RESEARCH COMMUNICATION

Cyclophilin-40: evidence for a dimeric complex with hsp90

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The expression of human cyclophilin 40 (CyP-40) as a glutathione S-transferase fusion protein has provided a means to identify cellular components that are in association with this ubiquitous protein. When the fusion protein was coupled to a GSH affinity matrix, heat-shock protein 90 (hsp90) was found to be the predominant associated protein in all tissue extracts examined. The relatively high concentration of each of these proteins in various tissues indicates that the dimeric complex exists in concentrations that exceed those of the inactive steroid receptors of which each protein is a component. Association does not occur with heat-shock protein 70 and is not affected by cyclosporin A (CsA). Independent expression of two domains of CyP-40 permitted dissociation of N-terminal isomerase and CsA binding activity from the hsp90 binding site, which is located at the FKBP-59-like C-terminal region. The biological association of CyP-40 with hsp90 in many tissues may reflect a conjoint role in protein folding and trafficking.

INTRODUCTION

The immunosuppressive drug cyclosporin A (CsA) made possible the identification of an ever growing ubiquitous family of proteins termed cyclophilins (CyP) (reviewed in [1]). The immunosuppressive activity of CsA appears to be primarily attributable to the strong inhibitory activity of the CyP/CsA complex on the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin [2]. The consequent failure to assemble the functional NFAT transcription factor suppresses interleukin-2 expression and antigen induced activation of T cells [3]. These immunosuppressive properties have made CsA a valuable drug and an excellent tool for the characterization of T-cell signalling pathways. A recent report suggests that a protein associated with other Ca^{2+}-associated events may be a natural ligand for cyclophilin A [4]. In contrast with these details of CyP function in lymphocytes, there is very limited knowledge of the natural roles and ligands of the ubiquitous cyclophilins in other cell types and organisms. Their ability to isomerize peptidyl-prolyl bonds suggested a role in protein-folding events and, indeed, it was found that the cyclophilin homologue ninaA is required for the processing of *Drosophila* rhodopsin [5] and that CsA, which inhibits the isomerase activity, slows collagen triple-helix formation *in vivo* [6].

Although most work has focused on the 18–22 kDa members of the cyclophilin family found either in the cytosol or in the mitochondrial matrix and endoplasmic reticulum [1,7,8], a CyP-40 has been characterized [9,10] and shown to be associated with the inactive non-DNA-binding form of the oestrogen receptor [11]. CyP-40 has N-terminal sequence similarity to CyP-18, the cyclophilin 'prototype' [12], and C-terminal similarities to FKBP-59, a heat-shock-inducible immunophilin of the FK-506-binding class [13] that is also associated with certain steroid-receptor complexes [14,15]. It is currently believed that this association occurs through the 90 kDa and 70 kDa heat-shock components (hsp90, hsp70) of the receptor [16]. The biological function of the immunophilins in these receptor complexes is not understood, but may involve assembling and localization.

MATERIALS AND METHODS

Materials

CsA was generously supplied by Dr. J. Borel of Sandoz (Basel, Switzerland). Antibodies against CyP-40, hsp90 and hsp70 are available from Affinity Bio-Reagents (Nehanic Station, NJ, U.S.A.). Human recombinant hsp90 was obtained from StressGen (Victoria, BC, Canada). Fresh calf thymus and brain were kindly provided by Porzio Inc. (Waterbury, CT, U.S.A.). Deep-frozen bovine uteri and kidneys were purchased from Pel-Freez (Rogers, AR, U.S.A.). RPMI medium and fetal-calf serum were obtained from GIBCO (Gaithersburg, MD, U.S.A.). Succinyl-Ala-Ala-Pro-Phe-nitroanilide was a gift from Miles Laboratories (West Haven, CT, U.S.A.). The CyP-18/GST (glutathione S-transferase) expression plasmid was kindly provided by Dr. P. Belshaw (Harvard University, MA, U.S.A.). The expression vectors were purchased from Bio-Rad, and T4-ligase and restriction enzymes were from New England Biolabs (NEB). PCR primers were synthesized in the William Keck Foundation, Yale School of Medicine, Oligonucleotide Synthesis Laboratory. All other chemicals were of the highest purity and were purchased from Sigma, unless otherwise stated.

Construction of the expression vectors

To construct the GST/CyP-40 fusion gene, the CyP-40 cDNA, inserted in the Bluescript vector KSII –, was amplified by PCR using the two primers 5′-CACGGATCCGATGTCGCACCCG-TCCCCCCAA-3′ (5′-primer) and 5′-ATTAACCCTCACTA-

Abbreviations used: CsA, cyclosporin A; CyP-40, cyclophilin-40; CyP-4018/GST and CyP-4059/GST, recombinant FKBP-59- and CyP-18-like domains of CyP-40 coupled to GST; FKBP, FK506-binding protein; GST, glutathione S-transferase; hsp90 and hsp70, heat-shock proteins of 90 kDa and 70 kDa.

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AAG-3′ (3′-primer) and inserted into the *Bam*HI/*Eco*RI sites of pGEX-1 that enables expression as an N-terminal GST fusion protein.

The CyP-18-like domain was generated with the 5′-primer 5′-CGTGGCATCCAGATGTCGCACCCG-3′ and the 3′-primer 5′-CGATGAATTCTCGCCAGAGCCATC-3′. For the FKBP-59-like region the 5′-primer 5′-CGTGGGATCCGCGACAGT-CATCCA-3′ and the 3′-primer 5′-ATTAACCCTCACTAAAG-3′ were used. PCR products were inserted into the *Bam*HI/*Eco*RI sites of pGEX-3X.

Expression and purification of the fusion proteins

After electroporation of the recombinant plasmids into *Escherichia coli* DH5α, a 20 ml overnight culture of a single colony was used to inoculate a 1-litre LB culture. For induction of expression, 1 mM isopropyl β-D-thiogalactoside was added at $A_{600} = 0.6$ for an additional 3 h at 25 °C. The cells were harvested by centrifugation and resuspended in buffer A (100 mM KCl, 10 mM Tris, pH 7.3, 5 mM β-mercaptoethanol, 1 mM phenylmethanesulphonyl fluoride, 0.05 % NaN_3). The cells were disrupted by French Press (103.5 MPa; 15000 lb/in^2) and debris was removed by centrifuging for 20 min at 37000 ***g***. The GST-coupled CyP-40 and truncated proteins were adsorbed on a GSH affinity matrix and eluted with 10 mM GSH in buffer A.

Production of polyclonal rabbit anti-CyP-40 antibodies

Antibodies against two peptides with CyP-40-derived sequences were prepared in collaboration with Affinity Bio-Reagents. Peptide I (G-I-G-H-T-T-G-K-P-L-H-F-K-G-C) represents residues 57–81 of CyP-40 and includes the sequence GKPLH that is absent from all other mammalian CyP sequences. This sequence is highly conserved, and to date has only been found in plants and to some degree in a *Toxoplasma gondii* [17]. Peptide II (C-A-Q-K-D-K-E-K-A-V-Y-A-K-M-F-A) represents the C-terminal amino acids 356–370 of CyP-40 with an additional N-terminal cysteine for conjugation to keyhole-limpet haemocyanin (KLH). After coupling the synthetic peptide to KLH, the conjugates were used for the immunization of New Zealand White rabbits. Antibodies against intact CyP-40 were prepared by using 600 μg of recombinant CyP-40 [18]. After resolution from minor contaminants by PAGE, the Coomassie-stained SDS/PAGE band was homogenized in PBS, emulsified with Freund's complete adjuvant and injected subcutaneously into two rabbits. After 14 days, the animals were boosted with the same amount of protein in incomplete Freund's adjuvant.

Western-blot procedures

Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) by using a Bio-Rad liquid blotting chamber with 20 % ethanol, 0.1 % SDS in Tris/glycine, pH 8.5, as the transfer buffer. After blocking with 5 % non-fat dry milk in TBST (10 mM Tris, pH 8.5, 150 mM NaCl, 0.05 % Tween-20), the nitrocellulose was incubated for 1 h at 37 °C with the primary antibody, diluted 1:1000 in TBST. After washing (3 × 15 min in TBST/5 % dry milk and 3 × 15 min in TBST), a goat anti-rabbit IgG coupled to horseradish peroxidase was used for detection. Signals were revealed with the ECL chemiluminescence kit and Hyperfilm MP from Amersham.

Isolation of CyP-40 associated proteins

A 2 mg portion of CYP-40/GST-fusion protein was coupled to 0.5 ml of GSH-conjugated agarose suspended in buffer A. Cytosolic tissue extracts were prepared by homogenization of 30 g of quick-frozen tissue in 60 ml of buffer A, followed by centrifugation for 1 h at 100000 ***g***. A 10 ml portion of the supernatant was incubated for 1 h at 4 °C with 1 ml of CyP-40/GST affinity resin. Incubation with purified hsp90 was done under the same conditions with 0.25 ml of CyP-40/GST resin and 50 μg of hsp90 in a final volume of 1 ml of buffer A. The column was washed until the eluates were free of protein, and bound proteins were eluted with 10 mM GSH in buffer A. The samples were analysed by SDS/PAGE and Western blotting.

RESULTS

The CyP-40 gene was originally isolated from a human pancreatic islet cell library and subcloned into the Bluescript KSII- vector [10]. This cDNA was used as template to construct the following vectors: pGEX3X40/GST to express CyP-40 as a GST-fusion protein coupled to the C-terminus of GST; pGEX3X4018/GST and GEX3X4059/GST for the expression of GST-coupled CyP-18-like and FKBP-59-like domains. The recombinant proteins were expressed in *E. coli* DH5α. To avoid the formation of insoluble inclusion bodies, expression was done at 25 °C with reproducible yields of 2–3 mg of protein/l of culture medium.

Affinity chromatography on GSH–agarose gave nearly pure protein, with the expected molecular masses for each protein (Figure 1). A minor amount of a truncated form with the same binding properties for CsA and GSH accompanied the expression of the GST fusion CyP-40, similarly to what we reported previously for recombinant CyP-40 (not coupled to GST) [18].

Peptidyl-prolyl *cis/trans*-isomerase activity of the recombinant proteins was determined by the chymotrypsin-cleavage assay [9] with succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. The CyP-40 and CyP-4018 fusion proteins showed CsA-sensitive isomerase activity comparable with that of the recombinant non-fusion CyP-40 [18]. No activity was detectable for the FKBP-59-like domain.

Three different antisera were developed, as described in the Materials and methods section. In Western-blot experiments antibodies against the peptide II and the whole protein showed high titres and specificity. Less than 4 ng of denatured CyP-40, blotted on to nitrocellulose, was detected. Under the same conditions the pre-immune sera gave no detectable immunoreaction. Despite the considerable sequence identities with CyP-

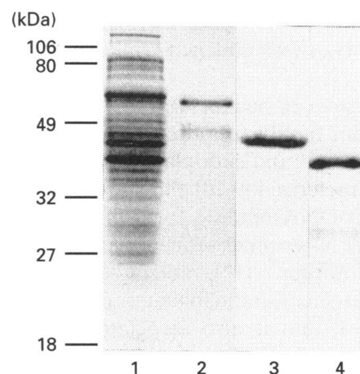

Figure 1 Coomassie-Blue-stained SDS/PAGE of the GSH–agarose-purified recombinant GST fusion proteins

Lane 1, 50 μg of *E. coli* whole cell lysate. Lane 2, 4 μg of CyP-40/GST. Lane 3, 3 μg of CyP-4018/GST. Lane 4, 3 μg of CyP-4059/GST. The 'kDa' values are derived from a Bio-Rad marker (low-molecular-mass, prestained).

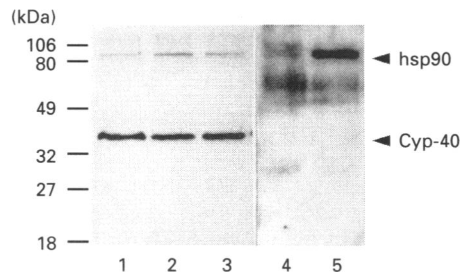

Figure 2 Expression of CyP-40 in Jurkat cells under stress conditions

Lanes 1–3 represent immunoblots with anti-CyP-40 antibodies; lanes 4 and 5 are controls with anti-hsp90 antibodies. Lanes 1 and 4, normal growth conditions. Lanes 2 and 5, heat-shocked (43 °C for 4 h). Lane 3, cadmium-treated cells (100 μM for 4 h).

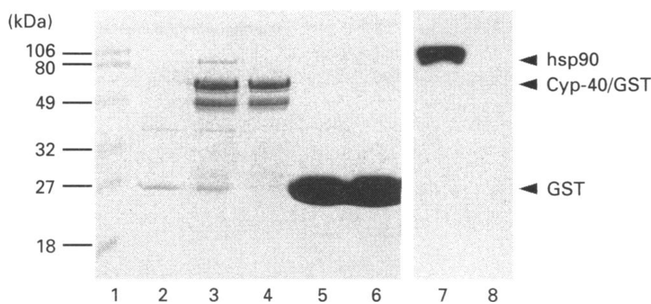

Figure 3 Identification of hsp90 as a CyP-40 associated protein

Lanes 1–6 are Coomassie-stained protein gels. Lane 1, molecular-mass standard (Bio-Rad). Lane 2, GSH eluate from GSH–agarose incubated with calf brain extract (control). Lane 3, GSH eluate from CyP-40/GST-resin incubated with calf brain extract. Lane 4, as lane 3, but no incubation with calf brain extract (control). Lanes 5 and 6, eluates from GST-resin incubated with and without calf brain extract, respectively (controls). Lanes 7 and 8, immunoblotting of lane 3 with anti-hsp90 and anti-hsp70 antibodies, respectively.

18 or FKBP-59, we saw no cross-reactivity with the anti-CyP-40 antisera.

The antibody to peptide II was used to determine whether CyP-40 belongs to the class of stress-inducible proteins. Jurkat cells were grown to 1×10^6 cells/ml in RPMI medium containing 10% fetal-calf serum at 37 °C under 5% CO_2. The cultures were stressed by shifting the temperature to 43 °C for 4 h or by addition of cadmium acetate (100 μM) and incubation for 4 h at 37 °C. Then 1×10^6 cells were analysed by Western blotting. As shown in Figure 2, no significant change was observed in the concentration of CyP-40 protein, whereas a major increase in hsp90 was detected with anti-hsp90 antibodies.

To discover natural ligands of CyP-40, the GST-fusion CyP-40 was coupled to GSH–agarose beads and incubated with calf tissue cytosolic extracts. SDS/PAGE of the GSH eluates of a column equilibrated with brain extract showed a band at 90 kDa (Figure 3, lane 3). This protein co-eluted with CyP-40 and was not detectable in the eluate of a GST-linked GSH–agarose column (lanes 5 and 6) or without incubation with brain extracts (lane 4) or GST/CyP-40 (lane 2). Similar amounts of the protein were found in uterus, kidney and thymus (results not shown). Specific antibodies against hsp90 and hsp70 only gave strong signals with a monoclonal anti-hsp90 antibody in all three tissue eluates (lane 7 for brain), but not in controls. The addition of 10 μM CsA to the tissue extracts did not affect complex-

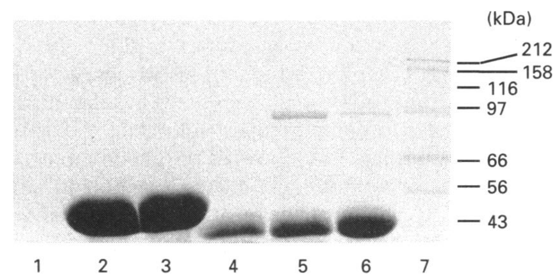

Figure 4 Localization of the hsp90 binding site of CyP-40

Lane 1, GSH eluate from GSH–agarose incubated with calf kidney extract (control). Lane 2, GSH eluate from CyP-4018/GST-resin without incubation with calf kidney extract. Lane 3, GSH eluate from CyP-4018/GST-resin after incubation with calf kidney extract. Lanes 4 and 5, same as lanes 2 and 3, but with CyP-4059/GST-resin. Lane 6, eluate from CyP-4059/GST-resin after incubation with pure hsp90. Lane 7, molecular-mass standard (NEB).

formation. In the presence of 5 mM ATP/Mg^{2+} there was a minor decrease in the amount of bound hsp90, but no evidence of association with additional proteins (results not shown).

To assess the contribution of each domain of CyP-40 to isomerase activity and hsp90 binding, constructs were prepared of the highly conserved CyP-18 domain (amino acids 1–199) and of the C-terminal region that shares 30% identity with FKBP-59 (amino acids 200–370). Each of these proteins was expressed as N-terminal GST fusion proteins and adsorbed on to a GSH matrix. Only the C-terminal protein retained hsp90 from tissue extracts (Figure 4). The identity of the 90 kDa band was confirmed by Western blotting. No associated proteins were found with the CyP-18 domain (lane 3) or with recombinant CyP-18/GST fusion protein (results not shown). To examine the possibility that cofactors in the tissue homogenates participate in the complex-formation, we incubated the FKBP-59 domain with purified recombinant human hsp90. As shown in lane 7, hsp90 bound equally well in the absence of homogenate. About 50% of 0.55 nmol of hsp90 bound to a column containing 11 nmol of CyP-4059/GST.

DISCUSSION

Heat-shock proteins hsp90 and hsp70 both appear to be natural and specific ligands of FKBP-59 [16,19], which itself has been reported to be heat-shock inducible [20]. However, in the system that we employed, CyP-40 associated only with hsp90. Cofactors seem not to be necessary, as purified hsp90 was binding to CyP-40 in the absence of tissue extracts. However, the hsp90/CyP-40 complex did not have sufficient affinity to remain associated in gel-filtration or membrane-exclusion experiments. These data are similar to previous reports for immunophilin binding to the complete inactive steroid-receptor complex [16].

Our assignment of the hsp90 binding site to the C-terminal region of CyP-40 demonstrates that peptidyl-prolyl *cis/trans* isomerase activity is not involved in hsp90 binding, a result consistent with the inability of CsA to affect complex-formation with intact CyP-40. Conversely, hsp90 did not affect the isomerase activity of CyP-40 (results not shown).

Recently, Johnson and Toft [21] reported that an anti-p23 antibody, JJ3, in the presence of rabbit reticulocyte lysate precipitates a complex of hsp90, CyP-40 and p23. The formation of this trimeric complex is Mg^{2+}/ATP-dependent and relates directly to assembly of the progesterone receptor. In our system addition of ATP was not necessary, and we could not detect p23

in the Coomassie-stained SDS/PAGE in the presence or absence of Mg^{2+}ATP. There were no quantitative differences in the amount of hsp90 complex-formation in tissues with widely varying concentrations of steroid receptors. Thus the function of the immunophilin/hsp complex does not relate exclusively to steroid receptors, but has a more common function in many cell types, where with or without p23 it may function to alter the conformation of selected proteins through either a catalytic or a stoichiometric mechanism.

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