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Cyclophilin-40 has a cellular role in the aryl hydrocarbon receptor signaling

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Abstract

CyP40 promotes the formation of the gel shift complex that contains AhR, Arnt and DRE using baculovirus expressed proteins. Here we reported that CyP40 plays a role in the AhR signaling. When the CyP40 content in MCF-7 cells is reduced, up-regulation of *cyp1a1* and *cyp1b1* by 3MC is also reduced, suggesting that CyP40 is essential for maximal AhR function. The CyP40 region containing amino acids 186–215, but not the PPIase and TPR domains, is essential for forming the AhR/Arnt/DRE complex. CyP40 is found in the cell nucleus after 3MC treatment and appears to promote the DRE binding form of the AhR/Arnt heterodimer.

Keywords

Cyclophilin-40; CyP40; aryl hydrocarbon receptor; AhR; Arnt

1. Introduction

CyP40 was first isolated as part of the ERα complex [1]. It was subsequently characterized as a CsA-binding protein with PPIase activity [2]. CyP40 has been found in different heterocomplexes of steroid receptors [3–6], although its precise role in the steroid receptor trafficking remains to be discovered. The X-ray structural data showed that CyP40 contains motifs that are found in the large cyclophilin family of chaperone proteins and the TPR domains of CyP40 interact with C-terminal MEEVD region of Hsp90 [7,8]. This CyP40-Hsp90 interaction may be important for the CyP40 function since it was reported that the TPR domain of Cpr7 (CyP40 homolog) is required for the glucocorticoid receptor signaling in yeast [9].

There is much evidence supporting that the cytosolic AhR complex contains a dimer of Hsp90, one molecule of p23 and one molecule of XAP2 (or Ara9 or AIP) [10–14]. Upon ligand binding, conformation change occurs to the cytosolic AhR complex, leading to nuclear translocation which appears to be an importin-mediated event [15]. Heterodimerization of AhR and Arnt in the nucleus is necessary for the AhR signaling because activation of AhR target gene transcription requires binding of this heterodimer to its enhancer element DRE to form the AhR/Arnt/DRE complex.

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We had expressed human AhR and Arnt as 6His fusions in a baculovirus system and found that formation of the AhR/Arnt/DRE complex in vitro requires protein factors [16]. With the use of the rabbit reticulocyte lysate, we identified CyP40 as a protein factor that allows the ligand-dependent formation of the AhR/Arnt/DRE complex in a gel shift assay [17]. At present, CyP40 has not been described as part of the AhR signaling, other than an interesting observation that the yeast homolog of CyP40 (Cpr7) appeared to affect AhR signaling in an artificial system [18]. In this paper, we have provided evidence to support that CyP40 is involved in the AhR signaling. It promotes the formation of the AhR/Arnt heterodimer that binds DRE in an Hsp90-independent manner.

2. Materials and methods

2.1. Cell Line, Antibodies and Nucleic Acids

MCF-7 cells were purchased from ATCC. Cell culture reagents were purchased from Sigma and Invitrogen. Antibodies against Arnt (H-172), CyP40 (PA3-022) and GAPDH (6C5) were purchased from Santa Cruz Biotechnology, Affinity Bioreagents and Ambion, respectively. CyP40 siRNAs were purchased from Ambion and all other oligonucleotides for gel shift assay and real time QPCR were purchased from Invitrogen (DRE: OL5, 5'-TCGAGTAGATCACGCAATGGGCCCAGC-3' and OL6, 5'-TCGAGCTGGGCCCATTGCGTGATCTAC-3'; CyP40 QPCR primers (182bp): OL270, 5'-AGGCATTGGACACACGACTGG-3' and OL271, 5'-AACCCTCCCGATCATGCTTGT-3'; CYP1A1 QPCR primers (340bp): OL90, 5'-GGCCACATCCGGGACATCACAGA-3' and OL91, 5'-TGGGGATGGTGAAGGGGACGAA-3'; GAPDH QPCR primers (147bp): OL291, 5'-GGCCTCCAAGGAGTAAGACC-3' and OL292, 5'-AGGGGTCTACATGGCAACTG-3'; CYP1B1 QPCR primers (165bp): OL333, 5'-CACCAAGGCTGAGACAGTGA-3' and OL344, 5'-GATGACGACTGGGCCTACAT-3'; CyP40 siRNA 111540: sense: 5'-GCAUGAUCGGGAGGGUUUAtt-3' and antisense: 5'- UAAACCCUCCCGAUCAUGCtt -3' and CyP40 siRNA 111541: sense, 5'- GGAUAUUGGAAAAUGUGGAtt -3' and antisense: 5'- UCCACAUUUUCCAAUAUCCtt -3').

2.2. Luciferase Activity With or Without CyP40 Knockdown

MCF-7 cells were grown in DMEM supplemented with 10 % FBS, 2 mM glutaMAX, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. The transfection mix (40 μl), which contained 1.2 μl of siPORT NeoFX (Ambion) and 2 μl of either water, CyP40 siRNA (111540 or 111541), or control siRNA (#5 siRNA) in Opti-MEM, was added to each well of a 24-well plate containing 7.2×10^4 cells in 0.36 ml of phenol red-free DMEM containing 10 % of charcoal stripped FBS (Gemini). The final concentration of the siRNA in each well was 30 nM. After 24 hours, the cells were transfected with reporter plasmids by addition of 25μl transfection mix containing 150 ng of pGudLuc 1.1, 50 ng of pCH110 (Amersham Pharmacia) and 0.4 μl of Fugene (Roche). Forty-four hours after siRNA treatment, 3MC (1 μM final) or DMSO was added and the cells were harvested 5 hours afterwards for luciferase assay according to manufacturers protocol (Applied Biosystems). The experiment was done in triplicate and luciferase activities were normalized by the internal β-galactosidase activities.

2.3. CYP1A1 induction by 3MC With or Without CyP40 Knockdown

MCF-7 cells were grown as mentioned above. Each data point was obtained from one well of a 6-well plate. The transfection mix (200 μl), which contained 6 μl of siPORT NeoFX (Ambion) and 10 μl of either water, CyP40 siRNA (111540 or 111541), or control siRNA (#5 siRNA, Ambion) in Opti-MEM, was added to a well containing 3.6×10^5 cells in 1.8 ml of phenol red-free DMEM containing 10 % of charcoal stripped FBS. The final concentration of the siRNA in each well was 30 nM. After incubating the cells for 44 hours at 37 °C, 3MC (1 μM final)

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was added and the cells were harvested 4 hours afterwards. RNA was obtained using the MasterPure RNA Purification kit (Epicentre) according to the manufacturer's protocol. MMLV reverse transcriptase (Epicentre) was used to generate the cDNA pool. Real-time QPCR was performed with iQ SYBR Green Supermix (Bio-Rad) in an iCycler thermal cycler (Bio-Rad). PCR conditions (40 cycles) were as follows: 90 °C for 10 seconds and 60 °C (annealing and extension) for 60 seconds. SYBR green fluorescence readings were taken at 60 °C when the fluorescence intensity corresponded solely to the PCR product of interest. The final PCR products were confirmed to give a single product on an agarose gel. Normalized fold increase of the endogenous transcript was determined by the $2^{-\Delta\Delta CT}$ method using GAPDH as the internal standard.

2.4. Generation of CyP40 Deletion Constructs

The cDNAs corresponding to amino acids 1–185, 1–215, and 186–370 of CyP40 were amplified by sequence-specific primers and then cloned into the BamHI and HindIII sites of pQE80 (Qiagen) to generate 6His fusion constructs. Purification of these constructs was described previously [17].

2.5. Gel Shift Assay

Gel shift protocol was described previously [17]. In brief, TALON purified baculovirus expressed Arnt (0.1 μl) was incubated with either TALON purified baculovirus expressed AhR (1–2 μl) or TALON purified bacterially expressed CΔ553 with or without thioredoxin fusion (0.1 μl) in the presence or absence of βNF (10 μM) in HEDG buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 10 % glycerol) for 10 minutes at 30 °C. Poly(dI-dC) (1 μg) and KCl (115 mM final) were added. After 10 minutes at room temperature, ^{32}P-DRE (100,000 cpm) was added, followed by another 10-minute incubation at room temperature before 4 % native gel electrophoresis.

2.6. Coimmunoprecipitation

^{32}P-CΔ553 was generated as described previously [19]. In brief, TALON purified bacterially expressed TH-CΔ553 was digested by thrombin to release thioredoxin, followed by HMK phosphorylation using ^{32}P-gamma ATP. ^{32}P-CΔ553 was incubated in a final volume of 35 μl with 80 μg of proteins (Sf9 soluble extract, bacterially expressed 6His-CyP40 or BSA) in the presence or absence of TALON purified baculovirus expressed Arnt (5 μl) in HEDG buffer for 10 minutes at 30 °C. Anti-Arnt pAb H-172 (5 μl) was then added, followed by a 30-minute incubation at room termpeature. The samples were then added to new tube containing 20 μl of dynabeads protein G (Invitrogen), followed by an 1-hour rotation at 4 °C. The dynabeads were washed three times with 0.5 ml of HEDG plus 0.1 % Tween-20. After wash, the immunoprecipitated proteins were directly eluted into the SDS treatment buffer and then analyzed by 10 % SDS-PAGE and autoradiography.

2.7. Statistical Analyses

The statistical significance between means (in triplicate) was determined by applying two-tailed t-tests in Excel 2000 software. Single asterisk represents a p value < 0.05.

3. Results and Discussion

Although CyP40 has been found in many Hsp90-containing chaperone complexes, it is unclear whether it functions as a cochaperone for general protein folding or it plays a different role in different complexes. We postulated that CyP40 has a specific function in the AhR signaling and the function involves the formation of the AhR/Arnt/DRE complex. In this paper, we presented evidence in support of our hypothesis and concluded that activation of the AhR-

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mediated gene transcription involves CyP40 in mammalian cells. CyP40 acts in the cell nucleus to promote the formation of the AhR/Arnt/DRE complex and this action appears to be Hsp90-independent and involve the formation the AhR/Arnt heterodimer that is capable of binding to DRE.

3.1. CyP40 is Involved in the AhR Signaling

We have previously shown that CyP40 is capable of restoring the formation of the baculovirus AhR/Arnt/DRE gel shift complex, suggesting that CyP40 might play a role in the AhR signaling. To explore this hypothesis, we used CyP40-specific siRNAs to knockdown its content in MCF-7 cells and addressed whether lowering of the CyP40 content might affect the AhR-mediated gene expression. We used two different CyP40 siRNAs (111540 and 111541) to knockdown the CyP40 content in MCF-7 cells. Either siRNA was capable of suppressing the CyP40 content in the presence or absence of 3MC. The CyP40 message and protein were significantly suppressed by at least 50 % and 75 %, respectively, in the presence of 3MC (Fig. 1A and B). Both siRNAs suppressed pronouncedly the 3MC-dependent luciferase expression by at least 75 % (Fig. 1C). When we examined the CyP40 effect on the endogenous AhR target genes, we found that both cyp1a1 and cyp1b1 messages were significantly suppressed by 40 and 30 %, respectively, when the CyP40 content was reduced (Fig. 1D and E). To our knowledge, this is the first evidence showing that CyP40 has a cellular role in the mammalian AhR signaling. Our immunofluorescence staining data showed that CyP40 is present in both cytoplasm and nucleus with distinctive nucleoli staining (Fig. 2). Other investigators have also observed CyP40 in the MCF-7 nucleoli using a custom anti-CyP40 IgG [20]. When the same anti-CyP40 IgG as ours were used, other investigators had reported that CyP40 was nucleolar in mouse Lcl3 [21] and rat pulmonary endothelial cells [22]. After 3MC treatment, CyP40 appeared to redistribute from nucleoli to nucleoplasm whereas the vehicle DMSO alone showed significant nucleoli staining after one hour. Other AhR ligands such as benzo[a]pyrene and β-naphthoflavone also showed less nucleoli staining after one hour, suggesting that this CyP40 redistribution occurs after AhR activation. We do not believe that this redistribution of CyP40 is specific to AhR since heat shock also showed a similar redistribution [20]. In addition, this redistribution is probably not caused by a direct interaction between AhR and CyP40 since the amount of CyP40 appears to be abundant as compared to AhR. Nonetheless CyP40 is present in the nucleus after AhR ligand treatment, making it possible that CyP40 assists the formation of the AhR/Arnt/DRE complex in the nucleus.

3.2. CyP40 Linker Region (Amino Acids 186–215) is Necessary to Form the AhR/Arnt/DRE Complex and the CyP40 Effect is Hsp90-Independent

We generated CyP40 deletion constructs to investigate the requirement of CyP40 for forming the βNF-dependent AhR/Arnt/DRE gel shift complex (Fig. 3A). Basically CyP40 contains PPIase (aa 1–185) and three TPR domains (aa 242–370) that are joined by the linker region (aa 186–215) which has been shown to possess chaperone activity [23]. We generated three deletion constructs (aa 1–185, PPIase only; aa 1–215, PPIase and the linker region; and aa 186–370, TPRs and the linker region) and used them to investigate whether PPIase, TPR and the linker region are necessary to restore the AhR gel shift complex. These deletion constructs were made as 6His fusions and expression of these proteins was confirmed by Western analysis (data not shown). Three μg of full length CyP40, aa 1–215, or aa 186–370 formed the βNF-dependent AhR/Arnt/DRE complex with comparable gel shift intensity; however, aa 1–185 only gave less than 50 % gel shift intensity of the full length CyP40 (Fig. 3B and C), suggesting that the amino acid region between 186 and 215 (the linker region) is necessary for the full CyP40 effect. Constructs with either PPIase or TPR deleted still retained the gel shift activity, suggesting that both PPIase and TPR are not necessary for forming the AhR/Arnt/DRE complex. Within the linker region of CyP40, there are some conserved acidic and hydrophobic residues which were suggested to be important for chaperone activity [23]. Further studies are

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necessary to examine whether these conserved residues are essential for forming the AhR/Arnt/DRE complex.

Realizing that CyP40 interacts with the C-terminal Hsp90 region through a number of hydrophobic and charged residues within its TPR domains [24], data from our deletion studies argued that Hsp90 is not involved in the CyP40 effect since the TPR domains are not necessary for CyP40 to form the AhR/Arnt/DRE complex. In order to further address the involvement of Hsp90, we used a deletion construct of human AhR fused to thioredoxin (TH-CΔ553) [19] in a gel shift assay. TH-CΔ553 does not interact with Hsp90 but still forms the AhR/Arnt/DRE gel shift complex in a ligand-independent manner. We hypothesized that if CyP40 does not require Hsp90 to form the AhR/Arnt/DRE complex, CyP40 should be able to form the CΔ553/Arnt/DRE complex which is Hsp90-independent but protein factor-dependent. Indeed, CyP40 caused the formation of the TH-CΔ553 gel shift complex (Fig. 3D). Interestingly we have identified another protein factor p23 which forms the AhR/Arnt/DRE complex in an Hsp90-dependent manner [25], revealing that there are multiple mechanisms to assist the formation of the AhR/Arnt/DRE complex.

Upon binding of CsA to CyP40, the PPIase activity of CyP40 is inhibited [26,27]. Formation of the CsA-CyP40 complex in turn inhibits the phosphatase activity of calcineurin [2]. Since PPIase of CyP40 is not necessary for the AhR/Arnt/DRE complex formation, it is unlikely that calcineurin would play any role here. In addition, phosphorylation may very well be involved after binding of the AhR/Arnt heterodimer to the DRE because protein kinase C-dependent phosphorylation was implicated in the AhR signaling and this effect did not affect the AhR/Arnt/DRE complex [28]. But this does not rule out the possibility of a mechanism that involves CyP40-mediated phosphorylation.

3.3. CyP40 Helps the AhR/Arnt Heterodimer Formation That Binds DRE

We performed a number of different gel shift experiments in an effort to gain mechanistic insights on how CyP40 allows the formation of the AhR/Arnt/DRE complex. First, we investigated whether the time of CyP40 addition to the gel shift sample is crucial. Typically in a gel shift experiment, we combined the baculovirus expressed human AhR and Arnt in the presence or absence of βNF and incubated them at 30 °C for 10 minutes (the activation step) to allow the heterodimerization of AhR and Arnt to occur. We then added the DRE to form the AhR/Arnt/DRE complex. Addition of CyP40 at or after the activation step would suggest whether CyP40 is important for forming the AhR/Arnt heterodimer or binding of the heterodimer to the DRE, respectively. Our gel shift data showed that CyP40 may be important in assisting the AhR/Arnt heterodimerization since CyP40 must be added at the activation step to give gel shift activity (Fig. 4A).

Realizing that the linker region of CyP40 has folding capability and can restore the AhR/Arnt/DRE complex, we postulated that CyP40 conforms the structure of the AhR/Arnt heterodimer in a way that would form the AhR gel shift complex. Our co-immunoprecipitation and gel shift data showed that CΔ553 interacts with Arnt in the presence of BSA alone but this heterodimer does not bind DRE. We believe that BSA possesses a general protein effect that helps proteins to interact with one another [29,30]. In contrast, in the presence of CyP40, the AhR/Arnt heterodimer binds DRE (Fig. 4B and C). This observation could be explained by the presence of two conformations of the AhR/Arnt heterodimer – CyP40 promotes the DRE binding form whereas the AhR/Arnt heterodimer that forms in the presence of a general protein (BSA) does not bind DRE. However, the mechanism of how CyP40 helps the formation of the AhR/Arnt heterodimer with DRE binding affinity is unclear. CyP40 appeared to interact with AhR very weakly and did not interact with Arnt from our co-precipitation studies (data not shown). It is conceivable that the linker region of CyP40 brings AhR and Arnt together in a conformation that binds DRE, although AhR or Arnt alone has weak affinity with CyP40. Another possibility

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is that there might be an inhibitory domain present on the AhR/Arnt complex that prevents the heterodimer to bind DRE and this inhibitory domain is “hidden” in the presence of CyP40. It is interesting that multiple forms of AhR have been reported in human breast cancer cells T47D [31], although the cause of different AhR forms is unclear. CyP40 may “fold” the nuclear AhR in a manner that promotes the formation of the AhR/Arnt heterodimer with high DRE binding potential.

Abbreviations

AhR, aryl hydrocarbon receptor
Arnt, AhR nuclear transclocator
CΔ553, AhR deletion construct with C-terminal 553 amino acids deleted
TH-CΔ553, thioredoxin fusion of CΔ553
DRE, dioxin response element
3MC, 3-methylchloranthrene
PPIase, peptidyl-prolyl cis-trans isomerase
TPR, tetratricopeptide repeat
βNF, β-napthoflavone
CsA, cyclosporin A
BaP, benzo[a]pyrene

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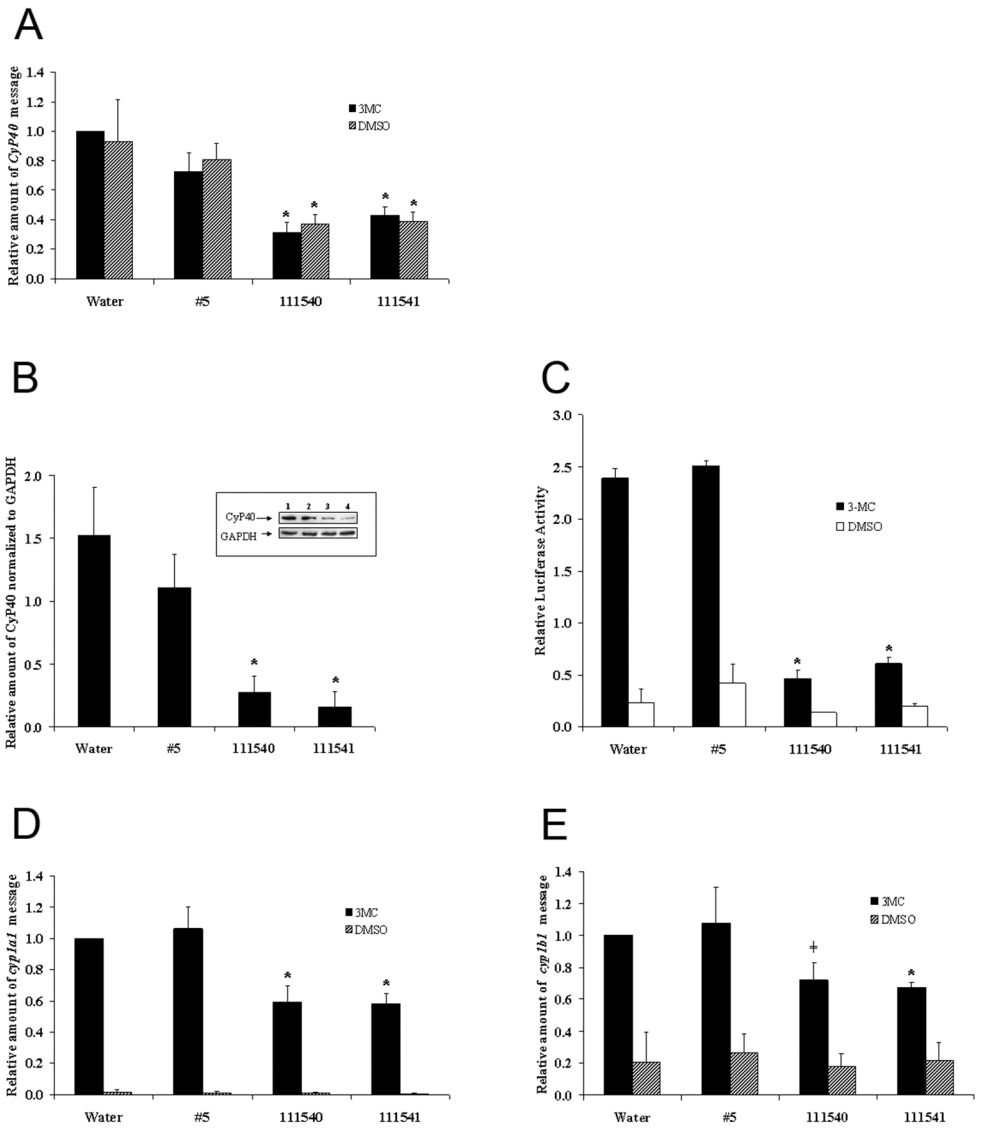

Fig. 1. Effect of CyP40 siRNA knockdown on AhR signaling

MCF-7 cells were transfected with one of the four conditions: water control, 30 nM of either scramble siRNA #5, or CyP40 siRNA 111540/111541. All data are in triplicate (n=3) and the error bars represent means ± SD. (A) Relative amount of the CyP40 message under the four conditions in the presence or absence of 1μM 3MC with *p-value < 0.05. The water control plus 3MC was arbitrarily set as 1. (B) Relative amount of the CyP40 protein under the four conditions in the presence of 1μM 3MC. The inset shows the CyP40 and GAPDH western. Anti-CyP40 pAb (1:5,000) and anti-GAPDH mAb (1:5,000) were used in TBST plus 5 % nonfat dry milk. Corresponding secondary IgG conjugated with AP (Sigma, 1:40,000) was used with Lumi-Phos substrate (Pierce). *p-value < 0.01 when compared to the siRNA #5 control. (C) Relative 3MC-driven luciferase expression under the four conditions with *p-value < 0.01. (D) Relative amount of the cyp1a1 message under the four conditions with *p-value < 0.01. (E) Relative amount of the cyp1b1 message under the four conditions with *p-value < 0.05 and ∓p-value < 0.07.

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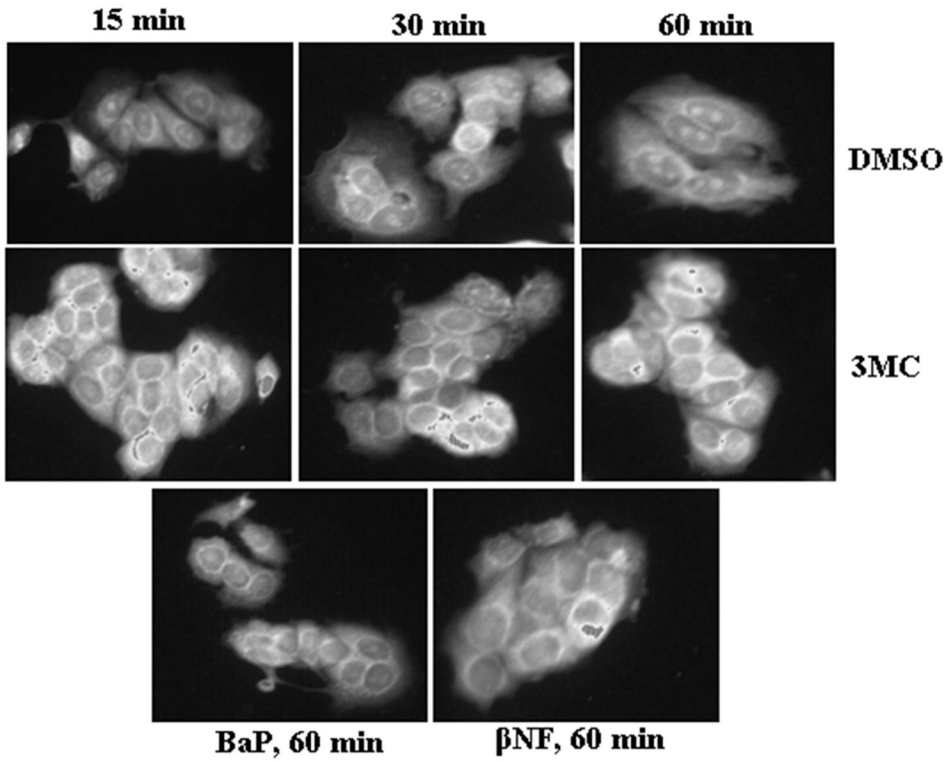

Fig. 2. Immunofluorescence staining results showing that redistribution of CyP40 after AhR ligand treatment

Cells were grown on coverslips in a 12-well plate and treated with 1 μM 3MC, 5 μM BaP, 10 μM βNF or DMSO for 15, 30, or 60 min at 37 °C. Cells were then fixed using 4 % formalin in PBS (30 minutes, room temperature) and methanol (4 minutes, −20 °C). Fixed cells were blocked with 5 % BSA in PBS (1 hours, room temperature), incubated with CyP40 polyclonal (1:250) for 1 hour at room temperature, and then incubated with anti-rabbit IgG conjugated with Alexa Fluor 488 (1:1000) in the dark for 1 hour at room temperature.

Fluorescence images were visualized under the same setting using a Leica DMIRE2 inverted fluorescence microscope.

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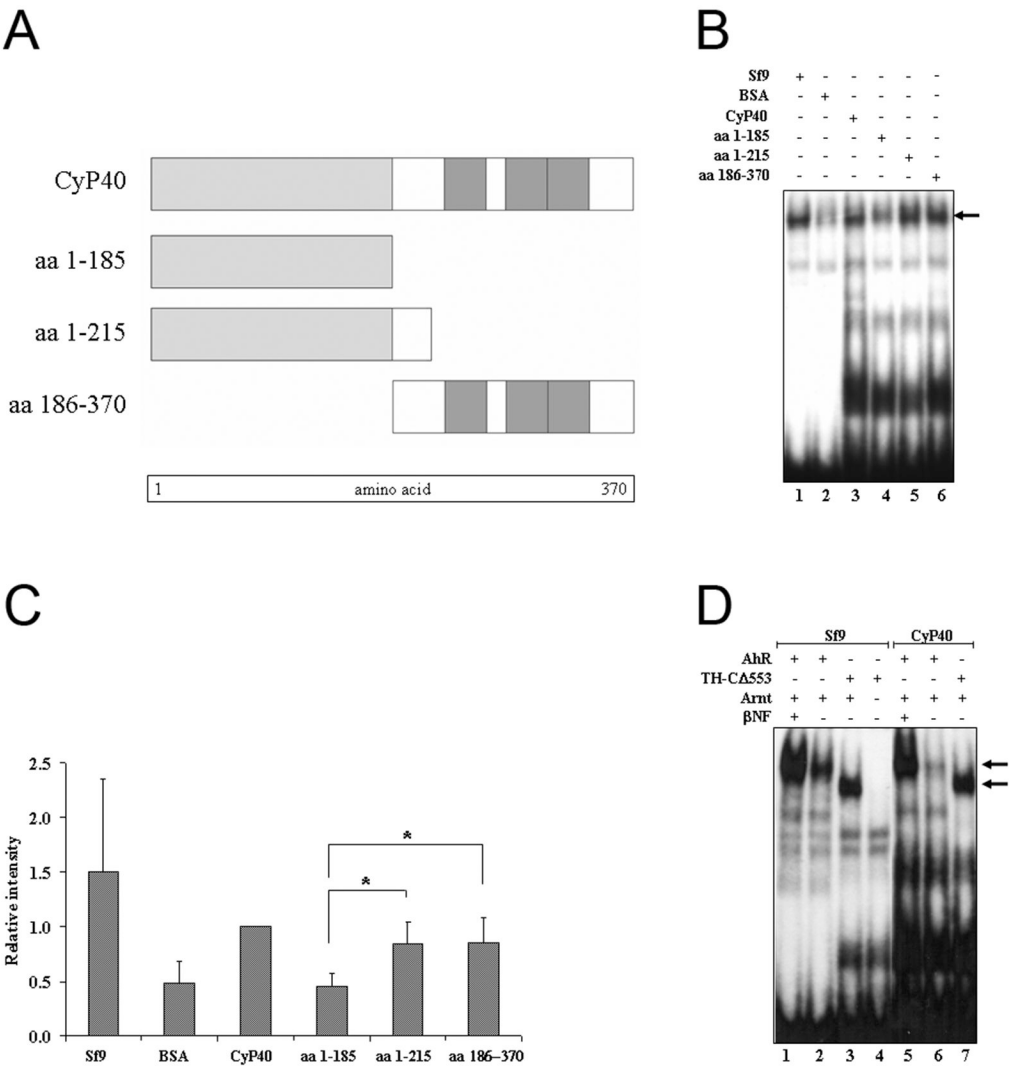

Fig. 3. The linker region of CyP40 is important for formation of the AhR/Arnt/DRE complex

(A) CyP40 deletion constructs. The shaded region on the left (aa 1–185) corresponds to the PPIase domain and the three shaded boxes on the right (>aa 215) correspond to the TPR domains. (B) Gel shift assay showing the effect of 6His-CyP40 constructs on the formation of the AhR/Arnt/DRE complex. All lanes contained baculovirus expressed AhR and Arnt, 10μM βNF, 1μg of poly(dI-dC), and 3 μg of protein (lane 1, Sf9 soluble extract; lane 2, BSA; lane 3, CyP40; lane 4, aa 1–185; lane 5, aa 1–215; lane 6, aa 186–370). (C) The gel shift experiment in (B) was repeated 5 times (n=5) and results were quantified by UN-SCAN-IT software and plotted normalized to CyP40. Error bars represented the standard deviation of the means, asterisks indicate a significant difference ($p < 0.02$) using student's t-test. (D) Gel shift assay showing that CyP40 restored TH-CΔ553 gel shift complex. All lanes used 3 μg of Sf9 soluble extract/TALON purified 6His-CyP40 and 1 μg of poly(dI-dC). Lanes 1 and 2, baculovirus expressed AhR and Arnt ± βNF + Sf9 soluble extract; lanes 3 and 4, bacterially expressed TH-CΔ553 ± Arnt + Sf9 soluble extract; lanes 5 and 6, baculovirus expressed AhR and Arnt ± βNF + CyP40; lane 7, bacterially expressed TH-CΔ553 + Arnt + CyP40. The upper and lower arrows indicate the AhR/Arnt/DRE and TH-CΔ553/ Arnt/DRE complexes, respectively.

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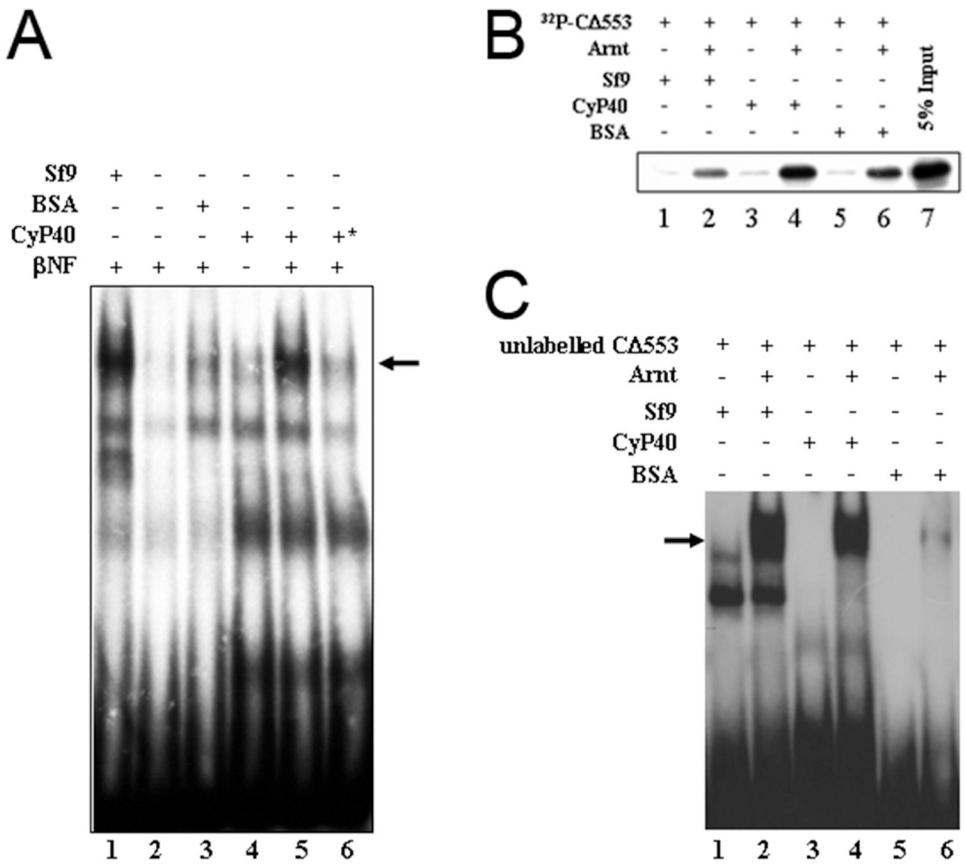

Fig. 4. CyP40 promotes the formation of the AhR/Arnt heterodimer that binds DRE

(A) Gel shift assay showing that the CyP40 action occurred at the activation step where AhR and Arnt dimerized. All lanes contained baculovirus expressed AhR and Arnt, and 1 μg of poly (dI-dC). Lane 1, Sf9 soluble extract (positive control); lane 2, no other protein added; lane 3, 5 μg of BSA; lanes 4 and 5, 5 μg of TALON purified 6His-CyP40 added before the activation step ± βNF; lane 6, 5 μg of TALON purified 6His-CyP40 added after the activation step + βNF. (B) Lanes 1 to 6 are co-immunoprecipitation data showing Arnt-dependent co-immunoprecipitation of ^{32}P-CΔ553 plus Sf9 soluble extract, TALON purified 6His-CyP40 or BSA. 5 % input (lane 7) refers to 5 % of ^{32}P-CΔ553 added to each sample. (C) Gel shift assay was performed by transferring 1 μl of co-immunoprecipitated sample mix in (B) before Arnt polyclonal addition (except that unlabelled CΔ553 was used) to a new tube with 10 μl of HEDG, 115 mM KCl and 2 μg of poly(dI-dC). The arrow indicates the CΔ553/Arnt/DRE complex.