Characterization of Cyclophilin 40: Highly Conserved Protein That Directly Associates with Hsp90

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Cyclophilin 40 (CyP40) is a recently identified member of the cyclophilin family that may be a component of unactivated steroid receptor complexes. It consists of an N-half portion that is highly homologous to cyclophilin A and has peptidyl prolyl isomerase (PPIase) activity, and a C-half portion that resembles the C-terminal portion of FKBP52 (FK506 binding protein 52), another component of unactivated steroid receptor complexes. To better understand the structure and functional characteristics of this new class of cyclophilin, we have raised monoclonal antibodies against the C-half portion of human CyP40. Immunostaining with the antibodies showed its preferential localization in cytoplasm. One antibody cross-reacted with a 45 kDa protein in yeast, suggesting high conservation throughout evolution. A CyP40-associated protein was isolated from rabbit reticulocyte lysate by means of an affinity resin, and was identified as hsp90. The C-half portion of CyP40 was necessary and sufficient for the interaction.

Key words: immunophilin; cyclophilin 40; hsp90; FKBP52; steroid receptor complex; tetrapetide repeat

Cyclophilins and FKBP52s (FK506 binding proteins) are highly conserved proteins that bind immunosuppressant cyclosporin A (CsA) and FK506 (hence called immunophilins). 2,3) Complexes between some of the members with cognate drugs are shown to strongly inhibit the signal transduction toward IL2 induction that leads to T cell activation. Although structurally different, cyclophilins and FKBP52s possess peptidyl prolyl isomerase (PPIase) activity, suggesting their possible endogenous functions in protein folding and protein trafficking in the cell. 4,5)

The cyclophilin family so far has six members. Among them, cyclophilin 40 (CyP40) has a unique feature in that its C-terminal region has homology with FKBP52, one of the FKBP52s. 4,5) The homologous region is not in a catalytic (PPIase) domain but contains three units of tetrapetide repeat (TPR). This sequence is postulated to be used for protein-protein interactions, and is found in several proteins for protein import, RNA synthesis and mitosis. 6) FKBP52 was isolated in unactivated steroid hormone receptor complexes together with hsp90, hsp70 and several other proteins. 7) CyP40 is also found as a component of unactivated estrogen receptor complexes. 8) Recently it has been shown that FKBP52 directly binds to hsp90, and its C-terminal region containing the TPR sequence is necessary for the binding. 9, 6) p60, which is often found in the complexes, also possesses TPR and was suggested to interact directly with hsp90 (although the PPIase activity is not reported). 9a)

Information about varying aspects of the functions of immunophilins in vivo (in the absence of a cognate immunosuppressant) have increasingly accumulated, mainly through studies of their associated proteins. FKBP12 has been shown to interact with two types of intracellular Ca2+ release channel receptors, 10,11) and a model was proposed in which it aids in the conformational changes that affect the gating properties of the channels. FKBP12 also interacts with a type I receptor of TGF-β. 12) CyP-A associates with the gag protein of HIV and may play a role in the proper folding of the protein. 13,14) CyP-B interacts with a novel participant in the calcium-signal transduction pathway, implicating CyP-B in calcium signaling. 15) A cDNA encoding CyP-C-associated protein was also isolated and characterized. 16)

To understand the function of immunophilins, an evolutionary point of view is quite helpful. Hsp90 and hsp70 are highly conserved from bacteria to mammals. Although their functions are not well understood, p60 is shown to have a yeast homologue Sst1. 17) and FKBP52 is also suggested to have a homologue in yeast. 18) It is possible that such accessory proteins have much more fundamental roles to be highly conserved before the emergence of steroid hormone receptors.

Several lines of evidence have recently suggested that components of the protein folding machinery play crucial roles in keeping the proteins of signal transduction in a functional conformation. 19) If yeast homologues exist, it is possible to speculate the function through genetic analysis, as were the cases with hsp90 and DnaJ. 21)

Here we report on cellular localization and evidence of a yeast homologue of CyP40, as well as the isolation and identification of a CyP40-associated protein, as a first step toward elucidating the functions of this immunophilin.

MATERIALS AND METHODS

Cell Culture LLC-PK1, a porcine kidney cell line, was obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.) and cultivated in Dulbecco's modified essential medium containing 10% fetal calf serum. Nanalwa KJM-1, a subline of human Burkitt lymphoma cell line Nanalwa, 22) was grown in RPMI 1640 medium containing 10% fetal calf serum. Yeast *Saccharomyces cerevisiae* Hf7c cells were grown in YPD medium 23) containing 20 μg/ml adenine. E. coli cells were
grown in LB medium.24) For immunoblot analysis, Namalwa KJM-1 cells were lysed by incubation in a lysis buffer (50 mM sodium phosphate [pH 7.5], 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.5% Triton X-100, 5% glycerol, 0.3 mM phosphomethylsulfonyl fluoride) at 4°C for 1 h, followed by centrifugation. Yeast cell lysate was prepared by suspending the cells in a YBL buffer (20 mM Tris–HCl [pH 7.4], 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 3 mM dithiothreitol, 5% glycerol, 1 mM phosphomethylsulfonyl fluoride, 1 μg/ml pepstatin A) followed by vortexing the suspension with acid-washed glass beads (425–600 microns; Sigma, St. Louis, MO, U.S.A.) 15 times for 30 s at 4°C and then by centrifugation. Protein concentration was determined by the method of Bradford25) with reagents from Bio-Rad (Hercules, CA, U.S.A.).

Preparation of Monoclonal Antibodies against Human CyP40 A portion of human CyP40 (amino acid 186–291) was expressed in E. coli as a fusion protein with maltose binding protein (MBP) and was used as an antigen (MCP-2 in Fig. 3). Oligomers QCY640F and QCY957R (5'-CTCGAC- TAAATAGCTTTACCTTGACATCCTT-3') were used to amplify the portion by PCR with the cDNA of human CyP40 as a template. Pfu polymerase (Stratagene, La Jolla, CA, U.S.A.) was used for PCR amplification according to instructions from the manufacturer. The PCR parameters were 94°C for 90 s, 50°C for 60 s, and 72°C for 120 s for 15 cycles. Amplified products were digested with BamHI and HindIII (Takara Shuzo, Shiga, Japan) and cloned into the maLE-fusion vector pMALS-c2 (New England Biolabs, Beverly, MA, U.S.A.). E. coli cells harboring the recombinant plasmid were grown, induced with isopropyl β-D-thiogalactopyranoside (IPTG), harvested, suspended in a column buffer (20 mM Tris–HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA) and disrupted by sonication. The fusion protein was purified with an amylose column as the manufacturer recommended. The fused protein was digested with Factor Xa (New England Biolabs). Since the fusion protein contains the recognition sequence of the protease, IEGR, at the junction of MBP and CyP40, this cleavage generates the CyP40 portion free of an MBP sequence. The CyP40 portion was purified with DEA–Sepharose (Pharmacia, Uppsala, Sweden) and used for immunization.

Procedures for the immunization of BALB/c mice, cell fusion and hybridoma selection were as described.26) The specificity of immunoglobulins in the culture supernatant from each of the hybridomas was confirmed by enzyme-linked immunosorbent assay. The IgG was purified from each culture supernatant through enrichment with caprylic acid followed by ammonium sulfate precipitation.27)

Immunofluorescence Confocal Microscopy LLC-PK1 cells cultured in chamber slides (Nunc, Roskilde, Denmark) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 8 mg/ml NaCl, 0.2 mg/ml KCl, 1.44 mg/ml Na2HPO4, 0.24 mg/ml KH2PO4) at 4°C for 30 min. After being washed with PBS, the cells were permeablized by immersing the slides in 0.05% Triton X-100 in PBS at room temperature for 10 min, then blocked with 1% bovine serum albumin (BSA) in PBS at room temperature for 15 min. The cells were incubated with 30 μg/ml mouse monoclonal IgG against human CyP40 or mouse normal IgG (Sigma) in 1% BSA–PBS at 37°C for 40 min, washed with PBS, then incubated with 20 μg/ml fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG + IgM (H + L) (Kirkegaard & Perry Lab., Gaithersburg, MD, U.S.A.) in 1% BSA–PBS at 37°C for 40 min. The stained cells were submitted to fluorescence microscopy using a Meridian In-SIGHT-IQ laser scanning confocal microscope (Meridian Instruments, U.S.A.).

Immunoblotting The rabbit antibodies against murine hsp84 (corresponding to human hsp90) and murine hsp86 (corresponding to human hsp90x) were from Affinity BioReagents (Jersey City, NJ, U.S.A.). Mouse monoclonal antibody N27F3-4 against hsp70 (hsC70 and hsp70) and purified human hsp90 were from StressGen (Victoria, Canada). Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibody was from Dako (Glostrup, Denmark).

Proteins were subject to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)28) and transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 1% BSA in PBS and incubated with the first antibody. These membranes were then washed with 0.05% Tween 20 in PBS and reacted with second, peroxidase-conjugated antibody. Immune complexes were visualized by the ECL system (Amersham, Buckinghamshire, U.K.).

Expression and Purification of the CyP40 Fusion Proteins Human CyP40 is expressed in E. coli as a fusion protein with an amino-terminal 6 histidine residue (His-tag: HCP-1 and HCP-2 in Fig. 3) or E. coli maLE (MCP-1 in Fig. 3).

Synthetic oligonucleotides (forward primer QCY88F; 5'-GGCGATCCTGAGTCGCTTCCGCCCTGCCGAC-3', reverse primer QCY1195R; 5'-CTCAAGTTGAATGCACCATTTGCAATTCGTCAC-3') were employed to amplify the entire CyP40 protein coding region, except the first methionine with the His-tag, using human CyP40 cDNA as template. An oligomer QCY636R (5'-CCAGCTTAAATGGACGAACTTCGATACT-3') was used as the reverse primer to prepare truncated CyP40 (amino acid 1–184) containing only the cyclophilin-like portion (HCP-2). The PCR conditions were as above. The amplified fragment was digested with BamHI and HindIII, cloned into pQE30 (Qiagen, Chatsworth, CA, U.S.A.), and used to transform E. coli M15/pREP4 (Qiagen). The nucleotide sequence of the insert of the recombinant plasmid was confirmed. The fused protein contains 16 amino acids (MRGSHHHH-HHHSIEGR–) prior to the amino terminal of the human CyP40. Transformants were grown, induced with IPTG, and harvested as Qiagen recommended. Cells were disrupted and the His-tagged recombinant human CyP40 was purified with a Ni2+–NTA agarose column according to Qiagen's protocol.

Oligomers QCY640F (5'-GGCGATCCTGAGTCGCTTCCGCCCTGCCGAC-3') and QCY1195R were used to amplify the C-half portion of human CyP40 (amino acid 186–370; MCP-1 in Fig. 3). The
conditions of PCR were as described above. The amplified product was digested with BanH I and HindIII, cloned into pMAL-c2, and used to transform E. coli DH5α. The recombinant fusion protein was expressed and purified as above.

Isolation of CyP40-Associated Proteins from Rabbit Reticulocyte Lysate Three μl of Ni²⁺-NTA agarose beads were mixed with recombinant human CyP40 (HCP-1 or HCP-2, 120 pmol) and rabbit reticulocyte lysate (40 μl; Promega, Madison, WI, U.S.A.) or purified human hsp90 (15 μg; StressGen) in 100 μl of TKMTI buffer (10 mM Tris–HCl [pH 7.4], 50 mM KCl, 3 mM MgCl₂, 10 mM thiglycerol, 20 mM imidazole [pH 7.4]), and incubated at 30 °C for 30 min. After incubation on ice for 30 min, the beads were washed 4 times with 300 μl of M buffer (10 mM Tris–HCl [pH 7.4], 20 mM NaMoO₄, 10 mM thiglycerol, 20 mM imidazole [pH 7.4]). Each wash involved intermittent mixing and incubation on ice for 2–5 min prior to centrifugation. The bound proteins were released by incubation with 40 μl of 400 mM imidazole in M buffer [pH 7.4] at 4 °C for 30 min. The released proteins were fractionated by SDS–PAGE, and the gel was silver-stained. When MCP-1 (480 pmol) was used, Ni²⁺-NTA beads were replaced with 6 μl of amylase beads, and bound proteins were released with 10 mM maltose in M buffer. Recombinant E. coli MBP (New England Biolabs) was used as the negative control.

RESULTS

Antibody against CyP40 In order to characterize CyP40, we raised specific monoclonal antibodies against human CyP40. Since the homology of other cyclophilin members to the N-half of the protein, i.e., the cyclophilin-like portion, is much higher than that of FKBP52 to the C-half portion, we have chosen a region in the C-half portion for the antigen to obtain specific antibodies (See Fig. 3). Several hybridoma clones whose culture supernatant had specific reactivity to CyP40 were obtained. Three of them (KM1166, KM1172, KM1175) were investigated by Western blot analysis using a human cell (Namalwa K3M-1) lysate, and all were shown to react with a single protein of 41 kDa and not with ubiquitously expressed FKBP52 or p60⁹,³⁰ (Fig. 1. for KM1172; data not shown for the rest), indicating high specificity toward CyP40. Similar results were obtained with the lysate of LLC-PK1 cells (data not shown; see below).

The localization of CyP40 was investigated using one of the antibodies (KM1175) with immunofluorescence microscopy (Fig. 2). The cells used in this study were LLC-PK1 (porcine kidney) which have a flat morphology and were employed in a study of the localization of FKBP52.³⁰ The monoclonal antibody preferentially stained cytoplasm in a somewhat fibrillar manner. The nuclei were stained to some extent, with the exception of the nucleoli. Confocal analysis showed that the staining was present in all planes of the nucleus. The same amount of mouse normal IgG gave no fluorescence (data not shown).

CyP40 Has a Homologue in Yeast Two mammalian proteins, FKBP52 and p60, which contain a homologous region with the C-half portion of CyP40 and make complexes with hsp90, were highly conserved throughout the evolution and were suggested to have homologues, even in yeast. In our molecular genetic studies, CyP40 was suggested to diverge from other classes of cyclophilins early in evolution, and was highly conserved thereafter (Yokoi et al., in preparation). We sought to obtain additional evidence of this conservation through immunological studies.

Yeast (Saccharomyces cerevisiae) cell lysate was immunoblotted with a monoclonal antibody KM1172. This antibody clearly recognized a single protein of 45 kDa (Fig. 1), suggesting the presence of a homologue of CyP40 in yeast.

Identification of a CyP40-Associated Protein Johnson and Toft have reconstituted the inactivated progesterone receptor complex, consisting of the receptor, hsp90, hsp70, and other associated proteins, by incubation in rabbit reticulocyte lysate in the presence of ATP/Mg²⁺.³¹ To identify proteins which are directly bound to CyP40, we first constructed a His-tag-CyP40 fusion protein (HrCyP40) with a pQE30 expression vector. This recombinant protein, expressed in E. coli and purified to near homogeneity with Ni²⁺-NTA beads, retained PPlase activity (k₅₀/Kₘ = 9.9 × 10⁵ μM⁻¹ s⁻¹) and its CsA sensitivity (IC₅₀ = 3 × 10⁻⁷ M). Then, HrCyP40 bound Ni²⁺-NTA beads were incubated with rabbit reticulocyte lysate and the associated proteins were eluted with imidazole (Fig. 4A).

A 90 kDa protein which associated specifically with the CyP40-bound beads but not with unbound beads was isolated (Fig. 4A, lanes 1, 2, and 4). As CyP40 was isolated as a member of unactivated estrogen receptor complexes, it was possible that this protein is hsp90. To test the possibility, the associated protein was immunoblotted with an antibody against murine hsp84 (corresponding to human hsp90β). The 90 kDa protein was reacted with the antibody (lane 2) as well as with that against murine hsp86 (corresponding to human hsp90α; data not shown). To further confirm this, purified human hsp90 was subjected to the affinity beads. Hsp90 bound to the HrCyP40-bound beads but not to the unbound beads (lanes 7, 9).

As CyP40 participates in the progesterone receptor complex in an ATP/Mg²⁺ dependent manner,³³ and the cyclophilin A-hsp90 binding in vitro also requires ATP,³² CyP40-hsp90 association may be enhanced by ATP. In the condition described here, however, ATP had instead an inhibitory effect (Fig. 4A, lane 3). The addition of chemicals that have an affinity with CyP40 or hsp90, including CsA (20 μM), herbimycin (500 μM),³³ and deoxypergualin (500 μM),³⁴ did not affect the complex formation nor generate another associate protein (data not shown). Hsp70 was not detected in the CyP40-bound fraction, even with immunoblot analysis (data not shown).

Next, we sought to identify the region of CyP40 responsible for the interaction with hsp90. With FKBP52, the C-terminal segment was shown to be necessary for the interaction. Therefore, it is conceivable that the C-portion of CyP40, having homology with that portion of FKBP52, may be responsible. Alternatively, as cyclophilin A interacts with hsp90,³³ the cyclophilin-like
portion, the N-terminal half of CyP40, may be involved. We have constructed fusion proteins with a portion of CyP40. They are expressed in E. coli and purified with affinity columns. HCP-2 has only the N-half portion (amino acids 2—184) and was shown to retain the PPIase activity (data not shown). As HCP-2 did not bind with hsp90 (Fig. 4A, lanes 5, 6, 9), the C-half portion was suggested to be necessary.

Next, we investigated whether the isolated portion was sufficient. As the fusion protein between His-tag and the C-half portion was not expressed easily in E. coli, we constructed a fusion protein with E. coli MBP (MCP-1). After confirmation that the fusion protein between MBP and the full-length CyP40 retained the affinity with hsp90 (data not shown), the possibility of the C-half portion to associate with hsp90 was investigated (Fig. 4B). Because only a fraction of the fusion protein had affinity with amylose resin for unknown reasons, the purification yield was low. As the E. coli resident MBP of 42 kDa was co-purified, the “MCP-1” contained approximately equal amounts of MBP (Fig. 4B, lane 3; the identity of MBP was confirmed by an immunoblot with anti-MBP antibody, data not shown).

The MCP-1 had the ability to interact with hsp90 from rabbit reticulocyte lysate (Fig. 4B, lane 2) and purified human hsp90 (lane 6), while purified MBP alone did not (lanes 4 and 7), indicating that the portion used is sufficient for the interaction with hsp90.

DISCUSSION

Recent studies have suggested pivotal roles of different components of protein folding machinery on the regulation of signaling pathways. In mammals, hsp90 function is suggested to be tied to the signaling pathway involving pp60src in addition to that of steroid hormones. Hsp90 was also shown to be necessary for two Ras-dependent signaling pathways in Drosophila. Through the genetic studies in yeast, DnaJ was suggested to play a potential role in the maintenance of steroid receptor complex assembly. As for CyP40, it has been reported that the addition of CsA potentiates glucocorticoid-induced transcription through a pathway which does not involve calcineurin, raising the possibility that a conformational or functional change in CyP40 affects the activity of an associated signal transduction molecule.

In this communication, we reported a 45 kDa protein in yeast that reacts with the monoclonal antibody raised against the non-cyclophilin part of human CyP40 (Fig. 1). Chang and Lindquist also reported a 45 kDa hsp90-

![Fig. 1. A Yeast Homologue of CyP40](image)

Cell lysate of Namalva KJM-1 (70μg) or Saccharomyces cerevisiae H7c (16μg) was subjected to 12% SDS PAGE, transferred to Immobilon membranes, and reacted with mouse monoclonal IgG KM1172 (1 μg/ml).

![Fig. 2. Immunofluorescent Staining of CyP40](image)

Fixed and permeabilized LLC-PK1 cells (from porcine kidney) were stained with the monoclonal antibody KM1175 and examined under fluorescence confocal microscopy.
associated protein in yeast whose partial amino acid sequence is homologous to cyclophilins, so these proteins may be identical and a yeast homologue of CyP40. This suggests that CyP40 diverged from other classes of cyclophilins very early in evolution and was highly conserved thereafter. This speculation is in accordance with our recent observations that the exon-intron organization of human CyP40 gene is different from that of human CyP-A, and that nucleotide sequences homologous with the human CyP40 gene were detected from non-vertebrates in conditions not allowing the detection of sequences of other cyclophilin members (Yokoi et al., in preparation).

Therefore, CyP40 may exist before the emergence of steroid hormone receptors. What are the functions of this immunophilin in the absence of the receptors? Recent observation indicates that a complex consisting of hsp90, p23, and immunophilins (CyP40 or FKBP52) can be formed without any steroid hormone receptors. Pratt has presented the notion that heterocomplexes between hsp90 and its associated proteins function as "transportosomes" which help many proteins fold properly and undergo trafficking through the cytoplasm. The existence of a yeast homologue may facilitate the elucidation of CyP40 functions in signal transduction with the help of yeast genetics, as was the case of DnaJ.

The immunostaining study showed that CyP40 localizes preferentially in cytoplasm (Fig. 2), in contrast to FKBP52, which locates dominantly in the nucleus. However, their localization is similar in that they are fibrous in cytoplasm and not observed in the nucleolus.

We also demonstrated that CyP40 directly binds to both types of hsp90 in an ATP-independent manner (Fig. 4). This result contrasts with the observation that the formation of a heterocomplex consisting of hsp90, p23, and CyP40 requires ATP/MgCl2. The responsible region of the interaction was the C-half part which contains a TPR sequence. This is consistent with the result that FKBP52 directly binds to hsp90, and the region around the TPR sequence is necessary for the binding. The TPR sequence, present in several proteins for protein import, transcription or mitosis, may function as an hsp90-binding domain.

During the preparation of this manuscript, Hoffmann et al. reported similar results using glutathione S-transferase (GST)-CyP40 fusion proteins and human cell

![Fig. 3. Construction of Fusion Proteins](image)

The numbers refer to amino acid positions. The hatched or black box represents His-tag or MBP, respectively.

![Fig. 4. Isolation and Identification of CyP40-Associated Protein](image)

A: Rabbit reticulocyte lysates lanes 1–3, 5–6) or purified human hsp90 (lanes 7–9) was mixed with Ni2+–NTA beads together with His-tagged CyP40 (HCP-1; lanes 2–4, 7) or its truncated product (HCP-2; lanes 5, 6, 8). In lane 3, ATP (6 mM) was added to the reaction mixture. The bound proteins were eluted with imidazole, subjected to SDS-PAGE (5–20%, ATTO, Japan), and stained with silver (upper panel). The eluted proteins were also subjected to immunoblot analysis with rabbit anti-hsp94 antibody after SDS-PAGE with 8% gel, followed by detection with peroxidase-conjugated swine anti-rabbit immunoglobulin antibody (Dako, lower panel). B: Rabbit reticulocyte lysates lanes 1, 2, 4) or purified human hsp90 (lanes 6–8) was mixed with amylase beads together with MCP-1 (lanes 2, 3, 6) or MBP (lanes 4, 5, 7). Purified hsp90 (500 ng in upper panel, 200 ng in lower panel) was applied to lane 10 (A) or 9 (B). F, N; or C denotes HCP-1, HCP-2, MCP-1, respectively.
lysate.\textsuperscript{(40)}

Recently, the vpr gene product of human immunodeficiency virus type 1 (HIV-1) was demonstrated to interact with a cellular protein of 41 kDa which associates with glucocorticoid receptors (GR) and converts the receptor into an active form without the ligand.\textsuperscript{(41)} Although it is not certain at present that the GR-associated protein of 41 kDa is Cyp40, the result is another example which suggests the importance of accessory proteins of steroid hormone receptors in controlling the activity of the receptors, as was the case with DnaJ.

Still much effort will be necessary to elucidate the functions of Cyp40. The observations reported here will be a foundation for such investigations.

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