Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital

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Abstract The nuclear receptor CAR, a phenobarbital (PB)-responsive transcription factor, translocates into the nucleus of hepatocytes after PB induction. In non-induced mice, CAR forms a physical complex with heat shock protein 90 (HSP90) in the cytoplasm. In response to PB induction, protein phosphatase 2A is recruited to the CAR:HSP90 complex. This recruitment may lead CAR to translocate into the nucleus, consistent with the inhibitions of nuclear CAR accumulation in PB-induced mouse primary hepatocytes by okadaic acid as well as by geldanamycin.

Key words: Nuclear receptor CAR; Phenobarbital; Nuclear translocation; Heat shock protein; Protein phosphatase

1. Introduction

The nuclear receptor CAR is a coordinate transcription factor that regulates the inducible expression of hepatic genes in response to xenochemical exposures [1–4]. CAR, retained in the cytoplasm of non-induced mouse livers or mouse primary hepatocytes, translocates to the nucleus in response to phenobarbital (PB) and PB-like inducers [5,6]. Upon translocation, CAR forms a heterodimer with the retinoid X receptor and binds to nuclear receptor sites within the PB-responsive enhancer module or PBREM that is conserved in various PB-inducible genes from mouse to human [7,8]. Thus, nuclear translocation is an initial response of CAR to PB and other PB-type inducers in liver in vivo. However, the regulatory mechanism of the nuclear translocation is poorly understood at the present time.

The roles of signal transduction pathways in the PB induction mechanism have become increasingly recognized [5,9–13]. Treatment with protein phosphatase inhibitor okadaic acid (OA) inhibited induction of the *CYP2B* genes by PB in rat as well as mouse primary hepatocytes [9,12]. Moreover, OA repressed the PB-elicited nuclear translocation of CAR in mouse primary hepatocytes [5]. It is well known that certain nuclear steroid hormone receptors such as the glucocorticoid receptor (GR) undergo agonist-dependent nuclear translocation and OA inhibits this GR nuclear translocation [14,15]. GR forms a large heteromeric complex in the cytoplasm with heat shock protein 90 (HSP90), 23 kDa protein (p23), and immunophilin-like protein [16,17]. In addition to these proteins, protein phosphatase 5 (PP5) is a component of the GR:HSP90 complex [18]. Whether CAR forms a complex somewhat similar to the GR remains an important question. Characterization of a cytoplasmic CAR complex may reveal the mechanism that regulates the nuclear translocation of CAR and any protein phosphatase that may be involved in the regulation [5]. Column chromatography, immunoprecipitation, and chemical cross-linking were employed to characterize a CAR complex in the cytosol of mouse livers. Here we present experimental evidence indicating that CAR exists as a complex with HSP90 in the cytosolic fractions from non-induced mouse livers and that protein phosphatase 2A (PP2A) is recruited to the CAR:HSP90 complex in PB-induced mice. The regulated interaction of CAR with PP2A provides a new insight into understanding the molecular mechanisms by which the receptor senses xenochemicals such as PB, and mediates the transcriptional activation of hepatic genes.

2. Materials and methods

2.1. Materials

Butyl Sepharose, DEAE Sepharose and Superose 6 HR 10/30 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA); OA and geldanamycin (GA) from Calbiochem (San Diego, CA, USA); 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) and ImmunoPure Immobilized Protein A from Pierce (Rockford, IL, USA); anti-GR, anti-HSP90 and anti-PP2A Aα subunit antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-HSP90, anti-PP1, anti-PP5 and anti-PP2A C subunit antibodies from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-hCAR antibodies were prepared in our laboratory [19]. A peptide antibody (anti-mCAR-N20) was produced in rabbits with the N-terminal MTAMLTLETMASEEEYGPRNC conjugated with keyhole limpet hemocyanin, and was purified using the peptide and SulfoLink kit (Pierce).

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Abbreviations: PB, phenobarbital; OA, okadaic acid; GR, glucocorticoid receptor; HSP90, heat shock protein 90; PP5, protein phosphatase 5; PP2A, protein phosphatase 2A; GA, geldanamycin; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; RT-PCR, reverse transcription-polymerase chain reaction; 2-ME, 2-mercaptoethanol

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2.2. Animals and primary hepatocytes

Cr1:CD-1(ICR)BR mice (5–7 weeks) were obtained from Charles River Laboratories (Raleigh, NC, USA). PB (Sigma, St. Louis, MO, USA) in saline or the saline vehicle was injected intraperitoneally at a dose of 100 mg/kg of body weight. One hour after injection, the livers were removed to prepare cytosolic fractions for immunoprecipitation. To prepare cytosol, mouse livers were homogenized in three volumes of TEGDM buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 15% glycerol, 1 mM dithiothreitol and 20 mM Na_2MoO_4) supplemented with 2 μg/ml of leupeptin, 2 μg/ml pepstatin A, and 200 μM phenylmethylsulfonylfluoride. The homogenate was centrifuged at 100 000×*g* for 60 min to obtain cytosolic fractions. Mouse hepatocytes were prepared and cultured as previously described [5]. Hepatocytes were pre-treated with 20 μM of GA, 10 nM of OA or dimethylsulfoxide for 1 h, washed with culture medium, then treated with 250 nM of TCPOBOP or dimethylsulfoxide for 8 h. Total RNAs and nuclear extracts were prepared from primary hepatocytes as described earlier [5].

2.3. Column chromatography

Cytosolic fractions prepared from 200 mouse livers were precipitated with 30%-saturated ammonium sulfate and the resulting supernatant was precipitated with 55%-saturated ammonium sulfate. The 55% precipitate was dissolved in TEGDM buffer and applied on a butyl Sepharose column (150 ml bed volume) in the presence of 0.5 M of ammonium sulfate. Proteins were eluted by a linear gradient of ammonium sulfate from 0.5 to 0.0 M. Using Western blot for selection criterion, CAR-containing fractions were pooled, concentrated with YM-100 membrane (Millipore), and dialyzed against TEGDM buffer at 4°C for 16 h. The resulting concentrate was loaded onto a DEAE Sepharose column (15 ml bed volume) and was eluted by a linear gradient of NaCl from 0.0 to 0.5 M. After DEAE chromatography, CAR-containing fractions were pooled, concentrated, and dialyzed against HEGMS buffer (20 mM HEPES-KOH (pH 7.6) buffer containing 1 mM EDTA, 15% glycerol, 100 mM NaCl, and 20 mM Na_2MoO_4). For gel filtration, Superose 6 HR 10/30 column was equilibrated with 20 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 150 mM NaCl, and 20 mM Na_2MoO_4.

2.4. Analytical methods

Proteins (in HEGMS buffer) were cross-linked in the presence of 5 mM DTSSP on ice for 2 h. The reaction was stopped by adding Tris–HCl buffer (pH 7.5) to a final concentration of 50 mM on ice. The resulting reaction mixture was briefly centrifuged and was subjected to further analysis. For immunoprecipitation, protein A resin was incubated with a specific antibody (anti-CAR or anti-GR) for 1 h at room temperature, washed with TEGDM buffer twice, and incubated with a given protein solution at 4°C for 12–24 h. Subsequently, the resin was incubated in SDS–PAGE sample buffer at 100°C for 10 min to extract proteins. For Western blot analysis, proteins were separated on a 4–12% gradient polyacrylamide gel (NuPAGE 4–12% Bis-Tris Gel, Invitrogen, Carlsbad, CA, USA), transferred to Immobilon-P membrane (Millipore), and visualized using primary antibodies, horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and an enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech). CYP2B10 mRNA was measured by quantitative real-time PCR using an ABI Prism 7700 (PE Applied Biosystems) and specific primers as previously described [5].

3. Results and discussion

GA, a benzoquinone ansamycin, binds to an ATP/ADP binding site of HSP90 interrupting its chaperoning function [20,21]. GA is known to inhibit the agonist-dependent nuclear translocation of the GR and the progesterone receptor [22–25]. To determine whether GA inhibited PB induction of *Cyp2b10* gene, mouse primary hepatocytes were treated with GA for 1 h prior to induction by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). To measure the levels of CYP2B10 mRNA in the hepatocytes, total hepatic RNA was subjected to real-time reverse transcription-polymerase chain reaction (RT-PCR). Pre-treatment with GA resulted in the profound reduction of the TCPOBOP-induced mRNA, as observed with OA-pre-treated hepatocytes (Fig. 1A). Thus, both GA and OA effectively inhibited the induction of CYP2B10 mRNA by TCPOBOP in the hepatocytes. To investigate whether GA inhibited the nuclear translocation of CAR, we performed Western blot analysis on nuclear extracts that were prepared from TCPOBOP-induced mouse primary hepatocytes with or without pre-treatment (Fig. 1B). The nuclear accumulation of CAR was dramatically reduced by GA pre-treatment, as also observed with the OA-pre-treated nuclear extracts. These results indicate that GA, similar to OA, inhibits the nuclear translocation of CAR, thus repressing

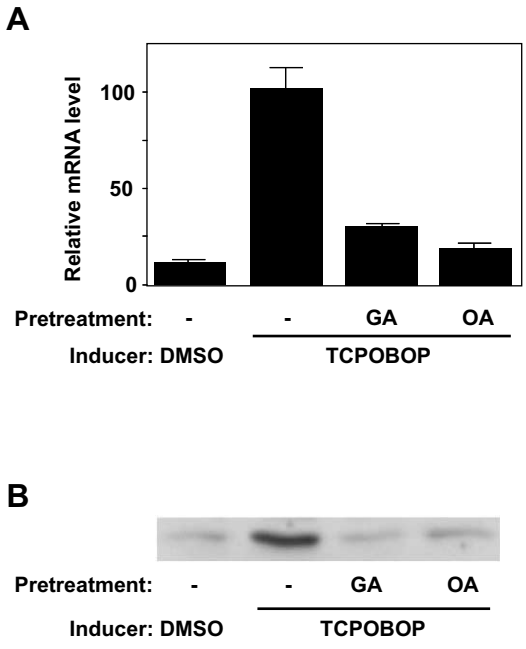

Fig. 1. Inhibition by GA and OA of PB induction. RNA and nuclear extract were prepared from the mouse primary hepatocytes treated with various chemicals as described in Section 2. The RNA samples (1 μg each) were used for RT-PCR for quantitative measurement of CYP2B10 mRNA, while the nuclear extracts (20 μg protein each) were subjected to Western blot analysis to determine the content of CAR protein. DMSO, dimethylsulfoxide.

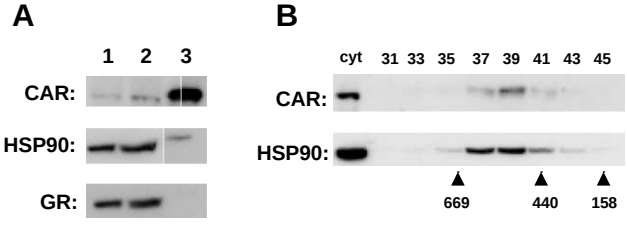

Fig. 2. A: Removal of GR from the CAR-enriched fraction. 80 μg of cytosolic fraction prepared from mouse livers (lane 1) and of 55% ammonium sulfate precipitate (lane 2) and 8 μg of CAR-enriched fraction after butyl Sepharose (lane 3) were subjected to Western blot analysis. B: Elution profiles of CAR and HSP90. A partially purified fraction after DEAE Sepharose column was separated through a Superose 6 column as described in Section 2. 20 μl of each 300 μl fraction and 80 μg of cytosol prepared from livers (cyt) was subjected to Western blot analysis. Molecular size standards used were thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa).

TCPOBOP-induced transcription of the *Cyp2b10* gene in hepatocytes.

Given the indication of HSP90 involvement in the nuclear translocation of CAR, column chromatography, chemical cross-linking, and immunoprecipitation were employed to investigate CAR in the cytosol from non-induced mouse livers. Ammonium sulfate fractionation concentrated CAR from the cytosol into the 30–55% precipitates. The precipitate, re-dissolved in TEGDM buffer, was subjected to sequential butyl Sepharose chromatography. CAR was highly concentrated in the fraction from the butyl Sepharose column, while only a small portion of HSP90 was retained and GR was undetectable (Fig. 2A). Subsequently, this fraction was applied on DEAE Sepharose for further purification of CAR. Elutes from the DEAE Sepharose column were pooled for later experiments, designated CAR-enriched solution. First of all, using the pooled elutes, gel filtration chromatography was performed to measure the molecular size of CAR (Fig. 2B). The size appeared to be approximately 500 kDa. Moreover, the co-elution of CAR with HSP90 suggested that they might be present as a complex.

The CAR-enriched solution was treated with the chemical cross-linker DTSSP to examine the presence of a CAR:HSP90 complex. 2-Mercaptoethanol (2-ME) can dissociate proteins that are cross-linked with DTSSP because the disulfide bond is sensitive under reducing conditions. Thus, proteins of the DTSSP-treated solution were electrophoresed on a SDS–polyacrylamide gel with or without 2-ME for subsequent Western blot analysis (Fig. 3A). CAR appeared as a high molecular weight band (>200 kDa) under the non-reducing condition (without 2-ME), while it migrated as a monomer under the reducing condition (with 2-ME). HSP90 was visualized on the same Western blot membrane using an anti-HSP90 antibody. DTSSP cross-linked HSP90 proteins of two different sizes: >200 kDa and 180 kDa. The >200 kDa bands overlapped, indicating that they represented a cross-linked complex containing CAR and HSP90. Based on its size, the 180 kDa band could be the HSP90 homodimer. To obtain further evidence for the presence of a CAR:HSP90 complex, the CAR-enriched solution was incubated with anti-CAR antibody and the resulting precipitates were subjected to Western blot. HSP90 was co-precipitated with CAR (Fig. 3B). These results clearly indicate that CAR forms a complex with HSP90 in the cytoplasm of non-induced mouse livers.

OA, at a low nM concentration, inhibits preferentially PP2A, although it may also inhibit the other phosphatases such as PP1 and PP5. Given the evidence that OA represses the nuclear translocation of CAR, we examined whether the CAR:HSP90 complex also contained PP2A by directly precipitating CAR with anti-mCAR-N20 antibody from the cytosolic fractions extracted from mouse livers and analyzing the resulting precipitates by Western blots (Fig. 4). The N20 antibody co-precipitated HSP90 from both the non-induced and PB-induced liver cytosolic fractions. The PP2A C (catalytic) and Aα subunits were co-precipitated from only the cytosols prepared from PB-induced livers, while PP5 was not detected in the precipitates prepared from either cytosols. Thus, these results suggest that PP2A, but not PP5, is recruited to the CAR:HSP90 complex in response to PB induction. Due to experimental limitations, however, the molar ratio of PP2A to CAR could not be determined. The possibilities that PP2A dissociated during immunoprecipitation

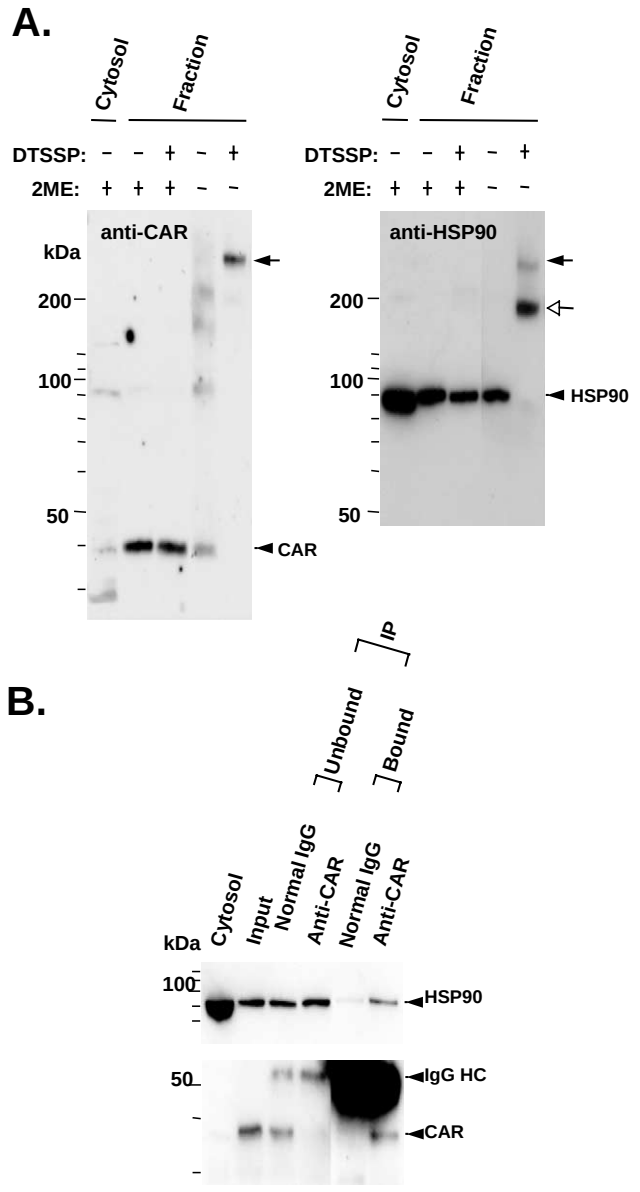

Fig. 3. A: Cross-linking with DTSSP: pooled fractions (0.4 μg protein) after DEAE Sepharose were incubated with or without DTSSP as described in Section 2. The DTSSP-treated and -untreated fractions were incubated at 100°C for 10 min in the presence or absence of 2-ME and were subjected to Western blot analysis. Cytosol prepared from livers (80 μg) was also run for analysis. Molecular weight (BenchMark™ Protein Ladder, Invitrogen) is shown on the left. The closed arrows indicate CAR:HSP90 cross-link, whereas the open arrow shows HSP90 homodimer. B: Immunoprecipitation: pooled fraction (0.4 μg protein, Input) after DEAE Sepharose was precipitated with anti-mCAR-N20 antibody or normal rabbit IgG. Equal volumes of the bound and of the unbound fraction (3 and 10 μl, respectively) were subjected to Western blot analysis. Cytosol prepared from livers (80 μg) was also run for analysis. The symbols IP and HC denote immunoprecipitation and heavy chain, respectively.

and/or that only a fraction of CAR:HSP90 complexes formed in PB-treated cytosol remain an important question in further investigations. The anti-GR antibody co-precipitated HSP90, PP5, and PP2A from the cytosols prepared from both non-induced and PB-induced mouse livers (Fig. 4), which agreed with the presumption that a pool of the GR:HSP90 com-

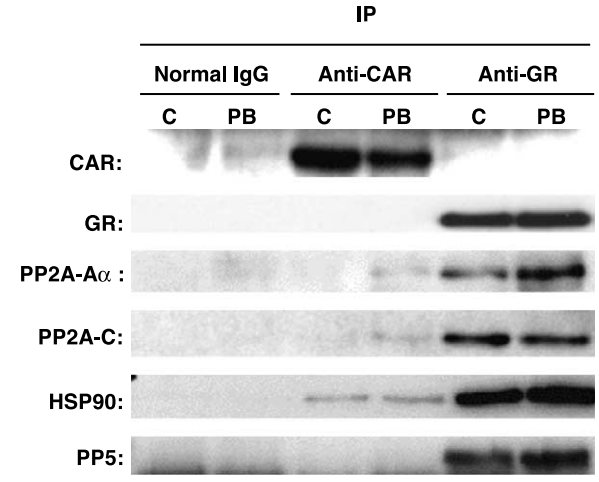

Fig. 4. Co-precipitation of PP2A and Hsp90 from the liver cytosols with CAR antibody. Cytosols prepared from livers (20 mg each) of the non-induced (C) and of the PB-treated (PB) mice were precipitated with anti-mCAR-N20, anti-GR or normal rabbit IgG. Resulting bound proteins were subjected to Western blot analysis.

plexes are always activated due to constant hormonal exposure and therefore are in dynamic nucleocytoplasmic shuttling in liver. The co-precipitation of HSP90 and PP5 was consistent with the previous findings in cytoplasm of rat liver [18]. Our present study was the first to show that the GR:HSP90 complex in cytoplasm of mouse hepatocytes may also contain PP2A. These results obtained with the GR antibody, in addition to the lack of precipitating of CAR, HSP90, or the PPs by normal IgG, provided us with the excellent controls to substantiate the specific interaction of PP2A with the CAR:HSP90 complex.

CAR undergoes OA-sensitive nuclear translocation in mouse primary hepatocytes treated with PB-type inducers [5]. Our present study reveals that CAR exists as a complex with HSP90 in the cytoplasm of non-induced mouse livers and that the CAR:HSP90 complex recruits PP2A in response to PB induction. Indicatively, PP2A may be required for the nuclear translocation of CAR and OA might inhibit PP2A activity to repress this translocation. Whether PP2A recruitment is a prerequisite in CAR nuclear translocation elicited by other PB-type inducers such as TCPOBOP remains confirmed in future investigations. When it was expressed in mouse liver, human CAR translocates into the liver nucleus of mice in response to TCPOBOP [6]. This translocation was also observed in CAR-null mice (unpublished data). Given the fact that TCPOBOP translocates human CAR but does not bind to human CAR, CAR nuclear translocation is not initiated by binding of inducers. Therefore, it is reasonable to speculate that, acting as a common regulatory step during nuclear translocation, recruitment of PP2A may occur in response to various PB-type inducers. However, the molecular mechanism of how the phosphatase regulates nuclear translocation remains unclear. CAR does not require its ligand-dependent activation function AF2 domain for PB-elicited nuclear translocation in the mouse liver in vivo and, instead, the translocation is dictated by the LXXLXXL motif near the C-terminus of the receptor molecule [6]. Whether CAR is the target for dephosphorylation is not known at the present time. Moreover, whether PP2A regulates the activity of the LXXLXXL motif requires investigation in the future. The possibility that the CAR:HSP90 complex may contain a yet unknown component that can be dephosphorylated by PP2A remains to be explored. Unlike steroid hormones that interact directly with the receptors to activate them, PB does not bind to CAR, making the activation mechanism very complex. In addition, the extremely low expression of CAR in the liver is problematic when executing experiments. Nevertheless, our present evidence of a CAR:HSP90 complex and of PP2A recruitment in response to PB provides the insights into understanding the PB-elicited nuclear translocation of CAR. Continuing these investigations may lead us to elucidate a novel mechanism of drug action and interaction with the genome.

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