Molecular Pharmacology Fast Forward. Published on December 23, 2005 as doi: 10.1124/mol.105.019505
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MOL 19505

Serine-202 regulates the nuclear translocation of constitutive active/androstane receptor CAR

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Molecular Pharmacology Fast Forward. Published on December 23, 2005 as DOI: 10.1124/mol.105.019505
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Running title: Serine-202 regulates the CAR nuclear translocation

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Number of Text Pages: 29

Number of Tables: 0

Number of Figures: 8

Number of References: 25

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Abbreviations: DBD, DNA binding domain; LBD, ligand binding domain; hPP2Ac, human protein phosphatase 2A catalytic subunit; CCRP, cytoplasmic CAR retention protein; hGRIP1, human glucocorticoid receptor interacting protein-1; CAR, constitutive active of androstane receptor

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Abstract

The constitutive active receptor CAR in mouse primary hepatocytes undergoes okadaic acid (OA)-sensitive nuclear translocation following activation by xenobiotics such as phenobarbital (PB) and 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). We have now mimicked this TCPOBOP-dependent and OA-sensitive translocation of mouse CAR (mCAR) in HepG2 cells and have demonstrated that protein phosphatase 2A regulates this nuclear translocation. Site-directed mutagenesis analysis of various Ser and Thr residues delineated the translocation activity to Ser202. Mutation of Ser202 to Asp (S202D) prevented mCAR translocation into the nucleus of TCPOBOP-treated HepG2 cells. Also, in the livers of *Car*$^{-/-}$ mice, the YFP-tagged S202D mutant did not translocate into the nucleus after PB treatment. To examine whether Ser-202 can be phosphorylated, flag-tagged wild-type mCAR or flag-tagged S202A mutant was expressed in HepG2 cells and subjected to Western blot analysis using an antibody specific to a peptide containing phospho-Ser-202. A high molecular weight phosphorylated form of CAR was detected only with the wild-type mCAR. These results are consistent with the conclusion that the dephosphorylation of Ser-202 is a required step that regulates the xenobiotic-dependent nuclear translocation of mCAR.

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Introduction

Upon activation by PB and various PB-type inducers, CAR up-regulates the expression of a large set of hepatic genes that encode drug metabolizing enzymes and transporters such as cytochrome P450 and multidrug-resistant protein (Sueyoshi and Negishi, 2001; Ueda *et al., 2002*; Kast *et al*., 2002). Thus, CAR coordinates a cellular defense mechanism elicited against xenobiotic insults by increasing hepatic capability to metabolize and excrete them. In addition to its role in drug metabolism, CAR is now implicated as a coordinating factor in regulating various types of hepatic functions, such as gluconeogenesis, fatty acid oxidation and the metabolism of steroid hormones and bilirubin (Ueda *et al.*,2002; Wei *et al*., 2000; Sugatani *et al.*,2001; Huang *et al.*,2003). For example, CAR cross-talks with the insulin response transcription factor FOXO1 to repress the genes that encode gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1 (Kodama *et al.,* 2004). CAR can also be an adverse factor, as it was recently shown that CAR promotes the development of hepatocellular carcinoma in PB-treated mice (Yamamoto *et al.,* 2004). While the diverse role of CAR in regulating various liver functions is becoming evident, the molecular mechanism of CAR activation still remains elusive. CAR is unique in which it can be activated by many drugs such as PB without binding directly to them. The presence of a signal transduction pathway has been suggested to regulate this so-called “ligand-independent” or “indirect” activation. However, phosphorylated CAR as a direct target of the signal pathway has not yet been demonstrated.

CAR is retained in the cytoplasm of non-induced mouse hepatocytes and translocates into the nucleus following activation by drugs such as PB and TCPOBOP (Kawamoto *et al.,* 1999). This nuclear translocation is the initial step that occurs during the process of CAR activation,

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providing an excellent target for investigations into whether a signaling pathway is involved in this activation. Our previous studies showed that the xenobiotic response signal (XRS), a peptide near the C-terminus of the LBD regulates PB-induced nuclear translocation of mCAR in mouse hepatocytes (Zelko *et al.*, 2001). We have shown that a protein phosphatase inhibitor okadaic acid (OA) represses the nuclear translocation of mCAR (Kawamoto *et al.*, 1999). Since CAR forms a complex with Hsp90 in the cytoplasm and recruits protein phosphatase 2A (PP2A) in response to PB (Yoshinari *et al.*, 2003), it has been speculated that PB treatment may dephosphorylate the LDB to translocate CAR into the nucleus.

Unlike in mouse hepatocytes (Kawamoto *et al.*, 1999), CAR diffuses passively into the nucleus when it is expressed in transformed cells such as HepG2. In particular, stable HepG2 cell lines such as Ym17 express CAR predominantly in the nucleus (Swales *et al.*, 2005). Thus, when CAR is co-transfected with a reporter gene in HepG2 cells, it activates fully the transcription of reporter gene in the absence of CAR activator such as TCPOBOP. This has been discouraging us to investigate CAR nuclear translocation at the molecular level using HepG2 cells. However, we have been observing that CAR first appears in the cytosol of transiently trasfected HepG2 cells prior to the receptor spontaneously accumulating in the nucleus. We have now investigated the mechanism of CAR nuclear translocation using transient transfection conditions under which HepG2 cells mimic the TCPOBOP-dependent and OA-sensitive CAR nuclear accumulation. With this HepG2 system, PP2A regulates the nuclear translocation of CAR. We then focused on serine and threonine residues of the LBD to identify the phosphorylation site that might regulate the nuclear translocation of CAR. To this end, we took advantage of our recent discoveries that the mouse pregnane X receptor (mPXR) translocates into the nucleus of mouse hepatocytes in response to its specific activator 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) but not to PB, the CAR activator (Squires *et al.*, 2004). However, when the LBD of mPXR is placed after the

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DBD of mCAR, the chimeric receptor is found to respond to PB, translocating into the nucleus of mouse livers. If the PB-induced signal and PP2A target a residue of mCAR, that residue may be a conserved serine or threonine in the LBD of both mCAR and mPXR. Taking these findings into consideration, we employed site-directed mutagenesis to delineate the residue that regulates the nuclear translocation of mCAR in HepG2 cells as well as mouse hepatocytes. Here we present our experimental observations that are consistent with the conclusion that the dephosphorylation of Ser-202 is a required step in the nuclear translocation of mCAR.

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Materials and Methods

Materials. 1, 4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was obtained from Sigma, okadaic acid from Calbiochem and Complete mini (Protease Inhibitor Cocktail Tablets) from Roche Diagnostics GmbH. Anti-V5 and Anti-V5 HRP conjugate antibodies were purchased from Invitrogen (Carlsbad, CA), anti-Flag HRP and anti-Flag M2-agarose from Sigma (St.Louis, MO) and anti-hsp90 monoclonal antibody from BD Transduction Laboratories (San Diego, CA). Antibody against the peptide MEDQIpSLLKGC (corresponding to residues 197-206 of mCAR with Ser-202 phosphorylated) was produced at Affinity Bio Reagent (Golden, Co). The specificity of this antibody (named pS202) to phosphorylated Ser-202 was confirmed by ELISA using phosphorylated and non-phosphorylated peptides, specifically binding to the phosphorylated peptide even at the concentration 20 μg/ml. In addition, Western blot analysis was performed and showed that pS202 antibody does not bind bacterially expressed GST-mCAR.

Plasmids. mCAR in pcDNA3.1/V5-His, pEYFP-c1 or pGEX4T-3, hCAR in pEYFP-c1, and $(NR1)_5$-tk-luciferase reporter were previously constructed (Zelko *et al.,* 2001; Kobayashi *et al.,* 2003; Sueyoshi *et al.,*1999). The full coding sequence of hCAR or hGRIP1 was amplified using appropriately primers and cloned into pcDNA3.1/V5-His-TOPO (Invitrogen). Catalytic subunit of human PP2A (hPP2Ac) was cloned into pcDNA3.1/V5-His-TOPO (Invitrogen). Flag-tag was added to the 3'-end of mCAR cDNA and cloned into pCR3 (Invitrogen). Using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA) and appropriately mutated primers, mutations were introduced and verified by nucleotide sequencing. A chimera, $mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$, was constructed bearing the mCAR DNA binding domain (1-116 of mCAR) and mPXR ligand binding domain (141-431 of mPXR) in pEYFP-c1.

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Cell culture and fractionation. HepG2 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics (100 unit/ml of penicillin and 100 µg/ml of streptomycin) on plastic dishes at 37 °C for 24h prior to transfection. At approximately 75% confluence, the cells were transfected with plasmid using LipofectAMINE 2000 (Invitrogen) according to a manufacturer's instruction. Cells were scraped from the plates, washed twice with phosphate-buffered saline, collected by centrifugation and homogenized in buffer A (10 mM HEPES buffer, pH 7.6, containing 10 mM KCl, 1.5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.3% nonidet P-40 and 1mM dithiothreitol) including Complete Protease Inhibitor Cocktail Tablets (Roche). The homogenate was centrifuged at 4,000 xg for 10 min and the resulting supernatant was centrifuged at 17,800 xg for 10 min to obtain the cytosolic fraction. The 4,000 xg pellet was washed three times with buffer A and suspended in lysis buffer; 10 mM HEPES buffer, pH 7.6, containing 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM Na_3VO_4, 10% glycerol, Complete Protease Inhibitor Cocktail. NaCl was added to a final concentration of 0.4 M and suspension was incubated with constant rotation at 4 °C for 1 hr, and centrifuged at 100,000 xg for 30 min to obtain a nuclear extract.

Immunoprecipitation. Anti-Flag M2-agarose (40 µl) was added to the cytosol (1 to 2 mg protein) isolated in 10mM sodium phosphate, pH7.4, containing 0.14 M NaCl, 10 mM KCl, 1,5 mM $MgCl_2$, 0.1mM NaF 20 mM Na_2MoO_4, 0.3% nonidet P-40, 1mM dithiotheritol and incubated at 4 °C for 16 hr. After centrifugation (800 xg for 2 min), the resulting pellet was washed five times with 1 ml of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 20 mM

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Na_2MoO_4 and 0.2% of Nonidet P-40. The remaining agarose was boiled for 10 min in NuPAGE LDS sample buffer (Invitrogen) to extract the proteins.

Expression of YFP-Tagged CAR in mouse liver. Plasmid was injected via the tail vein of CD-1 or CAR-null mice using the TransIT *in vivo* gene delivery system (Mirus, Maddison, WI) according to the manufacturer's instructions. By this method, generally around 10% of hepatocytes of the liver showed expression of YFP-CAR. The mice were twice injected with PB (100 mg/kg body weight) or DMSO at 3 and 6 hr after the injection of plasmid and sacrificed at 2 hr after the second drug administration. Liver sections 30-μm thickness were prepared and analyzed by confocal laser scanning microscopy using a Zeiss LSM510 system. A 63X oil immersion objective (1.4 numeric aperture) was used for scanning with a pixel size of 0.06 μm. YFP was excited at 514 nm and the emission was detected using a 530-nm band pass filter. Pinhole size was set for a 1.0 μm confocal slice. At least 100 cells from four different sections were examined for each treatment to determine the pattern of intracellular localization of the receptor either predominantly in the cytoplasm, present equal in the nuclear and cytoplasmic compartments or primarily nuclear localization.

Western Blot. Proteins were separated on a 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) or a 10% SDS-polyacrylamide gel and were transferred to Immobilon-P membrane. The membrane was blocked in 5% nonfat milk powder in 25 mM Tris-HCl, pH7.5, containing 137 mM NaCl and 0.1 % Tween 20 (TBS-Tween). When anti-pS202 was used as primary antibody, 5% BSA in TBS-Tween was used to block membrane. The blocked membranes were incubated for 1 h at 25 °C with primary antibodies; either anti-V5 (1:5000), anti-V5 HRP (1:5000), anti-Flag HRP

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(1:5000) or anti-pS202 antibody (0.5µg/ml in TBS-Tween). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5000; Santa Cruze Biotechnology, Santa Cruz, CA) was used as the secondary antibody. Finally, protein bands were visualized using ECL Plus Western blotting detection reagent (Amersham Bioscience). Blots were subsequently stripped and restained for Lamin β and Hsp90 as a loading control.

Luciferase reporter assay. Luciferase activity was measured in cell lysates using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as previously described (Sueyoshi *et al.*, 1999), promoter activity was determined in HepG2 cells from three independent transfections and calculated from firefly luciferase activity normalised against Renilla luciferase activity of an internal control pRL-SV40 plasmid.

GST Pull Down Assay. Glutation-S-transferase (GST)-mCAR, $mCAR_{S202A}$, $mCAR_{S202D}$ fusion proteins were expressed in *E.coli* BL21 cells and purified using glutathione-Sepharose (Amersham Biosciences). [^{35}S]-methionine labeled hGRIP1 was expressed using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) using pcDNA3.1/hGRIP1-V5-His. *In vitro* translated [^{35}S]-labeled hGRIP was mixed with GST-mCAR or the mCAR mutants in the presence of glutathione Sepharose in 50 mM HEPES buffer, pH 7.6, containing 0.1 M NaCl and 0.1% Triton X (HBST buffer). The mixture was incubated for 20 min at room temperature under gentle mixing; the resin was recovered by centrifugation and washed four times with HBST buffer. Subsequently, proteins were extracted in SDS-PAGE sample buffer, electrophoresed on a 4-12% gradient acrylamide Bis-Tris gel (Invitrogen). The gel was dried and subjected to autoradiography.

Gel shift assay. Proteins were produced using the *in vitro* transcription/translation system (TNT T7 quick-coupled system, Promega) and incubated with ^{32}P-labeled NR1 double strand DNA (40,000 cpm) probe in 10 µl of binding buffer containing 10mM HEPES, pH 7.6, containing 0.5 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 50 mM NaCl and 1.5 µg of poly(dI-dC). The proteins were separated on a 5% acrylamide gel in 7mM Tris-acetate buffer pH7.5, containing 1mM EDTA at 180 V for 1.5 hr and the gel was dried under vacuum and subjected to autoradigraphy at -70 °C.

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Results

Delineating translocation activity of mCAR to Ser-202. It is known that mPXR translocates into the nucleus after treatment with its activator PCN but not with PB the mCAR activator in mouse livers (Squires *et al.,* 2004). A strong nuclear translocation signal NLS within the DBD dictates this nuclear translocation of mPXR. Although a peptide sequence similar to NLS is present in the DBD of mCAR, this sequence does not regulate PB-induced nuclear translocation of mCAR (Zelko *et al*., 2001; Squires *et al.,* 2004). Given these facts, we hypothesized that the LBD of mPXR might respond to PB triggering nuclear translocation within the context of mCAR. To this end, the mPXR LBD was placed at after the DBD-hinge region of mCAR to construct the expression plasmid of YFP-tagged chimera $mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$. This chimera was directly expressed in the livers of CD-1 mice by injecting the plasmid via tail vein injection. Then its intracellular localization was determined and compared with those of mCAR and mPXR. Fluorescent images of liver cells that signify the expression of a given receptor are shown in Fig.1A. Quantitative analysis of the intracellular localization for a given receptor from at least 100 cells is summarized in Fig.1B. As expected, all three receptors were predominantly expressed in the cytoplasm of non-induced hepatocytes. Similar to mCAR, the chimera was expressed in the cytoplasm of over 80% of the cells analyzed, while over 60% of the cells that expressed mPXR showed the specific expression in the cytoplasm. In the PB-treated hepatocytes, chimera was expressed in the nucleus of nearly 90% of cells, which was similar to that of mCAR. On the other hand, mPXR remained expressed predominantly in the cytoplasm

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even after PB treatment. Thus, the LBD of mPXR acquires the activity of PB-induced nuclear translocation when it is placed in the context of the mCAR DBD.

If an OA-sensitive phosphatase dephosphorylates serine and/or threonine of mCAR to regulate the PB-induced nuclear translocation, this residue may be conserved in the LBD of mPXR. Amino acid sequence alignments of mCAR, mPXR and human CAR identified four serine and three threonine residues that are found in all three LBDs; Thr-176, Ser-202, Thr-218, Thr-224, Ser-279, Ser-331 and Ser-358 in mCAR (Fig. 2A). Thr-176 was previously identified as one of the conserved residue in these receptors (Ueda *et al*., 2005). The first six residues were mutated to Asp to mimic phosphorylation and examine whether the mutation altered the intracellular localization in mouse liver. The last residue Ser-358, which resides within the AF2 domain of mCAR, was excluded from investigations, since it is known that the AF2 is not involved in the PB-induced nuclear translocation (Zelko *et al*., 2001). Each of YFP-tagged mCAR mutants was expressed in the livers of *Car*$^{-/-}$ mice and its intracellular localization was evaluated by analyzing at least 100 hepatocytes (Fig.2B). All mutants, except S202D, translocated into the nucleus after PB treatment, as indicated by the fact that over 80% cytoplamic localization was reduced to 30%, while the nuclear localization was up from less than 10% to over 50% after PB treatment. In contrast, the S202D mutant expressed in the cytoplasm of nearly 100% hepatocytes analyzed before PB treatment and remained unchanged even after PB treatment.

To provide further evidence that the phosphorylation of Ser-202 may be involved in regulating the nuclear translocation of the mCAR, the expression of the S202R and

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S202A mutants was compared with that of S202D mutant in the mouse liver (Fig. 3). Similar to the case of wild-type mCAR, the expression of S202R and S202A in the nucleus increased from 10% to 60% of cells after PB treatment, with the number of the cells showing cytoplasmic expression decreasing from approximately 70% to less than 20%. Thus, placing hydrophobic or positively-charged side chain at position 202 did not change the ability of mCAR to translocate into the nucleus following PB treatment. As observed in Fig. 2, the S202D mutant was retained in the cytoplasm in 80% of the cells examined, even after PB treatment. When Asp with a negatively-charged side chain substituted Ser-202, did mCAR become unable to translocate into the nucleus. These results suggested that Ser-202 regulates the PB-induced nuclear translocation in the mouse livers, probably via its dephosphorylation.

Functional conservation of Ser-202. Ser-202 of mCAR is also conserved in hCAR and mPXR (Fig. 2A). Given the fact that Ser-202 regulated the PB-triggering nuclear translocation of mCAR, the corresponding Ser residues of hCAR and mPXR were examined as to whether these could also regulate their nuclear translocation. Ser-192 of YFP-tagged hCAR was mutated to Asp and Ala and the mutants were directly expressed in mouse livers. Similar to wild-type hCAR, the S192A mutant translocated into the nucleus after PB treatment. On the other hand, the S192D mutant remained in the cytoplasm even after PB treatment (Fig. 4A). Ser-271 of mPXR was also mutated to Asp and Ala in the $mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$ chimera and expressed in mouse livers. Similar to the hCAR mutants, S271D did not translocate into the nucleus in the PB-treated mouse livers (Fig. 4B). Thus, these results suggest that the Ser residues of mCAR, hCAR and mPXR (in the $mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$ chimera) are functionally

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conserved to regulate the nuclear translocation of the receptor. While mPXR is known to translocate into the nucleus of PCN-treated mouse liver (Squires *et al.*, 2004), it remains an interesting question whether Ser-271 regulates the PCN triggering nuclear translocation of mPXR.

Exogenously expressed PP2A-regulated nuclear accumulation of mCAR in HepG2 cells. V5-tagged mCAR was transiently expressed in HepG2 cells from which cytosolic and nuclear fractions were prepared. Subsequently, Western blotting analysis was performed to examine amount of mCAR in those fractions. Since PB does not bind directly to CAR and exhibited no effect on the intracellular localization of CAR in HepG2 cells, hereafter TCPOBOP was used to activate the receptor. TCPOBOP treatment decreased the amount of mCAR in the cytosolic faction and increased it in the nuclear faction at 24 or 45 hr after treatment (Fig. 5A). The amount of mCAR was higher in the cytosolic faction than the nuclear fraction of DMSO-treated cells, whereas TCPOBOP treatment reversed this distribution. Thus, mCAR undergoes TCPOBOP-dependent nuclear translocation in the transfected HepG2 cells. Co-treatment with the active inhibitor OA, but not with the inactive inhibitor nor-okadaone, dramatically increased the level of mCAR in the cytosolic fraction (Fig. 5B). These results suggested that HepG2 cells retain a degree of capability in mimicking the regulated nuclear translocation of mCAR, as observed previously in mouse hepatocytes (Kawamoto *et al*., 1999). To examine whether PP2A could regulate the nuclear translocation of mCAR in HepG2 cells, V5-tagged catalytic subunit of human PP2A was co-expressed with V5-tagged mCAR (Fig. 5C). Both hPP2Ac and mCAR were detected simultaneously in Western blots of the cytosolic fraction. Similar to TCPOBOP, hPP2Ac decreased

mCAR in the cytosolic fraction and increased it in the nuclear fractions. These results indicated that PP2A stimulates the nuclear translocation of mCAR in HepG2 cells.

Ser-202-regulated nuclear translocation of mCAR in HepG2 cells. To examine whether Ser-202 could regulate the TCPOBOP-dependent nuclear translocation, the V5-tagged S202D and S202A mutants were expressed in HepG2 cells in the presence or absence of TCPOBOP (Fig. 6A). The S202A mutant was predominantly expressed in the nuclear fraction in the absence of TCPOBOP and was unaffected after TCPOBOP treatment. On the other hand, the S202D mutant was preferentially expressed and retained in the cytosolic fraction even after treatment with TCPOBOP. The lack of nuclear translocation of the S202D mutant affected its trans-activation of the NR1 reporter construct in the HepG2 cells (Fig. 6B). Since V5 is tagged at the C-terminus of mCAR in the constructs used, the tagged receptor exhibits low constitutive activity that can effectively be activated by TCPOBOP in HepG2 cells (Swales *et al.*, 2005). Accordingly, all three receptors were similarly activated by TCPOBOP approximately 20-fold (Fig. 6B). The same activation rate suggested that Ser-202 is not directly involved in the trans-activation function of mCAR in the nucleus. Consistently, all three effectively formed a complex with NR1 in gel-shift assays (Fig. 6C). However, the basal as well as TCPOBOP-activated activities of the S202D mutant were approximately 50% lower than wild type mCAR and the S202A mutant (Fig. 6B). These results indicated that phosphorylation of Ser-202 may also be a factor regulating the nuclear translocation in the HepG2 cells.

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Recently, a co-regulator GRIP1 was reported to mediate the nuclear translocation of CAR in both mouse livers and HepG2 cells (Xia and Kemper, 2004). Experiments were performed to investigate whether the mutation of Ser-202 affected the GRIP1-dependent nuclear translocation of mCAR. To this end, human GRIP1 was co-expressed with the V5-tagged mCAR, $mCAR_{S202A}$ and $mCAR_{S202D}$ in HepG2 cells. Western blotting was carried out to investigate the expression of a given receptor in the cytosolic and nuclear fractions (Fig. 7A). As shown in Fig. 6A, the S202D and S202A were predominantly accumulated in the cytoplasmic and nuclear fractions, respectively. The co-expression of hGRIP1 did not affect their subcellular translocation. GST pull-down assays revealed no difference in the binding of mutants to hGRIP1 compared with that of wild type mCAR (Fig. 7B). hGRIP1 co-activated mCAR-mediated trans-activation of the NR1 reporter in the co-transfected HepG2 cells. As observed with the mCAR, all three mutated receptors exhibited the similar rate of co-activation by hGRIP1 in both the absence and presence of TCPOBOP (Fig. 7C). These results indicated that hGRIP1 does not play a role in the Ser-202-regulated translocation of mCAR.

Phosphorylation of Ser-202. Flag-tagged wild-type mCAR or the flag-tagged S202A mutant was expressed in HepG2 cells, from which cytosolic and nuclear fractions were prepared. Then, the wild-type mCAR was immunoprecipitated by anti-flag antibody. Using anti-pS202 antibody, Western blot analysis was performed on immunoprecipitates to examine whether Ser-202 is phosphorylated. Anti-pS202 detected an immunoreactive band for the wild-type mCAR in the cytosolic fraction (Fig.

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8). Western blotting using anti-flag antibody showed one major and minor band from this fraction, of which the major one corresponded to non-phosphorylated mCAR. The minor band, appeared above the major one, comigrated with the anti-pS202-reacting band and thus is suggested to represent the phosphorylated form of mCAR. This phosphorylated band was never detected in the nuclear faction, thus suggesting that Ser-202 can be phosphorylated only when mCAR is sequestered in the cytoplasm.
For control experiment, the Flag-tagged S202A mutant was also expressed, immunoprecipitated and subjected to Western blot analysis. As expected, no phosphorylated form of S202A mutant was detected by Western blotting with anti-pS202 antibody.

Discussion

Since PB triggering nuclear translocation of mCAR was found sensitive to protein phosphatase inhibitor OA, phosphorylation of mCAR has been a key subject of investigations. We have now identified Ser-202 as the phosphorylation site of mCAR. Ser-202 is phosphorylated when mCAR is sequestered in the cytoplasm; its dephosphorylation facilitates the nuclear translocation of mCAR. PP2A is a protein phosphatase that can catalyze the dephosphorylation of Ser-202. Mimicking phosphorylation, the mutation of Ser-202 to Asp makes mCAR no longer capable of PB triggering translocation into the nucleus in mouse liver. A key question arisen now is how PB and TCPOBOP trigger dephosphorylation of CAR. Because TCPOBOP can bind directly to mCAR, this binding may elicit signal to dephosphorylate Ser-202 of mCAR. However, the mechanism can be more complex for PB, since PB does not require direct binding to activate mCAR. Since our previous study shows that the cytoplasmic CAR:Hsp90 complex recruits PP2A upon PB treatment (Yoshinari *et al*., 2003), future investigations may lead us to uncover a drug-

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triggering signal transduction pathway that regulates PP2A and the nuclear translocation of mCAR.

Nuclear localization of mCAR can also be augmented by multiple factors including the ability of binding to RXR, co-regulators and/or DNA elements. The mutation of Ser-202 did not alter those binding abilities. Since the S202A and S202D mutants exhibited the same binding ability to RXR and NR1 as the wild-type mCAR in gel shift assays. GRIP1 has been suggested to accumulate mCAR in the nucleus through binding and stabilizing the receptor (Xia and Kemper, 2004). Western blot analysis confirmed the expression of GRIP1 in the HepG2 cells; however the S202D mutant still accumulated predominantly in the cytosolic fraction, while the S202A mutant accumulated in the nuclear fraction. While GRIP1 exhibited TCPOBOP-dependent binding to wild type mCAR in GST pull down assays, none of the mutations changed the binding. GRIP1 co-regulated the S202D mutant-mediated activation of the NR1 reporter in the presence or absence of TCPOBOP as effectively as the wild type mCAR and the S202A mutant. GRIP1 did not alter the inability of the S202D mutant to accumulate in the nucleus. No nuclear export signal is found in mCAR and proactive nuclear export has not been intensively investigated. Again, a strong tendency of mCAR to diffuse passively in cells is a major difficulty facing investigations. Once the proper nuclear export assay is established in the future, the role of Ser202 in the nuclear export of CAR should be revisited.

If the nuclear translocation of mCAR depended on only the dephosphorylation of Ser-202, it would be expected that the S202A mutant would accumulate in the nucleus without drug treatment. To some extent, this was observed in the HepG2 cells; the S202A mutant accumulated more effectively in the nucleus than the wild-type mCAR did in the absence of TCPOBOP.

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However, the S202A mutant is sequestered in the cytoplasm in non-treated mouse liver and translocates into the nucleus only after PB treatment, as dose wild-type mCAR. This suggests that the dephosphorylation of Ser-202 is required but not sufficient to regulate the nuclear translocation of mCAR. mCAR is retained in the cytoplasm by forming a complex with Hsp90 and a CAR specific co-chaperon CCRP (Yoshinari *et al.*, 2003; Kobayashi *et al.*, 2003). Receptors such as GR and AhR also form a complex with Hsp90 and contain the specific co-chaperones immunophilin and XAP, respectively; these co-chaperons regulate their nuclear translocations (Pratt, 1993; Pollenz *et al.*, 1994; Tai *et al.*, 1992; Carver and Bradfield, 1997; Pratt and Toft, 1997; Meyer *et al.*, 1998). Other factors such as CCRP and XRS may also be regulating the nuclear translocation of mCAR, maintaining mCAR under tight control in mouse liver. Some of these regulatory factors may be lost in HepG2 cells; thus Ser-202 becomes the dominant factor regulating nuclear translocation. Taken all together, there must be factors that regulate the nuclear translocation, in addition to Ser-202. Identifying those factors and their functions should greatly advance our understanding of PB triggering nuclear translocation of mCAR.

Two X-ray crystal structures of mCAR have now been reported; one is the ternary complex with RXR, co-activator peptide and TCPOBOP, while another is in the form of a homodimer (Xu *et al.*, 2004; Shan *et al.*, 2004). First, it is reasonable to have found that the mutation of Ser-202 never affected either CAR binding to RXR or the activation by GRIP1, since Ser-202 is not positioned to interact directly with either RXR or the co-activator. In the both structures, Ser-202 is located on the bottom of a shallow pocket and its side-chain oxygen is diagonally oriented toward the surface (loop consisted of residues 279 to 287) of the pocket. Accordingly, Ser-202 is positioned not to be phosphorylated in the active state of mCAR, suggesting that Ser-202 can

only be phophorylated in its inactive form. This structural constrain may explain why it has been so difficult to demonstrate the phosphorylation; during preparation of mCAR from cells, Ser-202 is rapidly dephosphorylated as the mCAR molecule takes the most stable structure. Due to a residual protein phosphatatse activity in the experimental conditions used, this dephosphorylation could not have been prevented. If Ser-202 should be phosphorylated, the loop needs to move away from Ser-202 and/or the side-chain oxygen of Ser-202 need to be re-oriented more toward the open surface of the pocket. When mCAR is sequestered in the cytoplasm, it forms a Hsp90:CCRP complex that is completely different from the nuclear complex with RXR and co-regulators. In the cytoplasmic complex or during the complex formation, Ser-202 may be accessible for phosphorylation by exposing its side-chain on the surface of the mCAR molecule. Once a three dimensional structure of CAR:CCRP complex is determined in the future, we should be able to answer the question as to whether Ser-202, in fact, undergoes this conformational change.

Molecular Pharmacology Fast Forward. Published on December 23, 2005 as DOI: 10.1124/mol.105.019505
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Molecular Pharmacology Fast Forward. Published on December 23, 2005 as DOI: 10.1124/mol.105.019505
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Legends and Figures

Fig. 1. **Intracellular localization of mCAR, mPXR and chimera in mouse livers. A.** mCAR, mPXR and a chimera molecule between them ($mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$) as YFP fusion proteins were expressed in the CD-1 mice livers by tail-vein injection of each expression plasmid. Mice were either untreated or treated with PB. Liver sections were prepared and examined by microscopy for YFP expression (in yellow) and Hoechest S33258 staining for nuclei (in blue). Two representative images are shown for each treatment group. **B.** Nucleo-cytoplasmic distribution of the YFP fusion proteins shown in panel A was categorized into three groups. Mice were either untreated (-) or treated (+) with PB. The percentage of cells with the YFP fusion proteins expressed predominantly in the cytoplasm (Cyt, closed bar), equally in the cytoplasm and the nucleus (Cyt & Nuc, gray bar), predominantly in nucleus (Nuc, open bar) were determined using a fluorescent microscope. At least 100 cells expressing the fusion proteins were examined for each treatment.

Fig. 2. **Intracellular localization of various Asp mutants of mCAR in mouse livers. A,** Conserved Ser or Thr residues are indicated in *colour*. **B,** Wild type mCAR and the mutants ($mCAR_{T176D}$, $mCAR_{S202D}$, $mCAR_{T218D}$, $mCAR_{T224D}$, $mCAR_{S279D}$ and $mCAR_{S331D}$) were expressed as YFP fusion proteins in $Car^{-/-}$ mouse livers by injecting the expression plasmids via the tail vein. Mice were either untreated (-) or treated (+) with PB and the cells expressing the YFP fusion proteins were categorized as in Fig. 2B. The percentage of cells with the YFP fusion proteins expressed predominantly in the cytoplasm (Cyt, closed bar), equally in the cytoplasm

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and the nucleus (Cyt & Nuc, gray bar), predominanlty in nucleus (Nuc, open bar) were determined. At least 100 cells expressing the fusion proteins were examined for each treatment.

Fig. 3. **Mutational analysis of Ser-202 for mCAR nuclear translocation in mouse livers. A,** mCAR, $mCAR_{S202D}$, $mCAR_{S202A}$ and $mCARS_{S202R}$ were expressed as YFP fusion proteins in *Car*$^{-/-}$ mouse livers by injecting the expression plasmids via the tail vein. Mice were either untreated (-) or treated (+) with PB. Liver sections were prepared and examined by microscopy for YFP expression (in yellow) and Hoechst S33258 staining for nuclei (in blue). Two representative images are shown for each treatment group. **B,** Nucleo-cytoplasmic distribution of the YFP fusion proteins shown in panel A was categorized into three groups. Mice were either untreated (-) or treated (+) with PB. The percentage of cells with the YFP fusion proteins expressed predominantly in the cytoplasm (Cyt, closed bar), equally in the cytoplasm and the nucleus (Cyt & Nuc, gray bar), predominantly in nucleus (Nuc, open bar) were determined. At least 100 cells expressing the fusion proteins were examined for each treatment.

Fig. 4. **Regulatory function of Ser-202 is conserved in hCAR and mPXR-CAR chimera. A,** Nucleo-cytoplasmic distribution of YFP-hCAR and its mutants in CAR null mouse livers *in vivo*. Plasmids encoding hCAR and its mutants as YFP fusion proteins were injected via the tail vein and cell counting of liver sections were carried out as described in Fig. 2B. Mice were either untreated (-) or treated (+) with PB. The percentage of cells with the YFP fusion proteins expressed predominantly in the cytoplasm (Cyt, closed bar), equally in the cytoplasm and the nucleus (Cyt & Nuc, gray bar), predominantly in nucleus (Nuc, open bar) were determined using a fluorescent microscope. At least 100 cells expressing the fusion proteins were examined for each treatment. **B.** Nucleo-cytoplasmic distribution of YFP- $mCAR_{DBD\text{-}Hinge}$-$mPXR_{LBD}$ Ser 271 mutant in CAR null mouse livers in vivo.

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Experiment was performed as in panel A using the mutant expression plasmid and protein localization in liver cells were categorized.

Fig. 5. **mCAR nuclear translocation in HepG2 cells. A,** HepG2 cells were transfected with pcDNA3.1/V5-His-mCAR and treated with 250 nM TCPOBOP at 3 and 24h after transfection so that treatment periods are 24h and 45h, respectively. Cells were harvested 48h after transfection for cell fractionation. Western blotting detection was performed for cytosolic, nuclear and whole cell extract proteins (30, 15 and 5 μg respectively), following electrophoresis on 10% SDS-PAGE gels using primary antibodies, anti-V5 for mCAR, anti-hsp90 and anti-laminβ. **B,** HepG2 cells were transfected with pcDNA3.1/V5-His-mCAR and cells were treated with 10 nM nor-okadaone (Nor-OK) or okadaic acid (OA) along with vehicle DMSO for 21h. Cells were harvested 24h after transfection and cytosol extracts were prepared. Western blotting detection of mCAR was performed with 30 μg of cytosolic proteins, after separation on a 10% SDS-PAGE gel using anti-V5 antibody. **C,** HepG2 cells were cotransfected with pcDNA3.1/V5-His-mCAR and pcDNA3.1/V5-His-hPP2A and cells were harvested after 24h. Cytosols (40 μg) and nuclear extracts (20 μg) were subjected to Western blot analysis with anti-V5 antibody for mCAR and hPP2A, anti-hsp90 and anti-laminβ.

Fig. 6. **Nuclear translocation and trans-activation of mCAR mutants in HepG2 cells. A,** HepG2 cells were transfected with pcDNA3.1/V5-His bearing mCAR, $mCAR_{S202A}$, or $mCAR_{S202D}$ and 3h after transfection cell were either treated with 250 nM TCPOBOP (+) or vehicle DMSO (-). Cytosolic (30 μg) and nuclear (10 μg) proteins were prepared 24h after transfection and subjected to Western blotting analysis as in Fig. 1. **B,** pcDNA3.1/V5-His bearing

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mCAR, $mCAR_{S202A}$, or $mCAR_{S202D}$ were cotransfected with $(NR1)_5$-tk-luciferase plasmid and pRL-SV40 into HepG2 cells. 24h after transfection cells were treated with 250 nM TCPOBOP or vehicle (DMSO) alone and subjected to luciferase assay 24h later. The luciferase activity level is indicated as an average from 3 wells with standard deviation. **C,** DNA binding activity of the mCAR Ser 202 mutants. mCAR and its mutants were prepared by in vitro translation and mixed with similarly prepared RXR for gel shift assays using ^{32}P-labeled NR1 oligonucleotide as a probe. Mock translated mCAR was used as the control.

Fig. 7. **Effect of hGRIP1 on the function of mCAR in HepG2 cells. A,** hGRIP1 shows no effect on localization of mCAR and its mutants in HepG2 cells. pcDNA3.1/V5-His bearing mCAR, $mCAR_{S202D}$, $mCAR_{S202A}$ were cotransfected with hGRIP1 expression vector (+) or empty pcDNA3.1 (-) in HepG2 cells. Cells were harvested and Western blotting was performed as in Fig. 1A. **B,** Interaction of mCAR and mCAR mutants with hGRIP1 in GST pull down assays. In vitro-translated ^{35}S-labeled hGRIP1 was incubated with GST-mCAR, GST-$mCAR_{S202A}$ or GST-$mCAR_{S202D}$ fusion protein on glutathione resin in the presence of DMSO (-) or TCPOBOP (+). After washing the resin, proteins were extracted and separated on a 10% SDS-PAGE gel. Five percent of the lysate used in the binding reactions was used as input. Bound proteins were detected by autoradiography. **C**, hGRIP1 co-activation of mCAR and S202 mutants in HepG2 cells. hGRIP1 was co-expressed with mCAR, $mCAR_{S202A}$, or $mCAR_{S202D}$ in HepG2 cells along with $(NR1)_5$-tk-luciferase reporter. 24h after transfection, cells were treated with 250 nM TCPOBOP or vehicle alone and subjected to luciferase assay 24h later. The luciferase activity level is indicated as an average from 3 wells with standard deviation.

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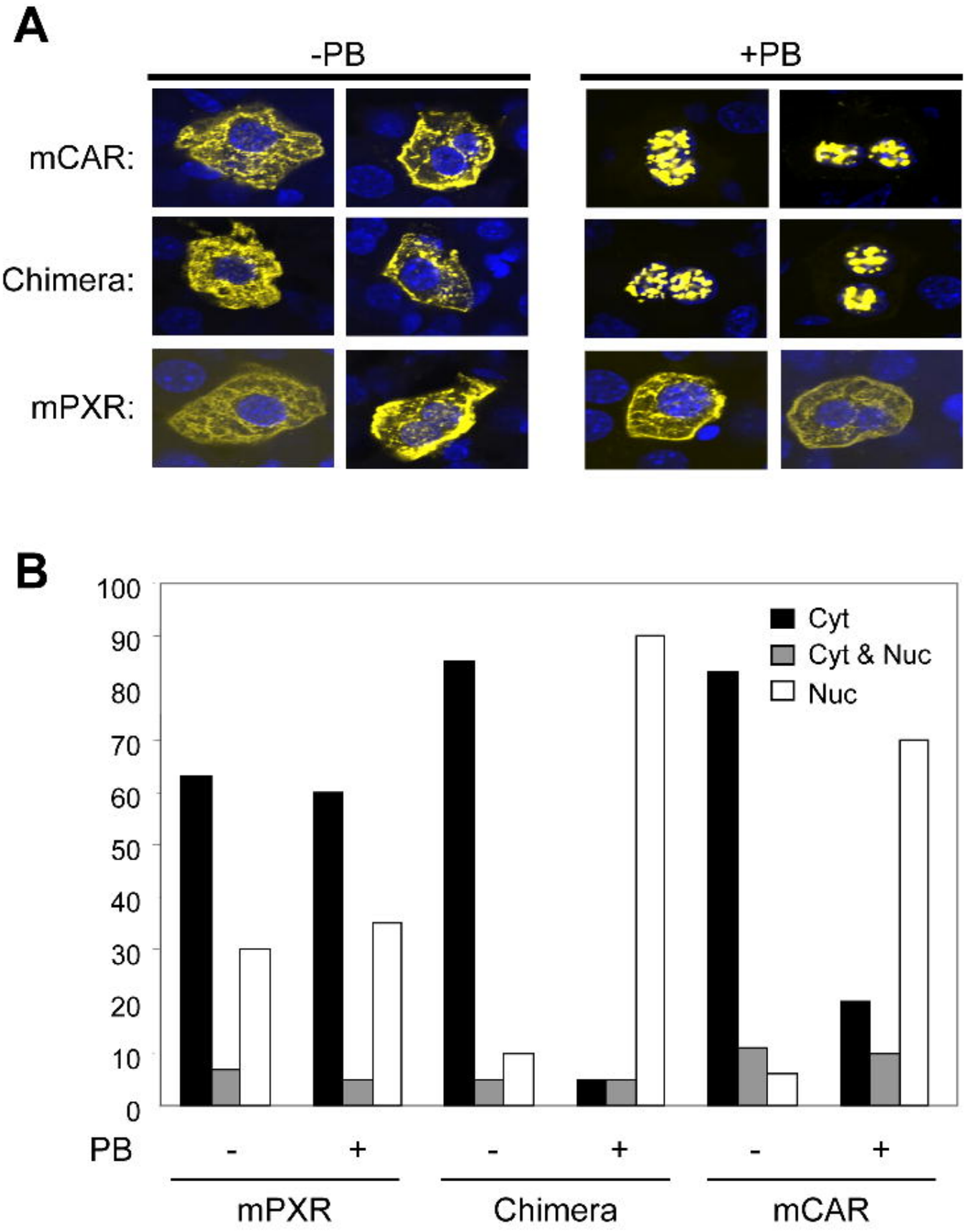
Fig. 8. **Phosphorylation of Ser 202 in HepG2 cells.** HepG2 cells were transfeced with Flag-mCAR/pCR3 and its S202A mutant. Immunoprecipitation was carried out by anti Flag-antibody agarose or normal mouse IgG agarose using 1.5 mg of cytosolic or nuclear extract isolated from transfected HepG2 cells. Precipitated samples were separated on a SDS-PAGE gel and transferred to a PVDF membrane. The same membrane was sequentially stained with anti pS202 antibody and then with anti-Flag HRP.

Fig. 1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mPXR:
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Cyt
Cyt & Nuc
Nuc
PB
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-
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mPXR
Chimera
mCAR

Fig. 2

A

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mCAR: 201 ISLLKGAAVE   ILHISLMTTF   GLQTENFFGG   171 FADINTFMVQ
hCAR: 191 ISLLKGAAVE   ICHIVLMTMF   CLQTQMFLCG   161 FADINTFMVL
mPXR: 270 ISLLKGATFE   MCILRFMTWF   DTETGTWECG   240 LADVSTYMFK

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B

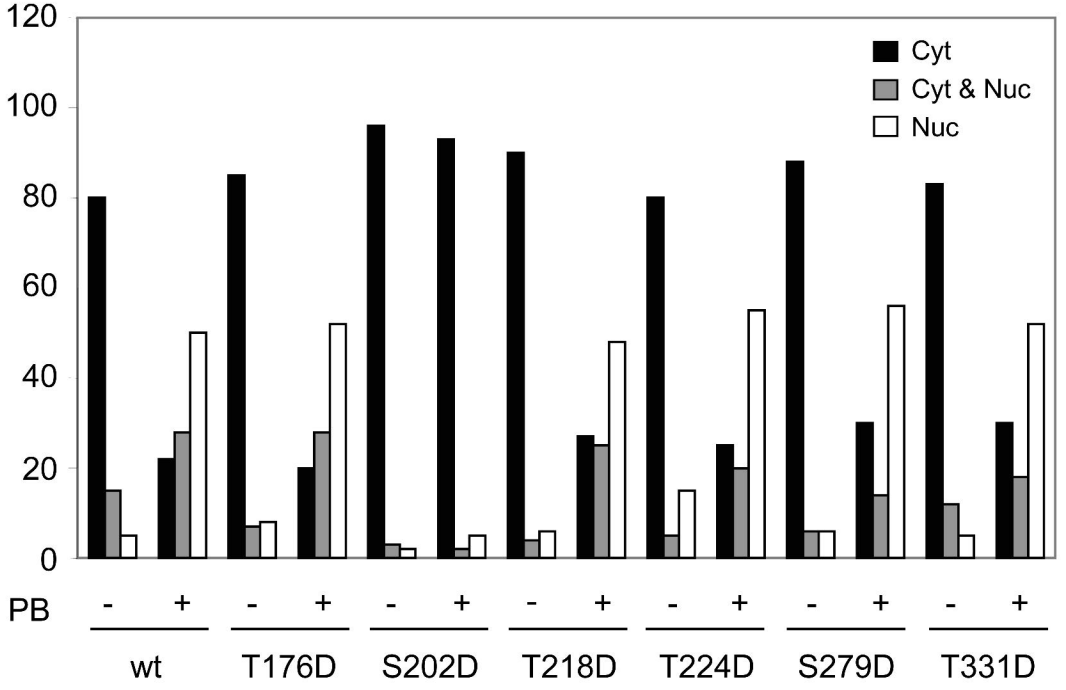
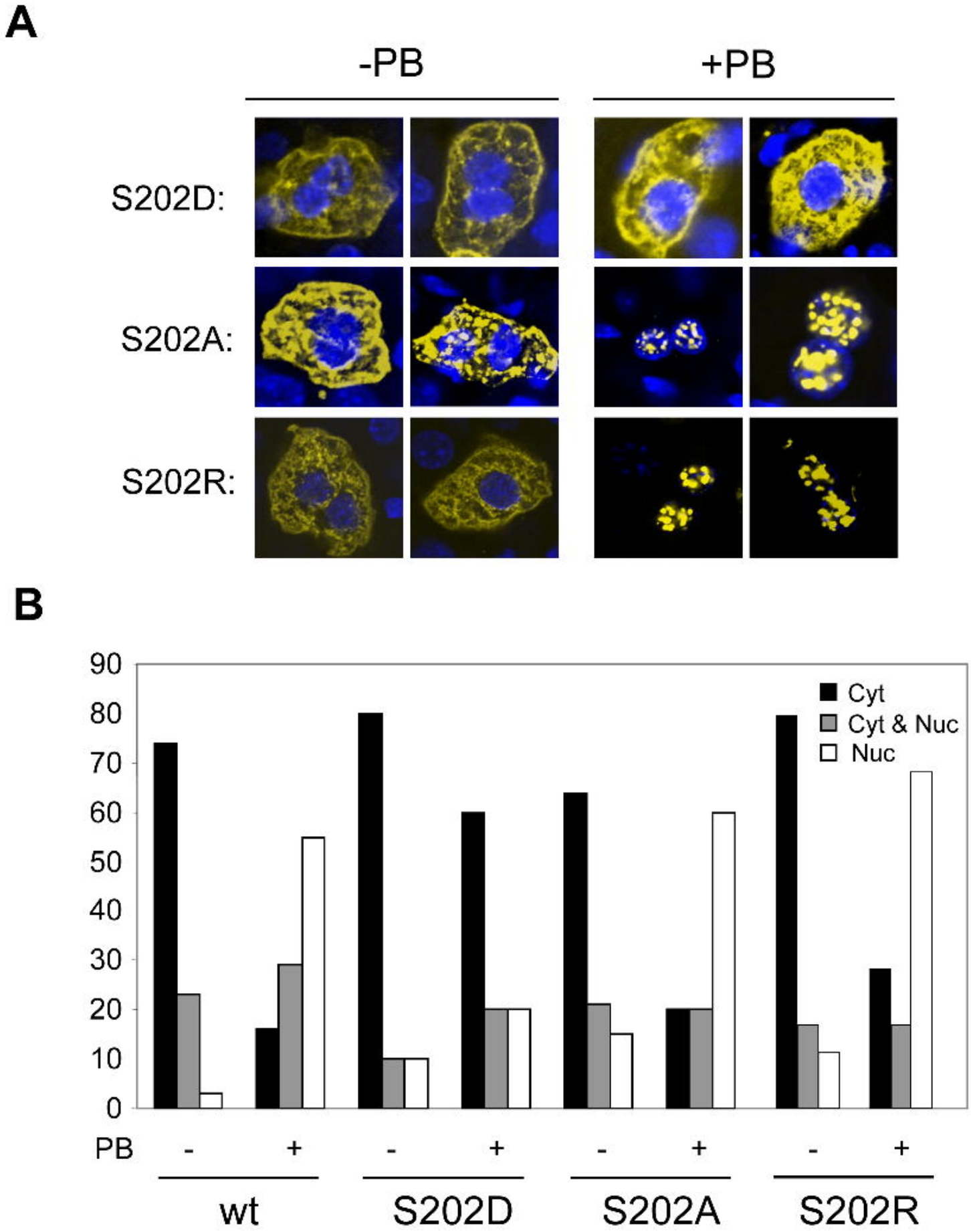


Fig. 3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80
70
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Cyt
Cyt & Nuc
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S202D
S202A
S202R

Fig. 4

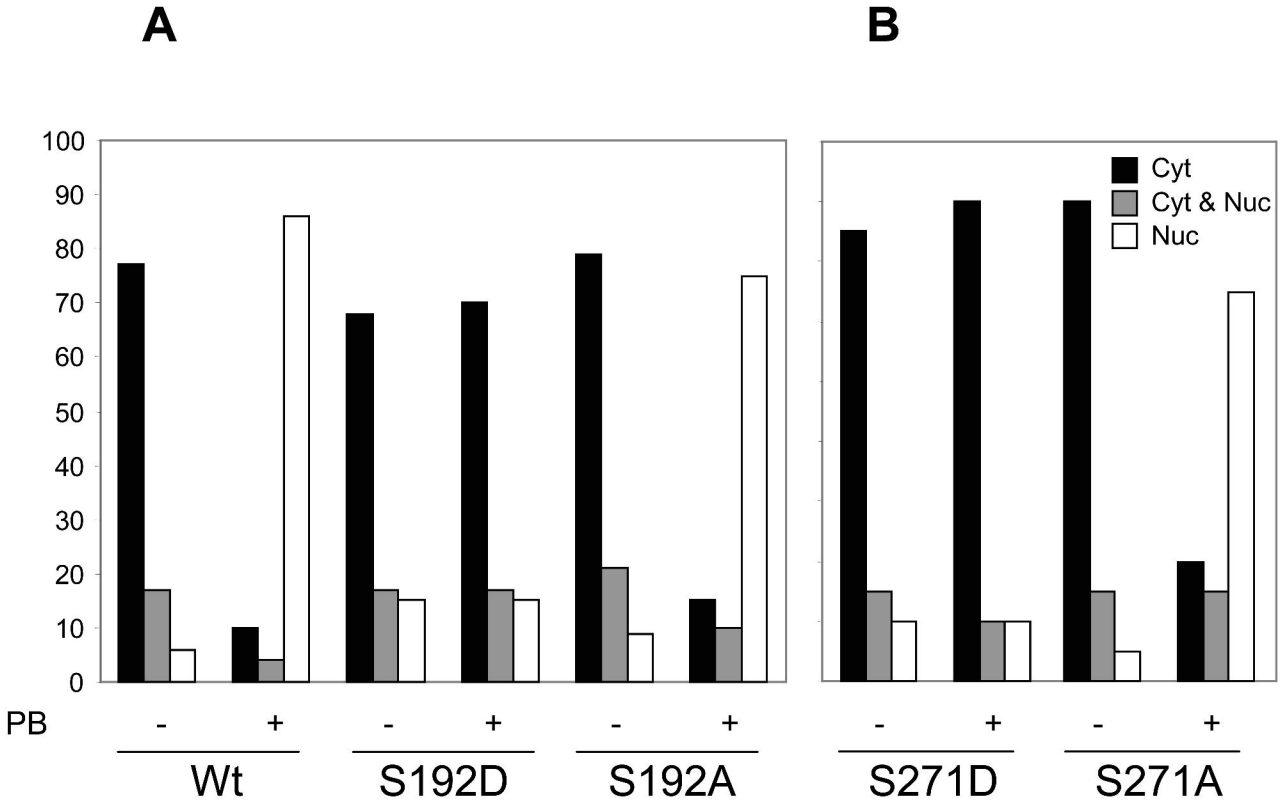
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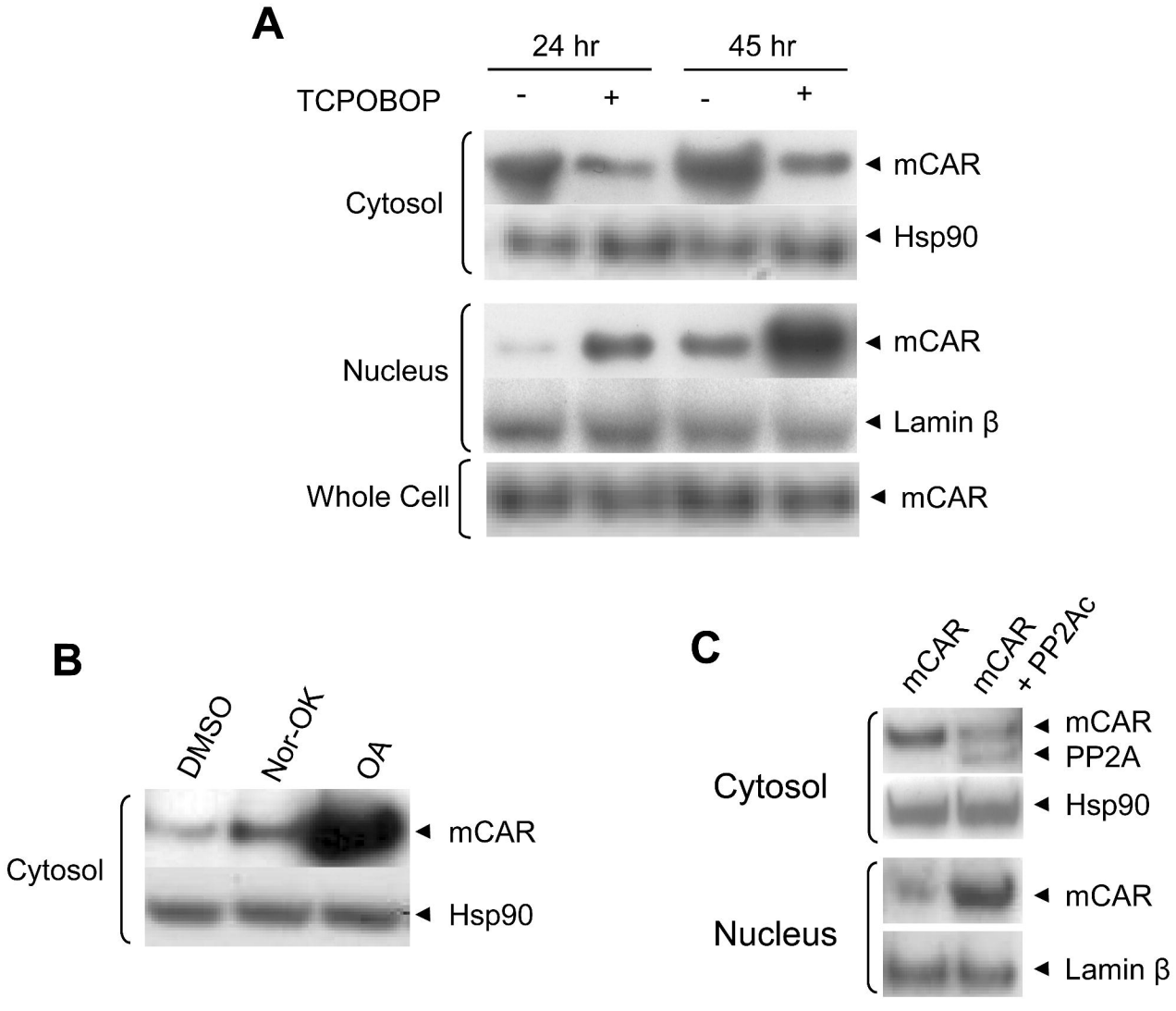
Fig. 5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B
DMSO
Nor-OK
OA
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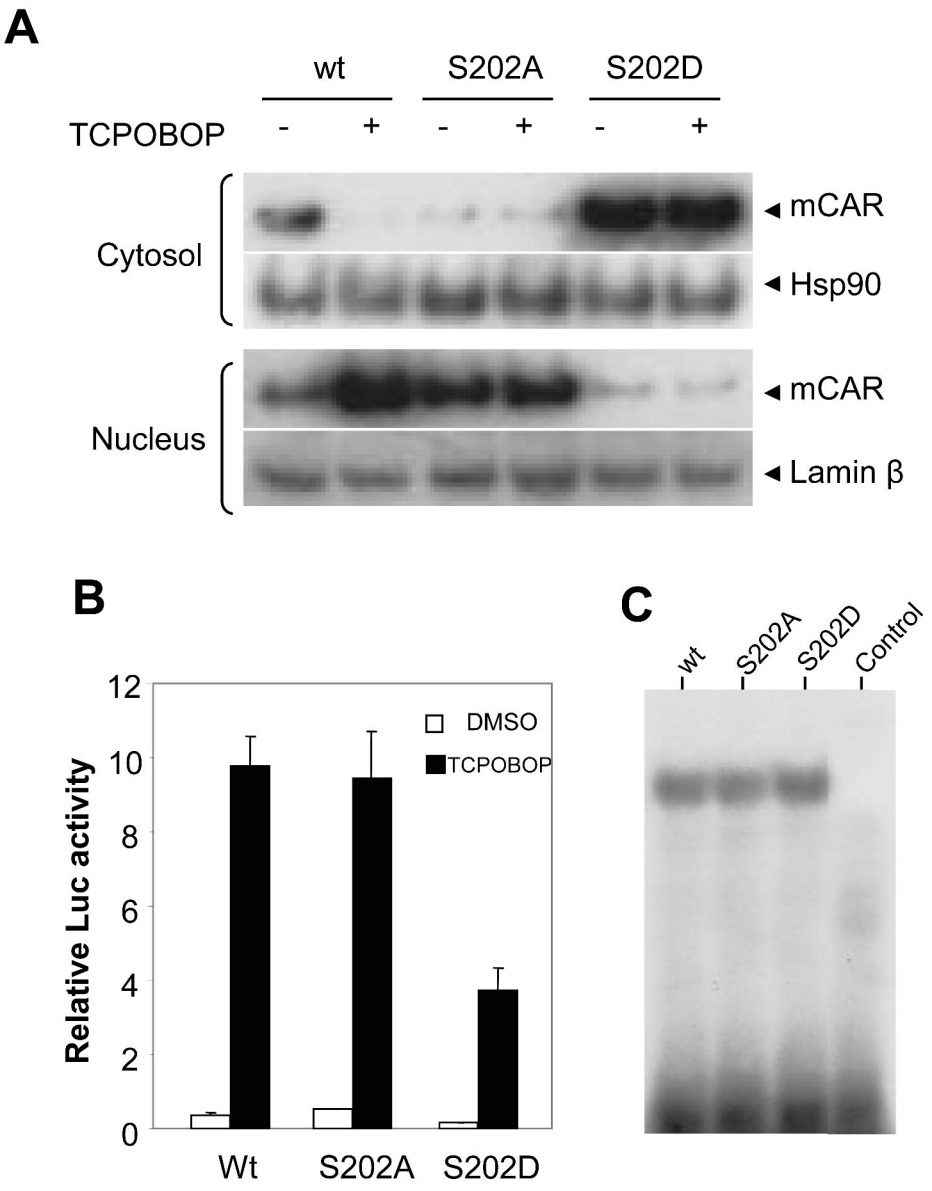
Fig. 6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B
Relative Luc activity
12
10
8
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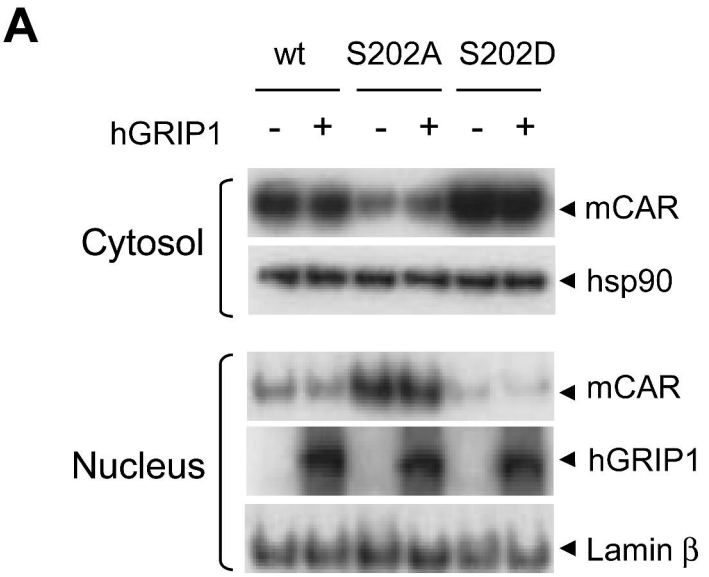
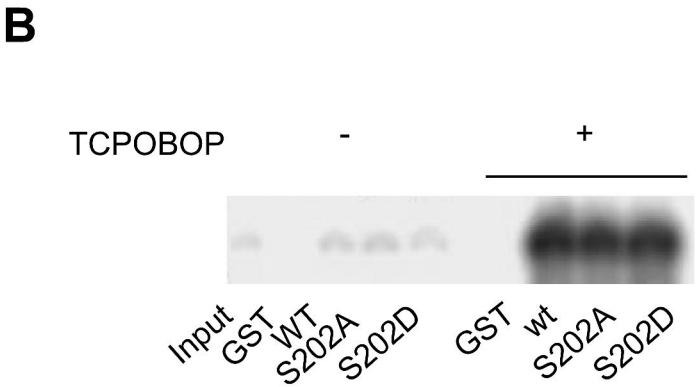
S202D
Control

Fig. 7





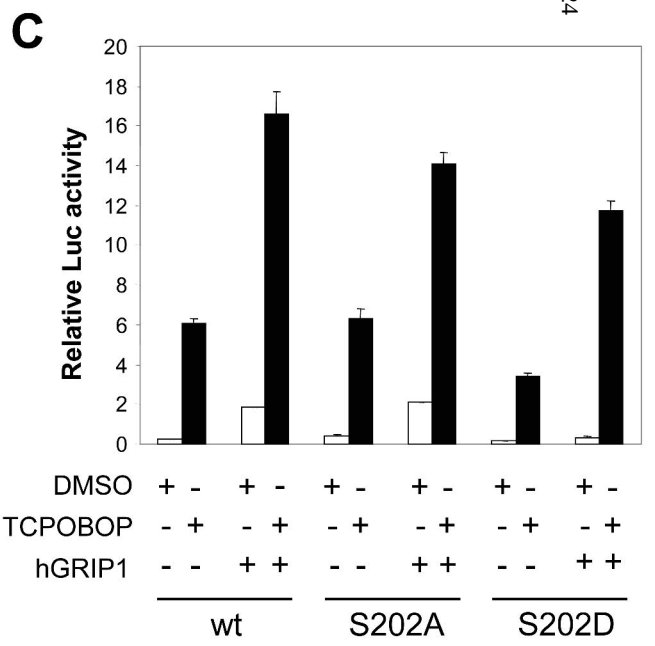
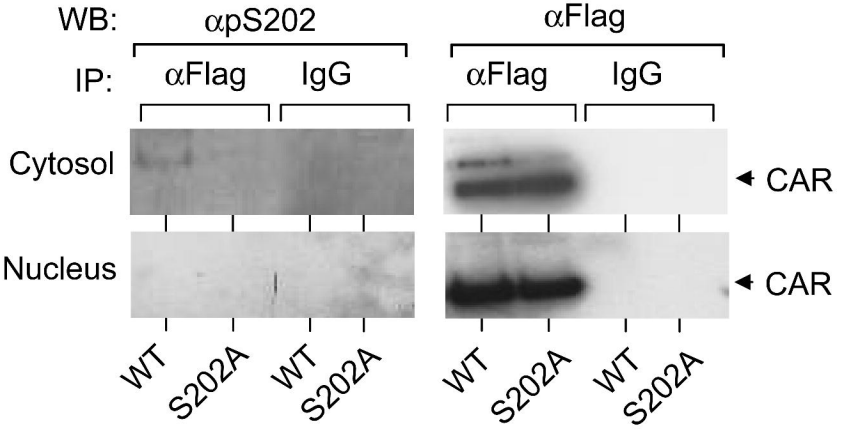


Fig. 8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