Heat Shock Protein-90 (Hsp90) Acts as a Repressor of Peroxisome Proliferator-Activated Receptor-α (PPARα) and PPARβ Activity†

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ABSTRACT: The nuclear receptor (NR) peroxisome proliferator-activated receptor-α (PPARα) mediates the effects of several hypolipidemic drugs, endogenous fatty acids, and peroxisome proliferators. Despite belonging to a class of NR not known to interact with cytosolic chaperone complexes, we have recently shown that PPARα interacts with heat shock protein 90 (Hsp90), although the biological consequence of this association was unknown. In the present study, PPARα directly associated with Hsp90 in vitro to a much greater extent than either PPARβ or PPARγ. This interaction is similar to other NR-Hsp90 complexes with association occurring between the middle of Hsp90 and the hinge (D) and ligand binding domain (EF) of PPARα. Using several different approaches to disrupt Hsp90 complexes within the cell, we demonstrate that Hsp90 is a repressor of both PPARα and PPARβ activity. Treatment with geldanamycin (GA) increased the activity of PPARα and in the presence of ligand in transient transfection assays. PPARα-response element (PPRE)-reporter assays in a stable cell line treated with GA resulted in enhanced expression of a known target gene, acyl-CoA oxidase. Similarly, overexpression of the tetratricopeptide repeat (TPR) of protein phosphatase 5 (PP5) increased PPARα or PPARβ activity in a PPRE-reporter assay and decreased the interaction between PPARα or PPARβ and Hsp90 in a mammalian two-hybrid assay. Finally, cotransfection with the C-terminal hsp-interacting protein (CHIP) construct, a TPR-containing ubiquitin ligase that interacts with hsp90, increased PPARα's and decreased PPARβ's ability to regulate PPRE-reporter activity upon ligand activation. All three methods to disrupt Hsp90 function (GA, PP5-TPR, CHIP) resulted in an alteration in PPARα or PPARβ activity to a much greater extent than PPARγ. While FKBP52 had no effect on PPARα activity, p23 greatly enhanced constitutive and Wy14 643 induced PPRE-reporter activity. Thus, we describe the chaperone complex as being a regulator of PPARα and PPARβ activity and have identified a novel, subtype-specific, inhibitory role for Hsp90.

Peroxisome proliferator-activated receptor α (PPARα, NR1C1 (*1*))[1] is a member of the nuclear receptor (NR) superfamily. NRs are ligand-activated, intracellular receptors that include the progesterone (PR) and glucocorticoid receptors (GR), as well as the thyroid hormone (TR) and retinoic acid receptors (RAR and RXR) (*2*). In addition to PPARα, the NR1C subset of receptors includes two closely related members, PPARβ (or δ, NR1C2) and PPARγ (NR1C3). The three isoforms of PPAR have been cloned from various species including humans (*3*), rodents (*4*−*6*), and amphibians (*7*). The PPARs show differential tissue distribution, and each subtype differs in physiological function (*8*). While PPARβ is ubiquitously expressed, PPARγ expression is predominant in white and brown adipose tissues, and PPARα is highly expressed in the liver, cardiac muscle, intestine, and renal cortex (*9*−*12*). PPARα is responsible for the modulation of the immune response and catabolism of fatty acids through peroxisomal and mitochondrial β-oxidation, microsomal ω-oxidation (*13*), and amino acid and carbohydrate metabolism (*14*). PPARγ is involved in adipocyte differentiation and lipid storage in the adipose tissue (*15*, *16*). Although not as well-studied as PPARα or γ, the physiological functions of PPARβ include embryo implantation, reverse cholesterol transport, colon and skin tumorigenesis, and epidermal wound healing (*12*, *17*−*19*).

All three PPAR subtypes activate gene expression in a ligand-dependent manner by recognizing and binding to peroxisome proliferator response elements (PPRE) that are composed of TGACCT-related direct repeats separated by one nucleotide (DR1) (*20*, *21*). PPARs binds to PPREs as a heterodimer with RXRα, the receptor for 9-*cis*-retinoic acid (*22*, *23*). The transcriptional activity of this heterodimer is

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[1] Abbreviations: PPAR, peroxisome proliferator-activated receptor; NR, nuclear receptor; Hsp, heat shock protein; MAPK, mitogen-activated protein kinase; GA, geldanamycin; PP, peroxisome proliferators; PR, progesterone receptor; GR, glucocorticoid receptor; TR, thyroid hormone; RAR, retinoic acid receptor, RXR, retinoid X receptor; VDR, vitamin D receptor; TPR, tetratricopeptide repeat; CHIP, C-terminal Hsp-interacting protein; XAP2, hepatitis B virus X-associated protein 2.

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regulated in part by the association of a family of proteins that are known to regulate NR activation (coactivators and corepressors (*24*)). PPARs also interact with other transcription factors such as AP1 and NFκB (*25*) as well as other members of the NR family such as the thyroid hormone (NR1A (*26*)). PPARs have multiple protein−protein interactions regulating the specificity and extent by which ligands affect gene expression, typical of the NR superfamily. Few PPAR subtype-specific interactions, at either the protein or the DNA level, have been described. Although several specific endogenous and pharmaceutical ligands of the PPARs have been described, many compounds are able to affect the activity of PPARα, β, and γ (*27*). Considering that several tissues express all three subtypes (*8*) and taking into account the overlapping ligands and signal transduction pathways, in order for the PPARs to manifest their distinct biological roles there must be cellular mechanisms that differentiate between PPARα, β, and γ that have not been described.

Despite many advances in understanding how PPARs regulate target gene expression, little is known about the PPARα complex prior to its activation with ligand. Heat shock protein 70 (Hsp70) was purified from rat liver cytosol using an immobilized PP (clofibric acid) affinity column (*28*, *29*). Subsequently, the association between rat PPARα and Hsp70 was described (*30*). These reports suggest that PPARα exists as a heteromeric complex similar to the NR3 subfamily (GR, PR, ER). We recently established the association between PPARα and Hsp90 in mouse liver cytosol (*31*). In addition, the cochaperone hepatitis B virus X-associated protein 2 (XAP2) was also complexed with PPARα and is capable of repressing PPARα-mediated transcriptional activity (*31*).

NRs such as GR and PR exist in the cytosol as multiprotein complexes that include Hsps 70 and 90, as well as p23 and certain immunophilins (*32*). These associated proteins appear to hold the receptor in a conformation that will allow for high affinity binding of the ligand (*33*). For example, the ability of PR to bind ligands is greatly compromised when the Hsp90-binding antibiotic, geldanamycin (GA), is added in a cell-free system (*34*). Unlike NR3 family members, NR1 subfamily members, which include TR, vitamin D receptor (VDR), and RAR, do not form a stable complex with Hsp90 (*35*, *36*). The involvement of Hsp90 in many NR1 receptor complexes (e.g., farnesoid X receptor (FXR), liver X receptor (LXR), and constitutive androstane receptor (CAR)) has not been examined. The goals of this paper were to determine the role Hsp90 plays in PPARα signaling as well as to examine if all three PPAR subtypes are equally sensitive. Using three different approaches to disrupt Hsp90 complexes, we demonstrate that Hsp90 is a repressor of PPAR activity, with the following subtype specificity: PPARα > PPARβ ≫ PPARγ. Thus, we describe a novel inhibitory role for Hsp90 in regulating the activity of PPARα and PPARβ. This represents a notable exception to widely held beliefs that NRs of the TR/RAR subfamily (NR1) do not enter stable Hsp90 complexes and that Hsp90 enhances the activity of the NRs it associates. In addition, the PPAR subtype-specificity of Hsp90 may have important biological consequences as it represents one mechanism by which one subtype may be preferentially regulated in a given cell type.

MATERIALS AND METHODS

Materials. Hy-Bond ECL nitrocellulose, [^{35}S]-methionine, and an ECL chemiluminescent detection system were purchased from Amersham (Arlington Heights, IL). Wy14 643 ([4-chloro-6-(2,3-xylindino)-2-pyrimidinylthio]acetic acid, CAS no. 50892-23-4, >98% pure) was purchased from Chemsyn Science Laboratories (Lenexa, KS). 15-Deoxy$\Delta^{12,14}$-prostaglandin J2 (PGJ2) was purchased from Biomol (Plymouth Meeting, PA). Geldanamycin, DH5α bacterial cells, and all media components were from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). The rat hepatoma FaO cell line was a generous gift from M. C. Weiss (Pasteur Institute, Paris, France). Sodium molybdate, MOPS, Tricine, bovine serum albumin (BSA), disodium ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), and Tri Reagent were obtained from Sigma (St. Louis, MO). Restriction endonucleases and all cofactors and nucleotides necessary for PCR were obtained from Promega (Madison, WI). Plasmid purification kits were purchased from Qiagen (Chatsworth, CA). All primers were designed using the Lasergene primer select program (DNASTAR, Madison, WI) and purchased from Operon (Gaithersburg, MD). NuSieve 3:1 agarose was obtained from FMC Bioproducts (Rockland, ME). Other chemicals and reagents were of the highest grade readily available.

Plasmids. The construction of pBK/CMV/mPPAR/FLAG and pBK/CMV/rPPAR/FLAG as well as the mammalian two-hybrid pM/PPARα constructs were described previously (*31*). The mammalian expression construct pCMV6/PP5/TPR/FLAG was provided by M. Chinkers (Oregon Health Sciences University, Portland, OR (*37*)), and pCI/FKBP52 was obtained from D. Smith (University of Nebraska Medical Center, Omaha (*38*)). The mammalian expression construct p6R/p23 was provided by K. Yamamoto (University of California, San Francisco). The full-length cDNA for mouse PPARβ and γ was PCR amplified and ligated in-frame to the GAL4 DNA binding domain (pM, Stratagene; pM/PPARβ and γ). Similarly, Hsp90 cDNA was PCR amplified and ligated in-frame into pVP16 (Stratagene; pVP16/Hsp90). A GAL4 responsive reporter, pFR-luciferase (Stratagene), and PPRE driven reporter pACO(x2)-luciferase (obtained from Dr. David Waxman, Boston University, Boston, MA) were used. The plasmid pDJM-βgal, a gift from M. W. McBurney (University of Ottawa, Ottawa, ON), and phRL-TK (Promega) were used as an internal transfection control as indicated in the figure legends. The Hsp90/GST domain plasmids pGEX-4T-3/N90, pGEX-4T-3/M90, and pGEX-4T-3/C90 were obtained from F. U. Hartl (Max-Planck-Institute for Biochemistry, Germany).

Hsp90/GST Fusion Protein Interaction Assay. A series of Hsp90/GST fusion proteins, Hsp90-N/GST (Hsp90 residues 9−236), Hsp90-M/GST (272−617), and Hsp90-C/GST (629−732) were generated in *Escherichia coli* and isolated as previously described (*39*). Equivalent amounts of each protein were incubated with glutathione-agarose (Sigma) in phosphate buffered saline (PBS) for 2 h and washed three times in PBS and two times in binding buffer (MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, and 10% glycerol, pH 7.3), 0.5% w/v CHAPS, 50 mM NaCl, and 20 mM sodium molybdate). [^{35}S]-Methionine-labeled in vitro

translated PPARs were then incubated in 0.7 mL of binding buffer overnight, washed three times in binding buffer and two times in MENG buffer, resuspended in an equal volume of 2× tricine sample buffer (TSB), resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), and Coomassie Blue stained to visualize Hsp90/GST fusion proteins. The [^{35}S]-methionine-labeled proteins were visualized by autoradiography. The Coomassie blue staining was used to ensure that equal amounts of each GST fusion protein were present on the gel.

Generation of a Stably Transfected FaO Cell Line. FaO hepatoma cells were grown in DMEM (Sigma) supplemented with 5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The pACO(-581/-471)Luc plasmid, a generous gift from Dr. Jonathon Tugwood (AstraZeneca, Maccelsfield, UK (*21*)) and pcDNA3 (Invitrogen, Carlsbad, CA) plasmids were transfected into cells using calcium phosphate precipitation (ProFection, Promega) following the manufacturer's protocol. A 10:1 ratio of reporter/selection plasmid (pACO(-581/-471)Luc:pcDNA3) was used. Selective pressure was applied to the cells 2 days after transfection by adding Geneticin (G418, 400 μg/mL) for 4 weeks. Surviving clones were expanded and tested for inducibility by Wy14 643 (50 μM for 24 h). The FaO-PPRE stable cell line used in these studies is a pooled population of Wy14 643-responsive cells.

Generation of FLAG/PPARα Mouse Stable Cell Populations. Immortalized hepatocytes from PPARα$^{-/-}$ mice were generated using a temperature-sensitive SV40 virus as described previously (MuSH, (*40*)). The MuSH cells in 6-well plates were transfected with FLAG tagged wild-type rat PPARα (pBKCMV/RPPARα) using LipofectAMINE (Life Technologies, Inc., Manassas, VA) according to the manufacturer's protocol. Cells were allowed to recover overnight. The media were replaced with standard cell media (α-MEM, 4% FBS) supplemented with 400 μg/mL G418 (Invitrogen). Cells were incubated at 34 °C until overt cell death occurred, whereupon G418 resistant colonies were allowed to grow to confluency. Each well of the 6-well plate was handled as an individual stable cell population. Each population was expanded and tested for PPARα/FLAG via Western blot. The population with the highest relative expression of PPARα/FLAG was used in subsequent experiments (MuSH 3.4).

Reverse Transcriptase Polymerase Chain Reaction. The rat hepatoma cell line FaO was grown to 75% confluence as previously described (*41*). Total RNA was isolated using Tri-Reagent. Quantitative reverse transcriptase PCR (RT-PCR) was performed as previously described to measure fatty acyl-CoA (ACO) mRNA (*41*). The PCR products were electrophoresed on 2.0% NuSeive agarose, and band densities were measured using the Eagle Eye Imaging System (Stratagene, La Jolla, CA) and Molecular Analyst software (BioRad, Hercules, CA).

Transient Transfections and PPRE-Reporter Assays. Both Cos-1 and HepG2 (human hepatoma) cells were grown in α minimum essential medium (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), and 1% penicillin streptomycin (Sigma, St. Louis, MO). For reporter assays, COS-1 cells were transfected at 80% confluency in 6-well tissue culture plates. A LipofectAMINE transient transfection procedure was employed according to the manufacturer's instructions (Life Technologies, Inc., Manassas, VA) with 500 ng of receptor, 200 ng of reporter, 100−200 ng of pDJM-βgal, and either 700 ng of pCI/FKBP52, pCMV6/PP5/TPR/FLAG, or pcDNA3/CHIP. The total amount of plasmid was maintained at 2 μg of DNA per well. Transfected cells were harvested 24−36 h after the transfection, and PPRE-driven reporter activity was measured with a luciferase assay system (Promega Biotech, Madison, WI) using a Turner TD-20e luminometer.

Mammalian Two-Hybrid Assay. Gal4 fusion plasmids, pM/PPARα, β, or γ, VP16/Hsp90, GAL4 responsive reporter, pFR-Luciferase, and internal transfection control renilla, pRLTK, were transfected into COS-1 cells using lipofectamine in 12-well plates. The TPR domain of PP5 in a mammalian expression vector pcDNA3 was used for competition studies. The total amount of plasmid was maintained at 1 μg/well. Twenty-four hours after transfection, luciferase and renilla activity were measured in a Turner LD20/20 luminometer using a Dual reporter assay system (Promega).

SDS−PAGE and Western Blots. To study the effect of GA on PPARα turnover, the MuSH 3.4 cell line, which stably expresses PPARα/FLAG, was treated with either 500 nM GA or carrier solvent for 4 h. Cytosol was subjected to SDS−PAGE analysis, and protein was transferred to a PVDF membrane. As a positive control, the AhR, which is highly sensitive to GA, (*42*) was used. The presence of PPARα/FLAG, AhR, Hsp90, and p23 were detected with an anti-FLAG rabbit polyclonal antibody, mAb RPT 1, rabbit anti-hsp 84 polyclonal antibody (each obtained from Affinity BioReagents), and mAb JJ3 (obtained from Dr. David Toft, Mayo Clinic, Rochester, MN), respectively. The antibody binding to each antigen was detected with ^{125}I-secondary antibodies.

Statistical Analysis. Where indicated, Minitab, Release 13 (State College, PA) was used to evaluate the data for statistical significance using One-way ANOVA and either Dunnett's test or Fisher's multicomparison *t*-test with significance at $p < 0.05$.

RESULTS

PPARα Binds to the Middle Domain of Hsp90. Although the association between PPARα and Hsp90 has been established in mouse liver (*31*), whether PPARβ or γ are capable of this interaction has not been determined. To test whether all three PPAR subtypes can bind to Hsp90, the N-terminal, C-terminal, and middle domains of Hsp90 were individually fused to GST and incubated with in vitro translated full-length PPAR subtypes. Following the GST-pull down assay, the binding of PPAR to Hsp90 was detected by autoradiography. As shown in Figure 1, both in vitro translated rat and mouse PPARα bind to the middle domain of the Hsp90. As expected, the ER under identical conditions also interacted with this portion of Hsp90. Neither PPARβ nor PPARγ significantly associated with Hsp90 under these conditions. As compared to the ER-hsp90 interaction, the association between hsp90 and PPARα appears to be less avid, although formal studies have not been performed to examine the stoichiometry or affinity in this system.

Geldanamycin Enhances PPARα and PPARβ-Mediated Transcriptional Activity. The ansamycin antibiotic geldanamycin (GA) specifically interacts with Hsp90 and is often used to study the function of Hsp90 in steroid hormone

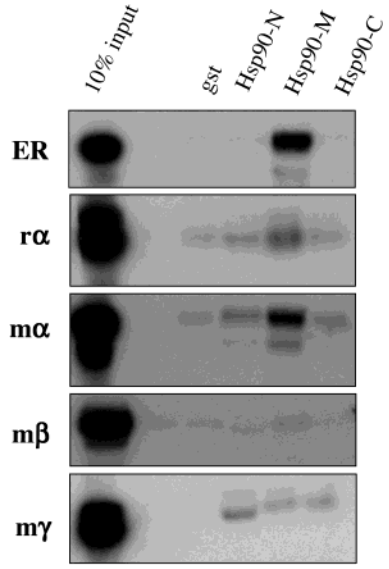

FIGURE 1: PPARα binds to the middle domain of Hsp90 with higher affinity than PPARβ and PPARγ. Hsp90-N/GST, Hsp90-M/GST, and Hsp90-C/GST were expressed in bacteria and immobilized on glutathione-agarose. Expression constructs containing full-length cDNAs for the estrogen receptor (ER), rat PPARα (rα), mouse PPARα (mα), mPPARβ (mβ), and mPPARγ (mγ) were in vitro translated in the presence of [^{35}S]methionine. The translated proteins were individually mixed with each immobilized Hsp90/GST fusion protein. After overnight incubation, each sample was washed and subjected to SDS−PAGE analysis. The resulting gel was dried, and the radiolabeled proteins were visualized by autoradiography.

receptor complexes (*43*). Acyl-CoA oxidase (ACO) mRNA induction has been demonstrated previously to be mediated by PPs in the rat hepatoma FaO cell line (*41*). FaO cells were treated for 60 min with GA (1000 ng/mL) followed by 50 μM Wy14643 for 6 h, after which total RNA was extracted, and ACO mRNA was examined via quantitative RT-PCR. Treatment of FaO cells with Wy14 643 resulted in an 8-fold increase in ACO mRNA over control cells (Figure 2A). When cells were pretreated with GA prior to Wy14643, the ACO message increased over 20-fold. GA alone had little effect on ACO mRNA levels relative to control levels. Similar results were seen in an FaO cell line that stably harbors a luciferase construct with the natural PPRE enhancer region of the ACO gene (Figure 2B). Under conditions described above, luciferase activity increased 7-fold with Wy14 643 and 9-fold with GA pretreatment followed by PPARα activation. These results suggest that the disruption of the hsp90 function enhances PPARα-mediated induction of an endogenous gene directly controlled by PPARα.

Transient transfection in HepG2 cells was used to determine subtype specificity of the GA response (Figure 3A). The ligands Wy14 643, L165041, and PGJ_2 are preferential ligands for PPARα, PPARβ, and PPARγ, respectively. All three subtypes showed enhanced ligand-induced luciferase activity in the presence of GA, although PPARα and β were more responsive than PPARγ. Following treatment of PPARα transfected cells with GA and Wy14 643, an increase of 2-fold in luciferase activity was seen relative to cells treated with Wy14 643 only; PPARβ transfected cells exposed to GA and L165041 had a 4.5-fold increase, while PPARγ transfected cells treated with PGJ_2 resulted in a 1.3-fold augmentation. However, the modest induction of PPARγ may reflect the endogenous PPAR subtype activity observed in the control transfections or an indirect effect of PGJ_2. The treatment of cells with GA results in a rapid decrease in Ah receptor (AhR) levels, as this receptor appears to require hsp90 to maintain its stability (*44*). We chose to test whether PPARα is rapidly degraded using the immortalized hepatocyte stable cell line MusH 3.4, which expresses significant levels of PPARα/FLAG and AhR. These cells were treated with 500 nM GA for 4 h, and cytosol was isolated. A quantitative protein blot analysis revealed only a 10% decrease in PPARα/FLAG levels, while the examination of an Ah receptor level revealed almost a complete loss of Ah receptor protein (Figure 3B). The levels of Hsp90 and p23 were also assessed to demonstrate that GA did not affect the levels of other proteins known to be present in receptor complexes.

PPARα and PPARβ-Mediated Transcriptional Activity Is Enhanced by TPR Domain of PP5. The tetratricopeptide repeat (TPR) is a degenerate 34 amino acid sequence identified in a number of cochaperone proteins that associate with Hsp90; overexpressing this domain causes disruption and possibly degradation of certain client proteins found in such complexes (*42*). To examine the functional involvement of Hsp90 in PPAR complexes, each PPAR isoform was cotransfected with increasing concentrations of the TPR domain of PP5 (TPR-PP5) in a COS-1 cell based reporter assay (Figure 4A). PPARα constitutive transcriptional activity was augmented with the transfection of increasing amounts of TPR-PP5 leading to greater than 6-fold increase in reporter activity at the maximum level of TPR construct utilized. The PPARβ or γ constitutive transcriptional activity was increased to a lesser extent under identical conditions. Overexpressing TPR-PP5 had a similar but more pronounced effect on ligand-induced PPRE reporter activity as previously observed with GA (compare Figure 3A with 4B). Cotransfecting TPR-PP5 with PPARα and treating with Wy14 643 increased the luciferase activity 6-fold as compared to cells transfected with PPARα alone. In PPARβ transfected cells treated with the L165041 and PPARγ expressing cells administered PGJ_2, increases of 3- and 2-fold in reporter activity in the presence of TPR was observed as compared with the expression of PPARβ or γ alone. These results suggest that within cells, hsp90 may actually interact with PPARβ, despite the fact that essentially no in vitro binding of PPARβ to hsp90 was observed in Figure 1. Mammalian two-hybrid assays were utilized to further assess this possibility.

We have previously reported the interaction of Hsp90 with PPARα in the mammalian two-hybrid assay and mapped the interaction to the hinge (D) and ligand binding domain (EF) of the NR (*31*). This work was extended to include PPARβ and γ and their sensitivity to TPR overexpression in COS-1 cells (Figure 5). The chimeric GAL4-PPARα construct (pM/PPARα) interacted with VP16/Hsp90 more avidly than pM/PPARβ (4-fold increase in GAL4-driven luciferase activity relative to 2-fold; Figure 5A). In contrast, pM/PPARγ cotransfected with VP16/Hsp90 actually decreased reporter activity as compared to VP16 alone. The interaction between PPARα and PPARβ with Hsp90 was significantly diminished by cotransfecting with the PP5-TPR plasmid. As expected, the D and EF domains of PPARα are responsible for the

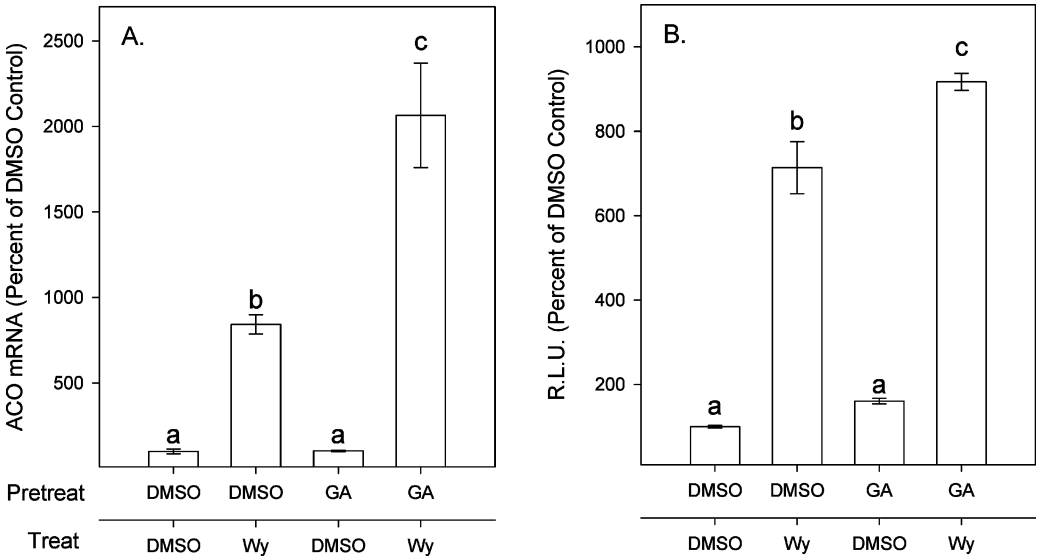

FIGURE 2: Geldanamycin enhances PPARα activity in the rat hepatoma cell line FaO. (A) FaO cells (seeded at 5×10^5 per well in 6-well plates) were pretreated with 1000 ng/μL GA (or 0.1% DMSO) for 60 min, followed by 50 μM Wy14 643 (or 0.1% DMSO) for 6 h. After treatment, cells were harvested as per supplied instructions. RNA was extracted as described in the Materials and Methods, and ACO mRNA levels were quantitated by RT-PCR. ACO levels are expressed as a percent of DMSO/DMSO treatment plus SEM ($n = 3$). Shown is one experiment that is representative of three independent experiments. (B) FaO cells that stably express pACO(-581/-471)Luc were seeded in 48-well plates (5×10^4 cells per well). Twenty-four hours after seeding, cells were pretreated with 1000 ng/μL GA for 60 min (or 0.1% DMSO), followed by 50 μM Wy14 643 (or 0.1% DMSO) for 24 h. Cells were harvested as per supplied instructions, and firefly luciferase was measured and standardized to protein content. Bars represent the mean percent induction over control ($n = 3$) plus standard error (SEM). Shown is one experiment that is representative of three independent experiments. Means with different letters (a, b, c) are significantly different from each other ($p < 0.05$, Fishers Multicomparison test, Minitab).

majority of the association with Hsp90, in particular when they are examined alone and without the C domain (Figure 5B). The TPR construct significantly decreased association between VP16/Hsp90 and the PPARα full-length (ABC-DEF), A/B, D, E/F, and ABCD constructs.

PPARα Transcriptional Activity Is Differentially Enhanced by CHIP. C-terminal Hsp-interacting protein (CHIP) is a E3 ubiquitin ligase that interacts with Hsp70 and Hsp90 via a TPR domain and alters Hsp90 chaperone complex stability (ref *45* and references within). For example, increased levels of CHIP within the cell leads to the ubiquitin-proteasome mediated degradation of the glucocorticoid receptor. Thus, we wanted to test what effect CHIP expression would have on PPAR transcriptional activity. Cotransfection with pcDNA3/CHIP resulted in a 4-fold increase in both the constitutive and the ligand-inducible PPARα transcriptional activity (Figure 6A). In the presence of cotransfected CHIP, while PPARα demonstrated a dramatic induction of transcriptional activity, a moderate induction of PPARγ transcriptional activity was observed. The transcriptional activity of PPARβ was repressed by CHIP (Figure 6B).

Effects of hsp90 Cochaperones FKBP52 and p23 on PPARα Activity. To further characterize the constituents of the PPARα/Hsp90 complex, the effects of the Hsp90 cochaperones FKBP52 and p23 were examined in transient transfection assays. Increasing amounts of FKBP52, an immunophilin that possess TPR motifs, were cotransfected with PPARα in a COS-1 cell-based reporter assay (Figure 7). In contrast to the TPR domain of PP5, FKBP52 did not affect PPARα activity, suggesting an inability to either sequester Hsp90 from the PPARα-Hsp90 complex or enter into PPARα complexes.

The hsp90 cochaperone p23 has been identified in a number of receptor-hsp90 complexes; whether p23 is also in PPARα complexes remains to be established. However, considering that hsp90 represses PPARα activity, we wanted to test if p23 could modulate this repression. Cotransfection of HepG2 cells with PPARα along with p23 constructs resulted in an increase in both constitutive and ligand inducible PPARα transcriptional activity (Figure 8). This p23-dependent increase was 6-fold in the absence of ligand and 5-fold when cells were exposed to Wy14 643, indicating that p23 can modulate PPARα activity, perhaps through influencing hsp90 activity.

DISCUSSION

The cytosolic chaperone protein Hsp90 has long been known to be essential for the function of certain nuclear receptors. In the absence of a ligand, newly synthesized NRs, such as GR and PR (*32*), as well as the Ah receptor (*46*) exist in complexes that contain a dimer of Hsp90. Hsp90 acts as part of a multiprotein complex that includes Hsp70 and other cochaperone proteins dependent on the status of the complex (e.g., initial synthesis and folding of the client protein). It has been implied that the predominant function of the Hsp90 complex in NR activation is in the cytosol prior to ligand binding (*47*). Recently, we have observed that the NR PPARα exists in mouse liver cytosol in association with Hsp90 as well as the cochaperone XAP2 (*31*). However, whether hsp90 influences the activity of PPARα was not determined. Although PPARα has previously been demonstrated to interact with Hsp70 (*30*), the association of PPARα with Hsp90 was unexpected since other closely related receptors such as TR (*35*), VDR (*48*), and RAR (*36*) apparently do not form stable complexes with this chaperone. While cochaperones associate with the N- and C-terminal domains of Hsp90, the middle domain binds to the client proteins such as NRs (*49*) and as described herein, PPARα.

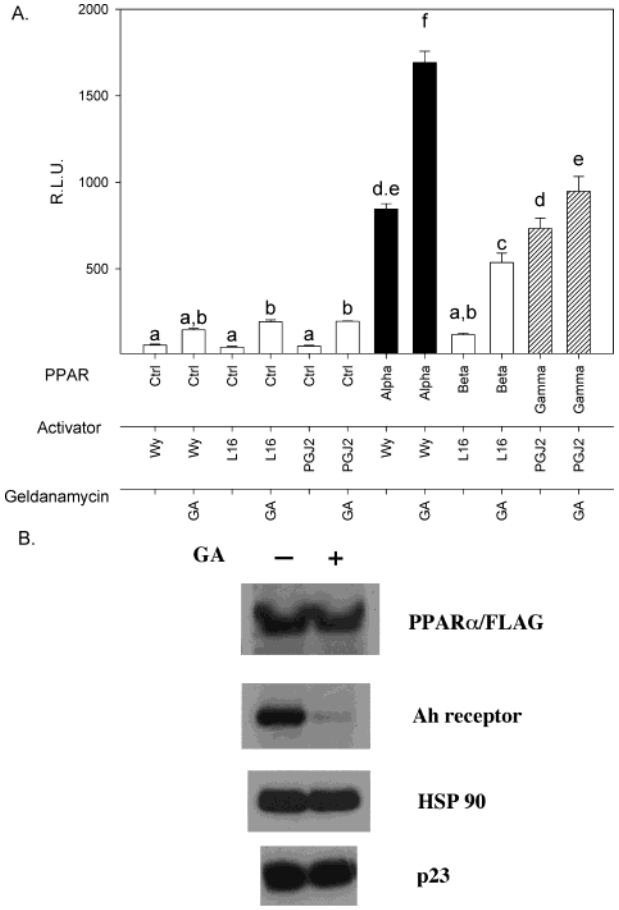

FIGURE 3: Differential activation of PPAR transcriptional activity by Geldanamycin. (A) HepG2 cells were cotransfected with either pBK/CMV or each subtype of pBK/CMV/PPAR/FLAG and pACO-(x2)-luciferase reporter. Cells were pretreated with 1 μM GA for 6 h and treated with their 50 μM Wy14 643 (Wy), 2.5 μM L165041 (L16), or 10 μM PGJ2 (PGJ2) for 24 h, and PPRE-driven reporter activity was measured. Data were corrected for the internal transfection control β galactosidase (R. L. U., relative luciferase units) ($n = 3$, representative experiment). Means with different letters (a−f) are significantly different from each other ($p < 0.05$, Fishers Multicomparison test, Minitab). (B) The mouse immortalized hepatocyte cells line, MuSH3.4, which stably harbors a PPARα/Flag construct, were treated with 500 nM GA for 4 h. Protein was isolated, equal amounts were loaded on SDS−PAGE, and Western blot analysis was performed using a Flag antibody, as described in Materials and Methods.

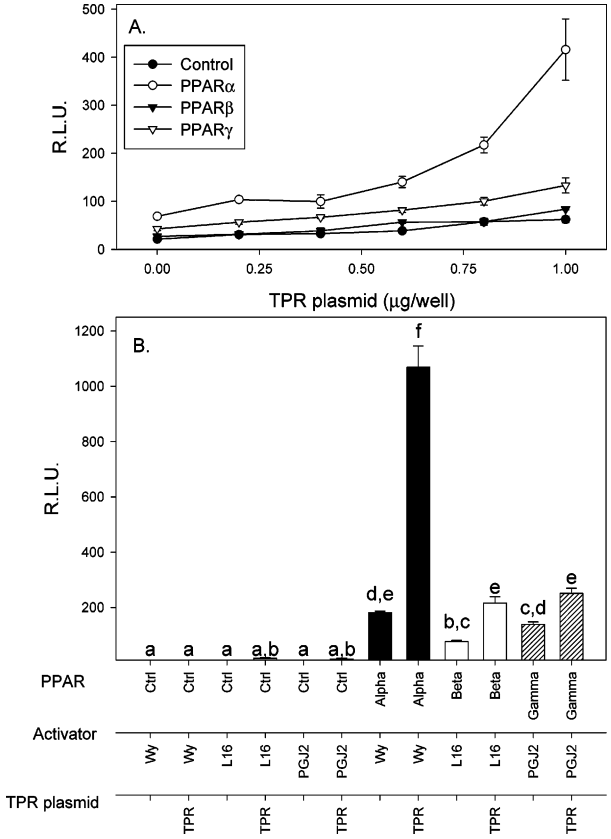

FIGURE 4: Differential activation of PPAR transcriptional activity by the TPR domain of PP5. (A) COS-1 cells were cotransfected with pACO(x2)-luciferase reporter, an individual PPAR subtype (either pBK/CMV (Ctrl) or pBK/CMV/PPARα, β, or γ/FLAG), and increasing concentrations of PP5-TPR. Twenty-four hours after the start of transfection, cells were lysed, and the PPRE-driven reporter activity was measured. Data were corrected for protein yield using the BCA assay. (B) HepG2 cells were cotransfected with pACO(x2)-luciferase reporter, either pBK/CMV (Ctrl) or each isoform of pBK/CMV/PPAR/FLAG, and 0.7 μg of PP5-TPR. Sixteen hours after the start of transfection, transfected cells were treated with either 50 μM Wy14 643 (Wy), 2.5 μM L165041 (L16), or 10 μM PGJ2 (PGJ2) for 24 h, and the PPRE-driven reporter activity was measured. Data were corrected for the internal transfection control β galactosidase ($n = 3$, representative experiment). Means with different letters (a−f) are significantly different from each other ($p < 0.05$, Fishers Multicomparison test, Minitab).

The interaction between PPARα and Hsp90 occurred predominantly in the carboxy terminus of the NR, reminiscent of ER (*50*) and GR (*51*). Neither PPARβ nor PPARγ formed stable complexes with Hsp90 in vitro. However, in the mammalian two-hybrid assays, both PPARα and PPARβ were able to interact with Hsp90, and PPARγ failed to interact with hsp90 in this assay system.

The functional consequence of this novel interaction between PPARα or β and Hsp90 was examined in more detail using three means to disrupt the chaperone complex: GA treatment, cotransfection with a PP5-TPR-containing construct, or overexpresssing the Hsp90-associating protein CHIP. Geldanamycin is an extensively utilized benzoquinone ansamycin drug that specifically binds to Hsp90 and disrupts its function. Treatment with GA hinders steroid-dependent GR translocation from cytoplasm to nucleus by blocking the receptor assembly in an intermediate state complexed with Hsp70 (*52*). As a result, treatment with GA inhibits glucocorticoid driven gene induction in several cell lines (*43*). GA also decreases the transcriptional activity of PR (*43*), ER (*53*), AR (*54*), and AhR (*44*). Hsp90-associated kinases such as v-src and p210bcr-abl are also inhibited by GA as a result of enhanced degradation through the ubiquitin−proteasome pathway (*55*). In contrast to these proteins, PPARα transcriptional activity was significantly induced by GA treatment. This was seen in vivo with a PPARα target gene ACO as well as in a stably transfected reporter gene and transient transfection systems. Consistent with the in vitro binding and mammalian two-hybrid assay results, disruption of Hsp90 with GA had a lesser effect on PPARγ. GA did increase PPARβ's ability to regulate a PPRE reporter, which supports the results obtained in the mammalian two-hybrid assay. Interestingly, the failure to observe an association between the hsp90 and PPARβ in the GST pulldown assay system may indicate that within the cell some other factor or modification (e.g., phosphorylation) is required to maintain this interaction. In contrast to other NRs and AhR, PPARα

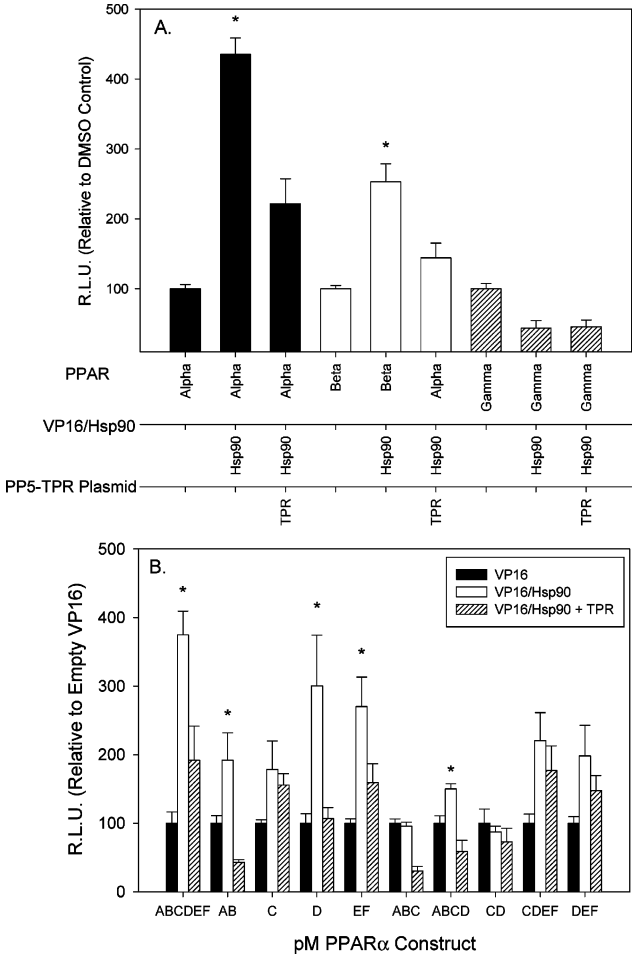

FIGURE 5: Interaction between Hsp90 and PPARα, β, or γ in a mammalian two-hybrid assay. (A) COS-1 cells were transfected with pM/GAL4/PPARα, β, or γ and pVP16/Hsp90, pRL, and PP5-TPR or appropriate control vectors to keep total transfected DNA constant. Twenty-four hours after transfection, cells were lysed and assayed for luciferase. Activity was corrected for transfection efficiency as described under Materials and Methods. All bars were standardized to the VP16 control plasmid. This graph is representative of two independent experiments ($n = 3$). (B) Determination of the PPARα domain that specifically interacts with Hsp90. COS-1 cells were cotransfected with plasmids containing PPARα domains fused to the GAL4 DNA binding domain, along with the PP5-TPR domain and pVP16/Hsp90. The domains included PPARα full length (ABCDEF), the A/B domain (AB), DNA binding domain (C), hinge region (D), ligand binding domain (EF), and various combinations of domains (A−C, A−D, C−D, C−F, and D−F). Cells were lysed and assayed for luciferase activity. Activity was corrected for transfection efficiency and expressed relative to values obtained with the expression of VP16. This graph is representative of two independent experiments ($n = 3$). Asterisks depict values that are significantly different than the VP16/Empty and PP5-TPR transfected cells ($p < 0.05$, Dunnett's t-test, Minitab).

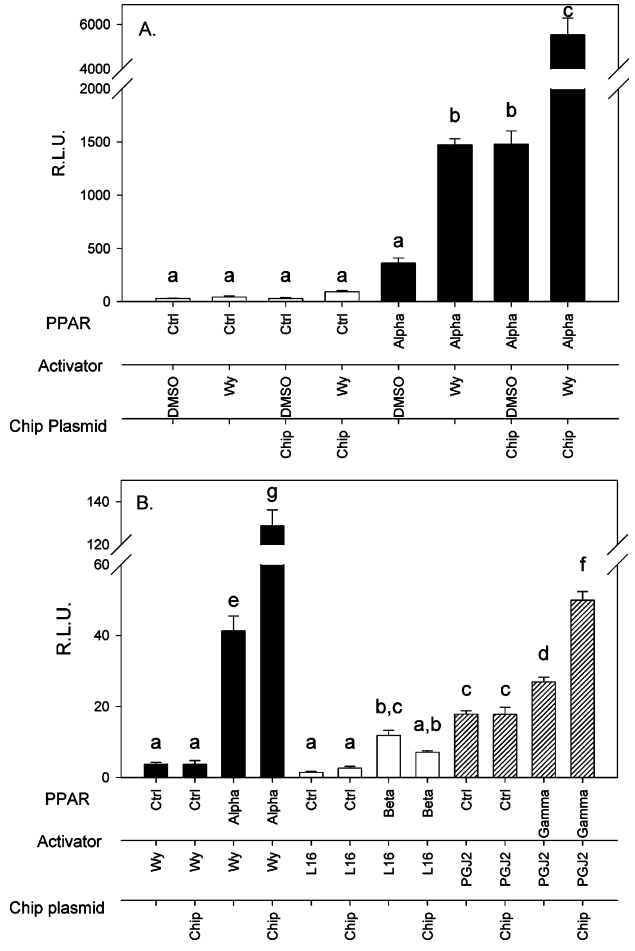

FIGURE 6: Differential activation of PPAR transcriptional activity by CHIP. (A) HepG2 cells were cotransfected with pACO(x2)-luciferase reporter, either pBK/CMV or pBK/CMV/PPARα/FLAG, and 0.7 μg of CHIP. The subset of cells was treated with Wy14 643 for 8 h. PPRE-driven reporter activity was measured, and the fold induction of PPARα transcriptional activity by CHIP was calculated. (B) HepG2 cells were cotransfected with either pBK/CMV or each subtype of pBK/CMV/PPAR/FLAG and pACO(x2)-luciferase reporter. The subset of cells was cotransfected with pCDNA3/CHIP. Cells were treated with their respective ligands for 24 h as described in the legend to Figure 4. Data were corrected for the internal transfection control β galactosidase ($n = 3$, representative experiment). Means with different letters (a−c) are significantly different from each other ($p < 0.05$, Fishers Multi-comparison test, Minitab).

levels were largely refractory to GA treatment (Figure 3B), which suggests that the observed transcriptional induction is not due to an alteration in receptor levels.

Many Hsp90 cochaperones are characterized by the presence of tetratricopeptide repeat (TPR) domains, which consist of 34-amino acid motifs that are known to be important for protein−protein interactions (*56*). The association of the TPR domain of serine phosphatase 5 and Hsp90 was first established in a co-immunoprecipitation experiment using GR from mouse L cell lysates (*38*). Both the GR (*37*) and the AhR (*42*) transcriptional activity is decreased by the TPR domain of PP5 cotransfection, presumably by masking the cochaperone site on the carboxy terminus of Hsp90 leading to disruption of Hsp90's ability to participate in the assembly of receptor complexes. This in turn results in an increased turnover of chaperoned receptors. In contrast, the transcriptional activity of PPARα was upregulated by the cotransfection of the TPR domain of PP5 in a dose-dependent manner, once again suggesting a repressive function of Hsp90 in the PPARα-Hsp90 complex. Similar to GA, TPR overexpression had a more pronounced effect on PPARα and β relative to PPARγ. In addition, we have shown that the increased activity resulting from TPR overexpression parallels a decreased association of PPARα or β and Hsp90 in the mammalian two-hybrid assays.

Finally, the effects of Hsp90 on PPARα activity were examined by cotransfecting with the TPR-containing chaperone cofactor, carboxyl terminus of Hsc70 interacting

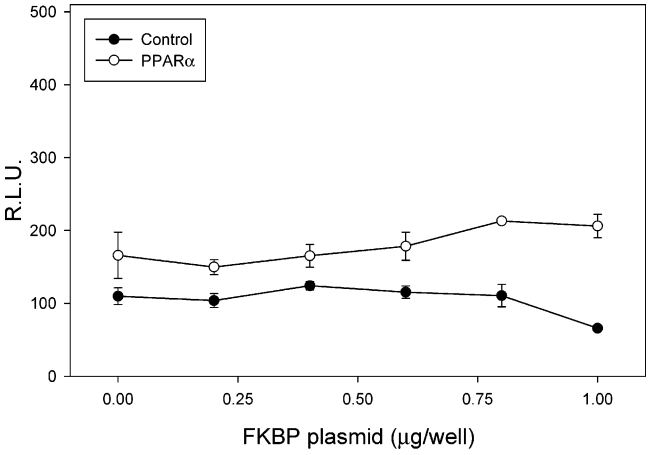

FIGURE 7: PPARα activity is not affected by FKBP52 expression. COS-1 cells were cotransfected with pACO(x2)-luciferase reporter, either pBK/CMV or pBK/CMV/PPARα/FLAG, and increasing concentrations of pCI/FKBP52. Twenty-four hours after the start of transfection, the PPRE-driven reporter activity was detected. Data were corrected for the protein yield using BCA assay ($n = 3$).

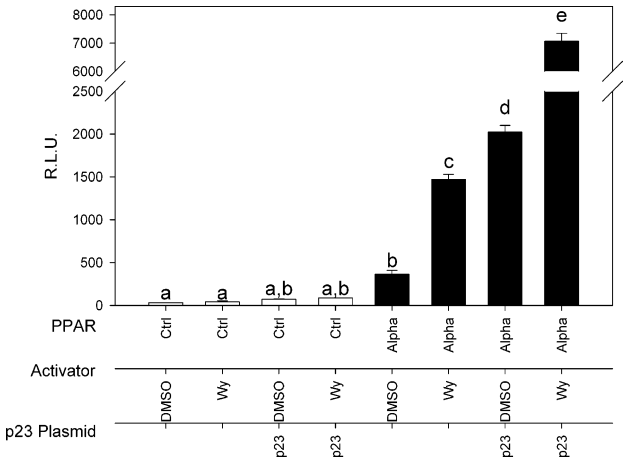

FIGURE 8: Activation of PPARα transcriptional activity by p23. HepG2 cells were cotransfected with pACO(x2)-luciferase reporter, either pBK/CMV or pBK/CMV/PPARα/FLAG, and 0.7 μg of p6R/p23. The subset of cells was treated with Wy14 643 for 8 h, and the PPRE-driven reporter activity was measured. Data were corrected for the internal transfection control β galactosidase. Means with different letters are significantly different from each other ($p < 0.05$, Fishers Multicomparison test, Minitab).

protein (CHIP). CHIP directly interacts with Hsp90 and aids in the destruction of misfolded proteins via the ubiquitin/proteasome degradation pathway (*57*). This protein has E3 ubiquitin ligase activity (*58*) and ablates the hormone binding activity and transactivation potential of GR, perhaps by binding to Hsp90 and targeting the receptor for degradation by a ubiquitin proteosome pathway (*59*). However, recent evidence from our laboratory suggests that CHIP's predominant function may be to alter Hsp90 function,[2] thereby affecting the turnover of its client proteins. In contrast to GR, the cotransfection of CHIP with PPARα resulted in an enhanced PPRE driven reporter activity. The other isoforms of PPAR are dramatically influenced by CHIP expression, once again suggestive of a different relationship between Hsp90 and PPARβ or PPARγ. Although, it is possible that the disruption of Hsp90 complex also leads to effects on other pathways (e.g., protein kinases) that indirectly affect PPAR activity.

Steroid receptors are in a complex containing Hsp90, p23, an immunophilin, and often Hsp70. The immunophilin FKBP52, a FK506- or cyclosporin A-binding protein, binds to Hsp90 via its TPR domain, and different receptor heterocomplexes exist based on which immunophilin (e.g., FKBP52, XAP2) occupies the TPR-binding region of Hsp90 (*60*). The cochaperone p23 stabilizes steroid hormone receptor complexes via binding to Hsp90 when Hsp90 is in the ATP bound state, and the p23-Hsp90 complex dissipates upon the hydrolysis of ATP (*61*). While TPR proteins bind near the C-terminus of Hsp90, the p23 binds to the N-terminal ATP-binding domain of Hsp90 (*38*). In receptor heterocomplexes, Hsp90 functions by altering the ligand potency (ligand binding affinity), while p23 functions by changing the ligand efficacy (ligand-induced gene transcription). The ER, MR, and TR transcriptional activity was repressed by the cotransfection of p23, whereas GR activity was augmented (*62*). In our study, PPARα transcriptional activity was enhanced severalfold by the cotransfection with human p23, suggesting that the PPARα-Hsp90-p23 complex behaves similarly to GR-Hsp90-p23. However, it remains to be established that p23 is actually in the PPARα complex.

The effects of Hsp90 disruption on a NR in the same family as PPARα (NR1) has been examined previously, although to our knowledge this is the first time that a direct association has been described for a mammalian NR1 member. In yeast engineered to express decreased levels of the Hsp90 homologue along with RARβ (NR1B2) and a retinoic acid-responsive reporter molecule, ligand binding and reporter expression were compromised (*63*). However, the mechanism through which Hsp90 alters RAR activity is unknown since these two proteins were not found in the same multimeric complex. The ecdysone receptor (ECR, NR1H1), a drosophilia NR, required Hsp90 and Hsp70 complexes for full activity and GA inhibited ECR transcriptional activity (*64*). As mentioned previously, TR (*35*), VDR (*48*), and RAR (*36*) do not form stable complexes with Hsp90. However, at least in the case of VDR, GA was examined and was shown to decrease receptor activity (*65*), possibly due to GA's ability to disrupt kinase signaling. In unpublished results cited in a recent article, the constitutive androstane receptor (CAR, NR1I3) was found in an Hsp90-containing complex, although no further details were given. Thus, the interaction between PPARα and Hsp90 is unexpected for an NR1 family member. Although GA does indeed affect the activity of several similar proteins, the enhancement of PPARα activity with GA has not been described elsewhere.

In summary, the data support a novel inhibitory role for Hsp90 in the PPARα complex. The hormone-induced activity of GR is inhibited by GA, PP5-TPR, and CHIP due to inappropriate protein folding, decreased ligand binding affinity, and enhanced receptor degradation (*60*). In contrast, Hsp90/PPARα complexes are not required for maintaining the receptor's stability or ligand binding potential. The AhR forms stable complexes with Hsp90 and XAP2 (*66*). Its ability to regulate gene expression is also inhibited by GA (*44*), PP5-TPR (*42*) and CHIP.[2] This receptor is exquisitely sensitive to GA as it rapidly degrades once dissociated from Hsp90 (*44*). PPARα also is found complexed with Hsp90 and XAP2

[2] Manuscript submitted.

(*31*). Unlike both GR and AhR, PPARα activity is increased by disrupting Hsp90, regardless of the means utilized. The treatment with GA had little effect on the intracellular levels of PPARα. Similar to GR, the cochaperone p23 increases the activity of PPARα (*25*). These data underscore the complexity of Hsp90 complexes, with multiple means of regulating the activity of its client proteins. Most importantly, interaction between Hsp90 and PPARα or PPARβ and its functional consequence is specific for these isoforms, and PPARγ activities are not regulated in this manner. It is quite possible that Hsp90 may serve to inhibit inappropriate activation of PPARα or β by low levels of fatty acids in tissues such as liver. Finally, heat shock factor 1 (HSF1) is repressed by association with Hsp90 and is activated by GA treatment (*67*). Hsp90 appears not to be required by the stabilization of HSF1 but functions as a suppressor protein until heat shock causes Hsp90 to dissociate and associate with misfolded proteins (*68*, *69*). Thus, in many ways PPARα and HSF1 appear to have a similar relationship with Hsp90.

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