-stranded (ss) DNA and double-stranded (ds) DNA. Almost no binding was observed using poly(C) or poly(G) columns. In contrast, specific binding was observed on poly(A) and poly(U) columns (see Fig. 4b), suggesting that hCyP33 is indeed an RNA-binding protein with a strong preference for A and U residues. It is interesting to note that only the 33 kDa band produced in reticulocyte lysates was able to bind. This could mean that a posttranslational modification is necessary for efficient binding to RNA. Phosphorylation and dephosphorylation of RNA-binding proteins are known to play an important role in regulation of the activities of these proteins [27].

To test whether the CyP domain (Fig. 2b) of hCyP33 is functional as well and therefore exhibits PPIase activity, a glutathione transferase fusion protein [22] containing hCyP33 at the carboxy-terminus was expressed in *E. coli*, purified using glutathione beads and tested in a peptide-coupled PPIase assay [24].

Indeed, this protein exhibits PPIase activity which can be inhibited by cyclosporin A (CsA; see Fig. 4c). The enzymatic activity of hCyP33 in terms of K_m/K_{cat} values is similar to that

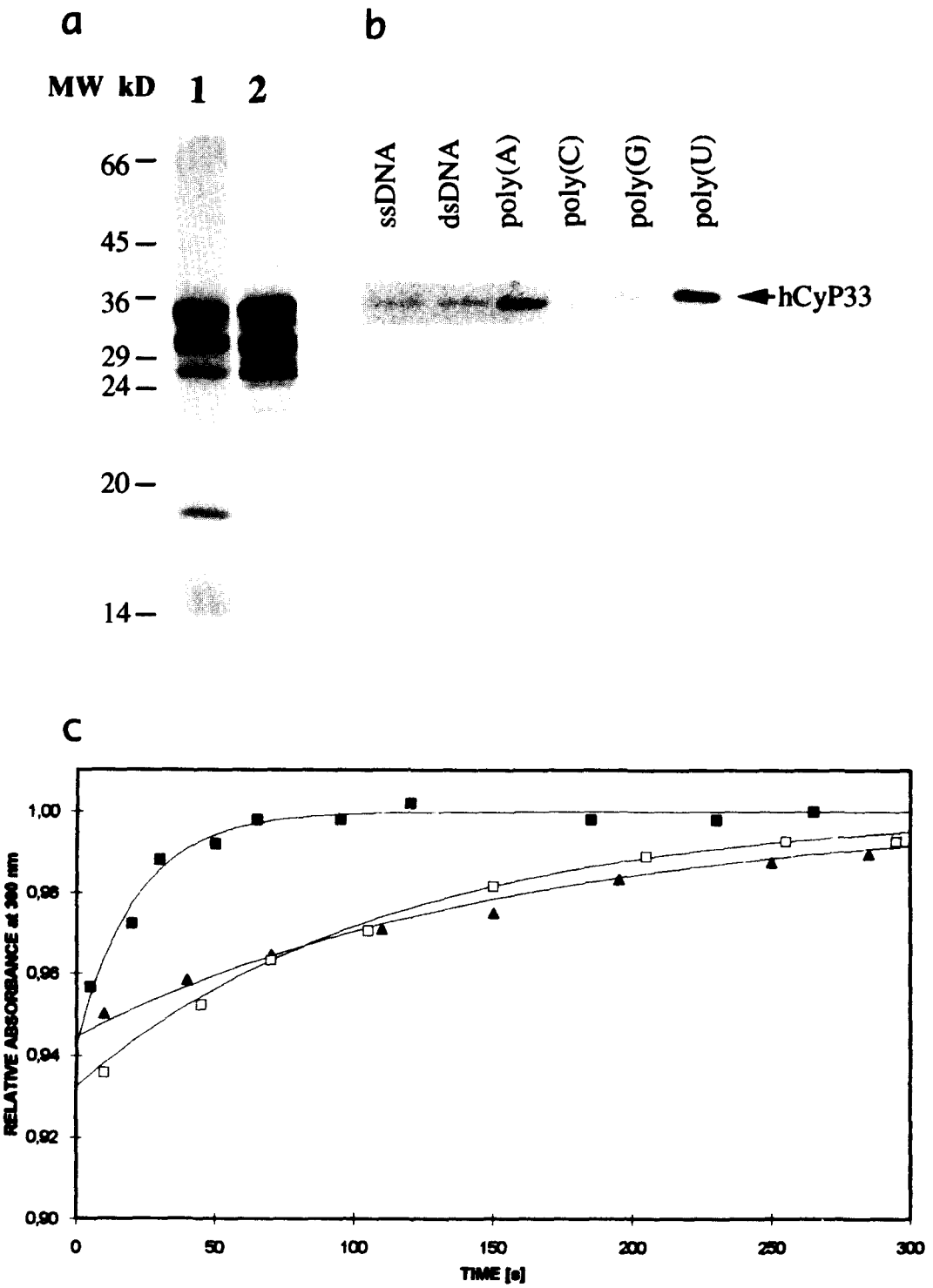

Fig. 4. Both the RNA-binding domain and the CyP domain are functional. a: Expression in vitro and antibody precipitation of hCyP33. A full-length cDNA coding for hCyp33 was subcloned into pGEM4. Coupled in vitro transcription/translation using [^{35}S]methionine as label was performed in a TNT/SP6 coupled reticulocyte lysate system (Promega) (lane 1). In order to confirm that the major bands appearing in lane 1 are related to hCyP33, immunoprecipitation using anti-hCyP33 antiserum was performed (lane 2). b: Nucleic acid binding assays were performed using ^{35}S-labeled hCyP33 from the in vitro expression system (see a, lane 1) as described [23]. Binding was monitored on single-stranded (ss) DNA, double-stranded (ds) DNA, poly(A), poly(C), poly(G) and poly(U), respectively, covalently bound to agarose. c: hCyP33 is active as a CsA-sensitive PPIase. Assays of PPIase activity of hCyP33: ▲ blank, ■ 17 nM GST-hCyP33, □ 17 nM GST-hCyP33 preincubated with CsA. Assays were performed in a standard peptide assay [24] using purified GST-hCyP33 and Suc-Ala-Ala-Pro-Phe-pNA as a substrate. 17 pmol of GST-hCyP33 (±1.70 nmol CsA) was preincubated at 10°C with 800 μg/ml chymotrypsin for 5 min in a volume of 995 μl. The assay was started by the addition of 5 μl of a 8 mM stock of the substrate. After mixing, the increase in absorbance at 390 nm was monitored.

of hCyP18 (data not shown). In addition to the peptide PPIase assay, we also tested whether hCyP33 is active as a true protein-folding enzyme. Using refolding of urea-denatured mouse dihydrofolate reductase (DHFR) as a model system [7,8], we found that hCyP33 accelerates DHFR refolding; this protein folding activity was in the same range as that for hCyP18 (data not shown) or *Neurospora crassa* NcCyP20 [7]. The enzymatic activities of all three cyclophilins were inhibited by CsA.

In summary, hCyP33 represents a novel nuclear T cell protein, which combines RNA binding and PPIase activities in the same molecule.

4. Discussion

Why might nature combine RNA binding and PPIase activity in one molecule? Known PPIases can play a dual role in protein folding and cellular signalling [15].

hCyP33 could be a regulatory protein which after binding to a specific RNA might bind another protein and perhaps stabilize or alter the folding state of this protein. This protein could in turn affect RNA metabolism. hCyP33 could have a function similar to hCyP40 which is present in nonactive steroid receptor complexes [9]. Transient interactions between PPIases and target proteins are known, as between hCyP18 and the gag protein of HIV. Disruption of this complex by CsA or mutations of critical prolyl residues in gag leads to defective virions [10,11]. Another example of a transient interaction between a cyclophilin and a target protein is the interaction between DmCyP26 (the *ninaA* gene product of *Drosophila melanogaster*), a CyP homolog present in the endoplasmic reticulum, and Rh-1 rhodopsin. DmCyP26 functions like a chaperone, forming a stable complex at least in the maturation process of rhodopsin [12].

hCyP33 could influence RNA processing, modification, transport or translation, either by itself or when bound to another factor. In this context, it is interesting to note that there exists a novel family of PPIases, the parvulin family [34], members of which are essential for regulation of human mitosis (Pin1 [33]) and for RNA maturation in yeast (Ptf1 [35]; H. Domdey and G. Fischer, personal communication). It is tempting to speculate that PPIases play not only a role in protein folding after protein synthesis or after proteins have crossed biological membranes but could also act as on/off switches due to *cis/trans* isomerization of regulatory proteins.

It will be of major interest to investigate which RNA(s) and/or protein(s) bind to hCyP33. A further question is how CsA, which acts as an immunosuppressant by suppressing transcription of specific T cell proteins, might influence these interactions.

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