Phosphorylation Analysis of 90 kDa Heat Shock Protein within the Cytosolic Arylhydrocarbon Receptor Complex†

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ABSTRACT: The arylhydrocarbon receptor (AhR) functions as a ligand-activated transcription factor that regulates the transcription of genes encoding xenobiotic metabolizing enzymes and also mediates most of the toxic effects caused by dioxins and polycyclic aromatic hydrocarbons. The cytosolic AhR complex exists as a transcriptionally cryptic complex, consisting of the 90 kDa heat shock protein (HSP90) and the hepatitis B virus X-associated protein 2 (XAP2). The posttranslational modifications, especially phosphorylation, of the cytosolic AhR−HSP90−XAP2 complex are poorly understood, although the phosphorylation of a transcriptionally active heterodimer of AhR and an AhR nuclear translocator is critically involved in AhR function. To reveal the phosphorylation status involved in AhR function, we used mass spectrometry to determine the site-specific phosphorylation of the steady-state cytosolic AhR complex, prepared from Chinese hamster ovary cells stably expressing mouse AhR. We identified phosphorylations of the HSP90 subunits within the AhR complex at Ser225 and Ser254 of HSP90β and Ser230 of HSP90α. By site-directed mutagenesis, these serine residues were substituted with alanine and glutamic acid to elucidate the role of the HSP90β serine phosphorylations in the AhR function. Immunoprecipitation assays using COS7 transfectants showed that the replacement of Ser225 and Ser254 by Ala, S225/254A, increased the binding affinity for AhR, as compared with the Glu replacement. In a ligand-induced AhR transcription activity assay using Hepa1 transfectants, the S255/254A mutant exhibited more potent transcription activity than the S225/254E mutant, which had activity similar to that of wild-type HSP90β. These results suggest that the phosphorylations in the charged linker region of the HSP90 molecule modulate the formation of the functional cytosolic AhR complex.

The arylhydrocarbon receptor (AhR)[1] is a ligand-activated transcription factor that is a member of the basic helix−loop−helix Per/ARNT/Sim (bHLH-PAS) family of proteins (*1*−*4*). The AhR, which is also known as the dioxin receptor, mediates the toxic effects of environmental pollutants. Exposure of laboratory animals to the most potent dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), results in immune system alterations, liver damage, and teratogenicity, as well as carcinogenesis (*5*−*10*).

Studies of AhR, especially the induction of xenobiotic metabolizing enzymes, revealed an induction mechanism of the cytochrome P450 1A1 (*CYP1A1*) gene by polycyclic

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[1] Abbreviations: ESI, electrospray ionization; MS, mass spectrometry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AhR, arylhydrocarbon receptor; ARNT, arylhydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; XAP2, hepatitis B virus X-associated protein; AIP, AhR-interacting protein; ARA9, AhR-activated 9; bHLH, basic helix−loop−helix; PAS, Per-ARNT-Sim homology; HSP90, 90 kDa heat shock protein; 3MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP, cytochrome P450; PBS, phosphate-buffered saline; DTT, dithiothreitol; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; αMEM, minimum essential medium alpha; IP, immunoprecipitation; IB, immunoblotting; ECL, enhanced chemiluminescence; PCR, polymerase chain reaction; LC, liquid chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; Hepa1, mouse hepatoma cell line 1; HA, hemagglutinin; IMAC, immobilized metal affinity chromatography; Pro-QD, Pro-Q Diamond phosphoprotein gel stain; CBB, Coomassie Brilliant Blue; ALP, alkaline phosphatase.

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aromatic carbons. In the cytosol, the unliganded AhR is present in a multiprotein complex containing one molecule of AhR, two molecules of 90 kDa heat shock protein (HSP90) (*11*−*14*), and an immunophilin-like protein, named hepatitis B virus X-associated protein (XAP2, also called AIP or ARA9) (*15*−*17*). Upon binding a ligand, such as dioxins and other polycyclic aromatic hydrocarbons, the AhR complex translocates into the nucleus, where the AhR dissociates from the HSP90 complex to form a heterodimer with another specific bHLH-PAS protein, the AhR nuclear translocator (ARNT). This heterodimer binds the xenobiotic responsive elements (XRE) in the promoters of the target genes, leading to their enhanced expression.

HSP90 is an abundant molecule representing up to 2% of the total cytosolic protein (*18*, *19*). Its role as a molecular chaperone, to prevent protein aggregation and to promote refolding of denatured proteins, has been studied in much detail (*20*, *21*). HSP90 also plays a key role in the conformational maturation of oncogenic signaling proteins, including HER-2/ErbB2, Akt, and Raf-1 (*22*−*25*). Moreover, nuclear hormone receptors, such as the glucocorticoid and progesterone receptors, are well-characterized substrates of the HSP90-mediated chaperoning process (*26*). In addition to steroid hormone receptors, HSP90 also regulates AhR function by retaining AhR as a complex in the cytoplasm. This prevents AhR from forming a transcriptionally active heterodimer with its nuclear partner ARNT, indicating that HSP90 may be important for the repression of AhR functions (*27*−*29*). Studies on AhR-transfected yeast cells with defective HSP90 expression revealed the absolute requirement of HSP90 for the formation of a fully functional AhR complex (*13*, *30*). Consequently, it was postulated that HSP90 has several distinct functional roles in the regulation of AhR activity, such as the maintainance of AhR in its correctly folded form for high-affinity ligand binding activity and the repression of AhR nuclear accumulation, dimerization with ARNT, and DNA binding activity.

The HSP90 family consists of highly conserved members, such as HSP90α and HSP90*β* in human, HSP84 and HSP86 in mouse, HSP83 in fly, HSC83 and HSP82 in yeast, and other members (*31*−*33*). HSP90 usually exists as a constitutive dimer, with its main intersubunit contacts within 12 kDa C-terminal domains. Previous experiments suggested that HSP90 existed as an α/*β* heterodimer as well as a homodimer and that neither HSP90α nor HSP90*β* had any preferential affinity for AhR (*14*, *34*). A highly conserved 25kDa N-terminal domain is the competitive binding site for ATP and geldanamycin, which is a specific inhibitor of HSP90. A conserved, though structurally flexible, 35 kDa middle domain is the binding site for the client proteins (*23*, *35*). A divergent charged sequence, the "charged linker region", separates the N-terminal domain from the other part of the molecule (*36*, *37*). The HSP90 chaperone activity requires its ATPase activity, which is coupled to a conformational cycle, involving the opening and closing of a dimeric "molecular clamp" via the transient association of the N-terminal domains in the dimer (*38*, *39*). HSP90 in tumor cells exists in a functionally distinct molecular form, whereas HSP90 from normal tissue is in an uncomplexed state (*40*). HSP90−protein complexes can be stabilized by molybdate and by other transition metal oxyanions, such as vanadate (*41*−*45*). Several studies have shown the phosphorylation of HSP90 (*46*−*48*). Especially, in exponentially growing HeLa cells, HSP90 is an abundant, cytosolic phosphoprotein (*49*, *50*). Taken together, the eukaryotic HSP90 molecule probably exists in various states, reflecting its functional diversity.

Protein phosphorylation is one of the most common cellular regulatory mechanisms. The accurate determination of phosphorylation sites is essential to fully understand phosphoprotein function and regulation in cellular signaling events. Although there are considerable data indicating that phosphorylations at both serine/threonine and tyrosine residues are necessary for the normal activity of the nuclear AhR−ARNT complex (*51*−*59*), little is known about the steady-state phosphorylation status of the cytosolic AhR complex (*60*). In the present study, we have specifically addressed the question of phosphorylation in the cytosolic AhR complex and its possible functional role in the AhR function. Our data suggest that the HSP90 phosphorylations within the cytosolic AhR complex modulate the affinity of HSP90 for AhR and may negatively regulate the ligand-induced transcription activity of AhR.

MATERIALS AND METHODS

Transient and Stable Transfection of Mouse AhR. Chinese hamster ovary (CHO) cells were cultured in minimum essential medium alpha (αMEM) supplemented with 10% calf serum. The expression plasmid pcDNA-mAhR was constructed as follows. A forward oligonucleotide primer was used in a standard PCR protocol with a reverse oligonucleotide primer that encodes a factor Xa cleavage site, a FLAG epitope, and a stop codon. The plasmid pBSKmAhR, encoding the full-length mouse AhR, was used as the template. The amplified DNA was cloned into pcDNA3.1/Zeo(+) (Invitrogen) to create the plasmid pcDNA-mAhR. The recombinant plasmid thus constructed was confirmed by DNA sequence analysis. CHO cells were transfected with the plasmid by the FuGENE6 (Roche) method, according to the manufacturer's recommendations. Stable transfectants were selected with zeocin, and then AhR expression was confirmed with an anti-FLAG M2 monoclonal antibody (Sigma).

Isolation of Chinese Hamster HSP90α and HSP90β cDNAs and Construction of the Human HSP90β Expression Plasmid. The cDNAs encoding Chinese hamster HSP90α and HSP90*β* were isolated by PCR from a Chinese hamster cDNA library prepared from CHO cells with an "RNeasy kit" (Qiagen) and "ReverTra Plus" (Toyobo). Each set of two primers (5′-ATAAGAATGCGGCCGCTAAGCCAAGATGCCTGAGGAAACCCAGACC-3′ and 5′-GCTCTAGAGCTTAGTCTACTTCTTCCATGCGTGATG-3′ for HSP90α; 5′-ATAAGAATGCGGCCGCTAATCAAGATGCCTGAGGAAGTGCACC-3′ and 5′-GCTCTAGAGCCTAATCGACTTCTTCCATGCGAGACGCATCCTCATCGC-3′ for HSP90*β*) was designed on the basis of the human HSP90α and HSP90*β* sequences to add *Not*I and *Xba*I recognition sequences at the 5′- and 3′-ends, respectively. The amplified Chinese hamster HSP90α and HSP90*β* cDNAs were cloned to analyze the DNA sequences. The expression plasmid, pcDNA-hHSP90*β*, was constructed as follows. A forward oligonucleotide primer was used in a standard PCR protocol with a forward oligonucleotide primer

that encodes an HA epitope. The plasmid pKN1-3 (kindly provided by Yasufumi Minami, Oita Medical University, Oita, Japan), encoding the full-length human HSP90β, was used as the template. The amplified DNA was cloned into pcDNA3.1/Hygro(+) (Invitrogen) to create the plasmid pcDNA-hHSP90β.

Purification of the Cytosolic AhR Complex. Stable transfectants producing recombinant mouse AhR were maintained using αMEM supplemented with 10% calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. For large-scale suspension cultures, the transfectants were grown in a 6 L bottle by a Cell Master system (Wakenyaku). The harvested cells were washed with phosphate-buffered saline (PBS) and stored in a 10% glycerol solution at −80 °C. All further steps were performed at 4 °C or on ice. After the addition of an equal volume of 40 mM Tris buffer (pH 7.5), 0.5 M sucrose, 1 mM EDTA, 4 mM $MgCl_2$, 40 mM sodium molybdate ($NaMoO_4$), 2 mM dithiothreitol (DTT), protease inhibitor cocktail (cocktails for mammalian cell and tissue extracts, Nacalai Tesque), and phosphatase inhibitors (40 mM β-glycerophosphate, 20 mM sodium fluoride, and 2 mM sodium orthovanadate), the cells were homogenized with 15 strokes in a Potter homogenizer. Nuclear pellets were removed by centrifugation of the cell lysates at 1000*g* for 15 min. Following the addition of 0.05% Triton X-100, the supernatant was further centrifuged at 3000*g* for 10 min to remove the residual cell debris. The cytosolic fraction was loaded onto a DEAE-Sephacel (Amersham Pharmacia) column equilibrated with 20 mM Tris buffer (pH 7.5), 20 mM sodium molybdate, 1 mM EDTA, and 0.05% Triton X-100 (buffer A). The column was washed with 50 mM NaCl in buffer A, and then the protein was eluted with 400 mM NaCl in buffer A. The eluent was passed three times over an anti-FLAG M2 affinity gel (Sigma) column equilibrated with 20 mM Tris (pH 7.5), 20 mM sodium molybdate, 1 mM EDTA, 400 mM NaCl, and 0.05% Triton X-100 (buffer B). Bound proteins were recovered by an elution with 100 μg/mL FLAG peptide in buffer B. Following the addition of 1 mM DTT, the eluent was loaded onto a HitrapDEAE (Amersham Pharmacia) column equilibrated with 20 mM Tris buffer (pH 7.5), 20 mM sodium molybdate, 1 mM DTT, and 0.1% CHAPS (buffer C). The column was washed with buffer C, and then the bound proteins were eluted with 400 mM NaCl in buffer C (buffer D). The eluent was concentrated with a Centricon-50 (Millipore) membrane and then applied to a Superdex 200 column equilibrated with buffer D. The peak containing the AhR complex was separated from the void peak containing the aggregated AhR and then was concentrated with a Centricon-50 membrane. The purified AhR complex was stored at −80 °C.

SDS−PAGE, Immunoblotting, and N-Terminal Amino Acid Sequence Analysis. The protein content was determined with a protein assay kit (Bio-Rad) using γ-globulin as a standard. SDS−PAGE and immunoblot analyses were performed according to the standard method. The samples were analyzed by the standard SDS−PAGE method, using 10% polyacrylamide gels. The gel bands were visualized with Coomassie Brilliant Blue (CBB) staining or zinc staining using a negative gel stain MS kit (Wako). To examine the relative ratio of the two HSP90 forms, α and β, SDS−PAGE under a maximum resolution condition was used with the CBB stain (*61*). For immunoblot analyses, proteins were transferred from the SDS−PAGE gel to a PVDF membrane. An anti-FLAG M2 monoclonal antibody (Sigma), an anti-HA monoclonal antibody (Roche), an anti-HSP90 polyclonal antibody (H-114, SantaCruz), an anti-HSP90α polyclonal antibody (HSP86 Ab-1, Neo Markers), and an anti-HSP90β polyclonal antibody (HSP84 Ab-1, Neo Markers) were used as primary antibodies. Primary antibody-bound protein bands were detected with HRP-conjugated secondary antibodies by the ECLplus system (Amersham Pharmacia). For N-terminal amino acid sequence analyses, the protein band of interest on the PVDF membrane, stained by amido black, was excised and analyzed with an Applied Biosystems protein sequencer equipped with an on-line 120A PTH analyzer.

Site-Directed Mutagenesis, Immunoprecipitation, and Reporter Gene Assays. The site-directed mutagenesis was performed with the QuickChange II XL site-directed mutagenesis kit (Stratagene), according to the manufacturer's recommendations. The mutation in the HSP90β sequence was confirmed by DNA sequence analysis. COS7 and Hepa1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. For the immunoprecipitation assays, the cells were cultured in a six-well plate and cotransfected with 0.5 μg of the HSP90β expression vector (wild type or mutant) and 0.5 μg of the AhR expression vector (wild type) by the FuGENE6 method, according to the manufacturer's recommendations. After a 36 h transfection period, the cells were washed with PBS and scraped in lysis buffer, consisting of 10 mM Tris buffer (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 20 mM sodium molybdate, 1 mM EDTA, 0.6% Triton X-100, 1 mM DTT, protease inhibitors (cocktails for mammalian cell and tissue extracts, Nacalai Tesque), and phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride, and 1 mM sodium orthovanadate), followed by freezing and thawing. The cell suspension was then incubated at room temperature for 10 min, followed by freezing and thawing again. The resultant lysate was centrifuged at 1000*g* for 5 min, and the supernatant was collected. After a 5-fold dilution with IP buffer, consisting of 10 mM Tris buffer (pH7.5), 10 mM NaCl, 3 mM $MgCl_2$, 20 mM sodium molybdate, 1 mM EDTA, 0.05% Triton X-100, protease inhibitors, and phosphatase inhibitors, the solution was recentrifuged at 1000*g* for 5 min to remove the insoluble materials. The collected supernatant was incubated with 30 μL of anti-FLAG or HA agarose (50% slurry, Sigma) for 2 h with gentle agitation. The resin was then washed with the IP buffer three times, and the bound proteins were separated by SDS−PAGE and analyzed by immunoblotting. For the reporter gene assay using the Hepa1 cells or stably transfected CHO cells expressing AhR, the cells were cultured in a six-well plate and cotransfected with 0.5 μg of the HSP90 expression plasmid (wild type or mutant) and 0.5 μg of the pXRE4tRLuc reporter plasmid, containing the luciferase gene under the control of four tandem copies of the XRE element, by the FuGENE6 method, according to the manufacturer's recommendations. For the reporter gene assay using the CHO cells that did not express AhR, the cells were cotransfected with 0.5 μg of the HSP90 expression plasmid, 0.5 μg of the AhR expression plasmid, and 0.5 μg of the pXRE4tRLuc reporter plasmid. In the experiments using the Hepa1 cells intrinsically expressing AhR or the CHO cells stably expressing AhR, the AhR expression plasmid was omitted. After an 18

h transfection period, the cells were treated with 1 μM 3-methylcholanthrene (3MC) or with an equivalent volume of vehicle (0.01% DMSO). Following an incubation for 18 h, the cells were washed with ice-cold PBS and collected for the luciferase assay using the Promega luciferase assay system with reporter lysis buffer. Experiments were carried out in triplicate, and the luciferase activities were normalized for the total protein contents. To examine the expression levels of the different HSP90 forms, equal amounts of cell lysates, without the 3MC stimulation, were subjected to an immunoblotting analysis with anti-HA antibodies.

Phosphoprotein and Phosphopeptide Mapping. For the phosphoprotein mapping, the normal SDS−PAGE system was utilized along with the Pro-Q Diamond phosphoprotein gel stain (Pro-QD, Molecular Probes), according to the manufacturer's recommendations. To identify the phosphorylation modification, the purified AhR complex (50 μL of 1 mg/mL) was divided into two aliquots, of which one was subjected to phosphatase treatment, prior to the electrophoresis. One-tenth volume of calf intestinal phosphatase (3 units/μL, Toyobo) was mixed with the analyte, and the reaction mixture was incubated at 37 °C for 2 h. The phosphate content of the HSP90 subunit within the purified AhR complex was estimated by a densitometry analysis on a Pro-QD stain gel, according to the manufacturer's product information. To construct a calibration curve, the following standard phosphoproteins were used: ovalbumin (2 phosphates/mol, Sigma), pepsin (1 phosphate/mol, Nacalai Tesque), and bovine serum albumin (0 phosphate/mol, Sigma).

For the phosphopeptide mapping, a tryptic digest was separated with a 40% alkaline polyacrylamide gel system under nondenaturing conditions (*62*), fixed in 30% formaldehyde, and detected by the Pro-QD stain, as follows. The purified AhR complex (about 50 μg) was precipitated by addition of 10% trichloroacetic acid and then was washed with cold acetone. After the solvent was evaporated, the dried residue was dissolved in 8 M urea containing 100 mM NH_4HCO_3 and was treated with 5 mM DTT at 56 °C for 15 min. The solution was then treated with 10 mM iodoacetamide at room temperature for 15 min, in the dark. After a 4-fold dilution with 100 mM NH_4HCO_3, the protein sample was treated with 10 ng/μL trypsin (sequencing grade modified trypsin, Promega) at 37 °C overnight. After the addition of 100 mM Tris buffer (pH 8.8), the tryptic peptide solution was divided into two aliquots, of which one was treated with six units of alkaline phosphatase at 37 °C for 2 h. The resolving and stacking gel solutions, the reservoir buffer, and the sample buffer were as described by West et al. (*62*). Following electrophoresis for 60 min at 20 mA/gel constant current, the gel was soaked in 30% formaldehyde and incubated at room temperature with gentle agitation for 60 min. This fixation step was repeated once more. The gel was then incubated in water for 30 min, and this step was repeated for a total of three washes. Stain and destain steps were carried out with the Pro-QD, according to the manufacturer's recommendations.

In-Gel Digestion and Phosphopeptide Fractionation. In-gel digestion of the gel-separated proteins was performed according to a standard procedure (*63*−*65*). The CBB-stained or zinc-stained gel pieces containing the AhR or Hsp90 proteins were washed with acetonitrile/20% NH_4HCO_3 (1:1) or destaining solution (from the "negative gel stain MS kit") several times. After dehydration by soaking in acetonitrile and evaporation of the solvent, the gel was treated with 10 mM DTT in 25 mM NH_4HCO_3 at 56 °C for 60 min. After being washed with 25 mM NH_4HCO_3, the gel was treated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature for 45 min, in the dark. After being washed with 25 mM NH_4HCO_3 and dehydrated in acetonitrile, the gel was treated with 20 ng/μL trypsin (sequencing grade modified trypsin, Promega) or V8 protease (sequencing grade endoproteinase Glu-C, Sigma) in 25 mM NH_4HCO_3 at 37 °C overnight. The peptides were extracted with 0.1% acetic acid and then acetonitrile/0.1% acetic acid (1:1). The extracts were concentrated to about 25 μL by evaporation, mixed with 25 μL of 5% acetic acid, and then stored at −80 °C until the LC-MS/MS analysis. To selectively fractionate the phosphopeptides from the peptide mixture, immobilized metal affinity chromatography (IMAC) was performed using a "phosphopeptide isolation kit" (Pierce), according to the manufacturer's recommendations, except that 0.2 M ammonium carbonate buffer (pH 9.2) was used for elution. The peptide mixtures were desalted on a C18 column (50 mg of SPE cartridge, Silicycle) with 2% acetonitrile containing 0.1% trifluoroacetic acid,and were recovered by elution with 70% acetonitrile containing 0.1% formic acid. The eluents were concentrated to about 10 μL by evaporation, mixed with 10 μL of 2% acetonitrile containing 0.5% formic acid, and then stored at −80 °C.

LC-MS/MS Analyses. Electrospray ionization (ESI) analyses were performed on a Q-Tof Ultima (API or Global type, Micromass-Waters) equipped with a Z-Spray nanoflow electrospray ion source. The mass spectrometer was operated in the electrospray positive ion mode with a source temperature of 80 °C, a cone voltage of 80−100 V, and a cone gas flow of 50 L/h. A voltage of 3.5−4.2 V was applied to the nanoflow probe tip. A Waters nano-LC system, CapLC system, was used with a PepMap C18 analytical column (75 μm i.d. × 150 mm, LC Packings-Dionex) and a PepMap C18 precolumn (300 μm i.d. × 5 mm, LC Packings-Dionex). The flow from pump C was used to load and wash the sample with an aqueous solution of 0.1% formic acid, and the bound peptides were then passed through the analytical column using a 0.1% formic acid/acetonitrile gradient elution. Mobile phases A and B consisted of 0.1% formic acid in acetonitrile/water (5/95) and 0.1% formic acid in acetonitrile/water (95/5), respectively. The flow from pumps A and B was 5.5 μL/min, and this was split to reduce the flow rate through the column to approximately 200 nL/min. The LC eluent was directed to the nanoflow ESI source on the mass spectrometer. To identify the protein constituting the cytosolic AhR complex, tryptic peptides were analyzed by the peptide sequence tag method (*66*). The product ion spectrum data were searched using the MASCOT program (Matrix Science) against a mammalian database (NCBInr).

To detect and identify the phosphopeptides, the precursor ion discovery method was used (*67*). Mass spectra from the first survey mode were acquired at a low collision energy (10 V), and the collision energy was increased to 30 V for the second survey mode so that fragment ions were generated from the precursor ions present in the first survey mode spectrum. Scanning for neutral loss of phosphoric acid (97.9769 Da) under higher collision energy condition was employed to detect phosphoserine- or phosphothreonine-

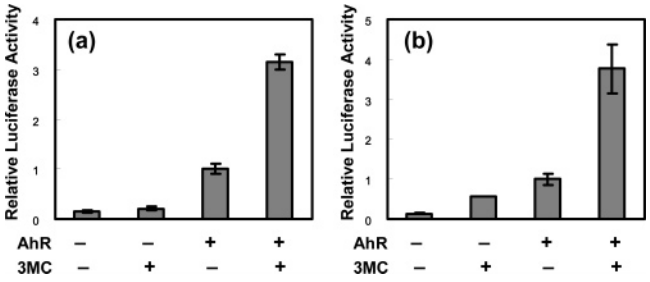

FIGURE 1: Transfected mouse AhR is responsive to 3MC in CHO cells. CHO cells were transiently cotransfected with an XRE-driven luciferase reporter gene, pXRE4tRLuc, and the mouse AhR expression vector, pcDNA-mAhR (or the empty expression vector as a control) (a). CHO cells stably expressing mouse AhR (or normal CHO cells as a control) were transfected with pXRE4tRLuc (b). These cells were pretreated with 1 μM 3MC, and the luciferase activity and the protein concentration of the cell lysates were measured. Each transfection was performed in triplicate. Error bars denote standard deviations.

containing peptides (*68*). From the product ion spectrum of the candidate precursor ion, the presence of phosphoserine or phosphothreonine was confirmed, and the sequence information was provided. The product ion spectrum data were searched using the MASCOT program (Matrix Science) against a FLAG-tagged mouse AhR sequence and the Chinese hamster HSP90α/β sequences.

RESULTS AND DISCUSSION

Recombinant Mouse AhR Associates with Chinese Hamster Endogenous HSP90α/β and XAP2 in CHO Cells. To determine the posttranslational modifications of the steady-state cytosolic AhR complex, we generated a recombinant mouse AhR, using CHO cells, which hardly express any intrinsic AhR (*69*), to facilitate the large-scale preparation. CHO cells were transfected with a mammalian expression vector, which directs the synthesis of the full-length mouse AhR with a C-terminal FLAG tag. Following selection with zeocine, the CHO cells stably expressed mouse AhR. The expression level was almost equivalent to that of the intrinsic AhR in Hepa1 cells (data not shown). Both transient and stable CHO transfectants expressing FLAG-tagged mouse AhR were responsive to 3MC, as detected by a luciferase assay (Figure 1). These results indicated that the FLAG-AhR was functional in CHO cells and capable of activating an XRE-luciferase reporter following induction by an agonist.

After the cytosolic fraction was isolated from a large-scale culture of the stable CHO transfectants, the AhR complex was purified using anion-exchange chromatography, affinity chromatography with an anti-FLAG antibody, and gel permeation chromatography. About 100 μg of the cytosolic AhR complex was purified from 5 L of the suspension culture. At least three proteins, 96, 88, and 36 kDa, were detected in the purified AhR complex, indicating that the recombinant mouse AhR was complexed with two species of Chinese hamster endogenous proteins (Figure 2a). In several experiments, some other bands were also detected, although these were variable among the experiments. Immunoblot analyses, N-terminal amino acid sequence analyses, and LC-MS/MS analyses, by the peptide sequence tag method, were used to help to identify the endogenous Chinese hamster proteins associated with the mouse AhR. In the immunoblot analysis, the 96 kDa protein reacted with

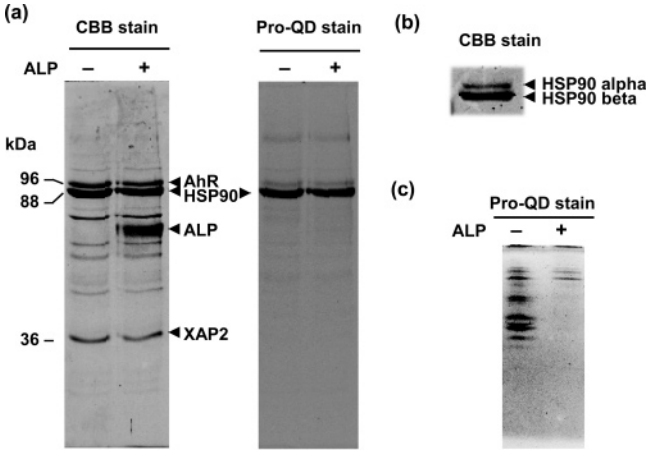

FIGURE 2: HSP90 is the major phosphoprotein constituting the cytosolic AhR complex. Cytosolic fractions were isolated from CHO cells stably expressing mouse AhR. The AhR complex was purified and resolved by SDS−PAGE with CBB or Pro-QD stain (a). The two HSP90 forms in the purified AhR complex were separated under a high-resolution SDS−PAGE condition (b). The purified AhR complex was digested with trypsin, resolved by 40% PAGE, and visualized with the Pro-QD stain (c). Prior to SDS−PAGE analysis, the sample (20 μg) was treated without or with 6 units of alkaline phosphatase (ALP) at 37 °C for 2 h.

the anti-FLAG antibody, and the 88 kDa protein reacted with the anti-HSP90 antibodies, which react with HSP90α and HSP90β from mouse, rat, and human origins (Table 1). In the N-terminal amino acid sequence analysis, the first four amino acids (P, E, E, V/T) were identical to those of human HSP90α/β. In the LC-MS/MS analysis, the 88 and 36 kDa proteins were matched to mouse HSP86/84(α/β) and human XAP2 with MASCOT scores of 907/666 and 513, respectively (Table 1). In addition, the apparent molecular masses were almost the same as the expected molecular masses of AhR-FLAG, 92 kDa, mouse HSP90α/β, 85/83 kDa, and human XAP2, 38 kDa. Therefore, we identified the endogenous 88 and 36 kDa Chinese hamster proteins as the HSP90α/β mixture and XAP2, respectively. This observation is consistent with previous studies by many groups, demonstrating that HSP90 and XAP2 functionally associate with AhR to form the unliganded cytosolic AhR complex (*12*−*17*, *70*). From densitometry of the CBB-stained protein bands on the SDS gel, the relative ratio of these three subunits (AhR/HSP90s/XAP2) in the purified AhR complex was determined as $0.80 \pm 0.11/2.0/0.84 \pm 0.27$ (three different preparations). Furthermore, the two HSP90 forms in the purified AhR complex were separated under a high-resolution SDS−PAGE condition, as described by Vamvakopoulos (*61*) (Figure 2b). In the immunoblot analyses, the upper and lower bands reacted with the anti-HSP90α and anti-HSP90β antibodies, respectively (data not shown). Densitometry of the CBB-stained protein bands revealed that the β form predominated in the complex by the ratio of $\alpha/\beta = 1.0/3.0 \pm 0.1$ (three different preparations).

HSP90 Is the Primary Phosphoprotein Constituting the Cytosolic AhR Complex. It is important to study events such as phosphorylation in the context of the cell, because the products of phosphorylation reactions carried out in vitro may not be detected under more physiological conditions. First, an experiment was conducted to determine which subunit within the cytosolic AhR complex is a phosphoprotein. The purified AhR complex was resolved by SDS−

Table 1: Identification of Proteins Constituting the AhR Complex[a]

mass (kDa)	reacting antibodies	N-terminal amino acid sequence	MS/MS analysis followed by MASCOT search (total score)	identification
96	anti-FLAG	nd	human AhR (474)	FLAG-tagged mouse AhR
88	anti-HSP90	PEEV/T	mouse HSP84 (907), mouse HSP86 (666)	endogenous Chinese hamster HSP90α and HSP90*β*
36		nd	human XAP2 (513)	endogenous Chinese hamster XAP2

[a] Each subunit constituting the purified AhR complex was digested with trypsin, and the resulting peptides were subjected to LC-MS/MS analysis. The obtained peptide sequence tags were searched against NCBInr. nd = not determined.

PAGE, and the gel was stained with Pro-QD (Molecular Probes) to detect the phosphoproteins selectively, according to the manufacturer's recommendations. The 88 kDa band (HSP90) was strongly stained, while the 96 kDa band (AhR) was only slightly detected, and the 36 kDa band (XAP2) was not stained (Figure 2a). Treatment of the purified AhR complex with alkaline phosphatase prior to the SDS−PAGE analysis minimally affected the stained image (Figure 2a). To examine the possibility that the alkaline phosphatase was not able to access the phosphorylation site because of steric hindrance, the purified AhR complex was digested with trypsin, and aliquots were treated with or without alkaline phosphatase. The resultant peptides were analyzed by the convenient phosphopeptide mapping method we developed. Many of the tryptic peptides resolved by 40% PAGE with the Pro-QD stain were no longer detected after the phosphatase treatment (Figure 2c). These results indicate that the cytosolic AhR complex is phosphorylated in the steady state and that the HSP90 subunit is the primary phosphoprotein among the subunits constituting the cytosolic AhR complex.

HSP90β within the Cytosolic AhR Complex Is Phosphorylated at Ser225 and Ser254 in the Charged Linker Region. LC-MS/MS is increasingly becoming the method of choice for identifying phosphorylation sites, because radioactivity is not required for detection, the method is inherently sensitive, and the mixture analysis is readily performed. To identify the phosphorylation sites by the MS/MS method, we first determined the amino acid sequences of Chinese hamster HSP90α/*β*. On the basis of the nucleotide sequences of human HSP90α and HSP90*β* in the N- and C-terminal regions, two sets of oligonucleotide primers were designed and used to amplify the Chinese hamster HSP90α and HSP90*β* cDNAs from a CHO cDNA library by the PCR method. The amino acid sequences of Chinese hamster HSP90α and HSP90*β*, deduced from their cDNA sequences, showed the highest similarity (99.7%) to mouse HSP90α and human HSP90*β*, respectively (data not shown).

The purified AhR complex (about 100 *μ*g) was subjected to SDS−PAGE and then visualized by zinc staining. After destaining, each of the gel slices containing AhR and HSP90 was reduced, alkylated to modify the cysteine residues, and digested with trypsin or V8 protease. To determine the precise phosphorylation sites, it is important to reduce the interference from the vast majority of nonphosphorylated peptides. The peptides extracted from the gel were further subjected to IMAC to enrich for phosphopeptides. The IMAC eluent was desalted and concentrated for a subsequent LC-MS/MS analysis. At this time, the phosphopeptide mapping technique described above, which combined the 40% PAGE separation with the Pro-QD staining, was very useful for monitoring the phosphopeptide fractionation. The specific precursor ion discovery method for phosphopeptide detection in a peptide mixture utilizes the preferred loss of a phospho group upon low-energy collisional activation (*67*). In the positive ion mode with a positional analysis by the MS/MS sequence, scanning for the neutral loss of phosphoric acid (97.98 Da) was employed for peptides containing phospho-Ser/Thr (*68*). The MS/MS sequence analysis of the HSP90 band digested with trypsin revealed that Ser225 and Ser254 of HSP90*β* and Ser230 of HSP90α, corresponding to Ser225 of HSP90*β*, were phosphorylated (Figure 3). This observation is consistent with a previous study on the phosphorylation of HSP90α/*β* purified from exponentially growing HeLa cells. Using in vivo labeling with ^{32}P, Lees-Miller et al. revealed that the purified HSP90α/*β* mixture contains 2 mol of phosphate/mol of HSP90 and that Ser230/225 and Ser262/254 of HSP90α/*β* were constitutively phosphorylated (*46*). Furthermore, the phosphate content of the HSP90 subunit within the purified AhR complex was estimated by comparison to the standard phosphoproteins on a Pro-QD stain gel. Assuming that the molecular mass of 1 mol of the purified AhR complex, containing 2 mol of HSP90, 1 mol of AhR, and 1 mol of XAP2, is about 300 kDa, we estimated that 1 mol of the HSP90 subunit contained 1.7 ± 0.3 mol (three different preparations) of phosphate. The present study suggested that the majority of the HSP90 associated with the AhR protein in vivo was phosphorylated at the same position as in the free HSP90 dimer in vivo, although we could not detect the Ser262 phosphorylation of HSP90α. Although HSP90 is a highly conserved protein that is required for viability in eukaryotes, the N- and C-terminal ends have variable lengths in eukaryotes. The charged linker connecting the highly conserved N-terminal domain with the middle and C-terminal domains of HSP90s is also variable among eukaryotic cells (Figure 3d) and almost completely absent from the prokaryotic HSP90 homologues. This charged linker diversity suggests that it plays some roles in the development of different HSP90 functions among various eukaryotes. This view was strengthened by our observation that, in mammalian cells, the HSP90s that were functionally associated with a client protein were posttranslationally phosphorylated in the charged linker.

Mutations at the Phosphorylation Sites of HSP90β Modulate the Interaction with AhR. To examine the possibility that the charged linker phosphorylations of HSP90s are involved in the association with AhR, we constructed two phosphorylation mutants, S225/254A and S225/254E, of human HSP90*β*. For the immunoprecipitation assay, we used transfected COS7 cells that transiently expressed either wild-type or mutant HA-tagged human HSP90*β* (HA-HSP90*β*), together with the wild-type FLAG-tagged mouse AhR (AhR-FLAG). The lysates prepared from the transfected cells, which expressed the different HA-HSP90*β* forms almost equally (Figure 4), were immunoprecipitated with anti-FLAG

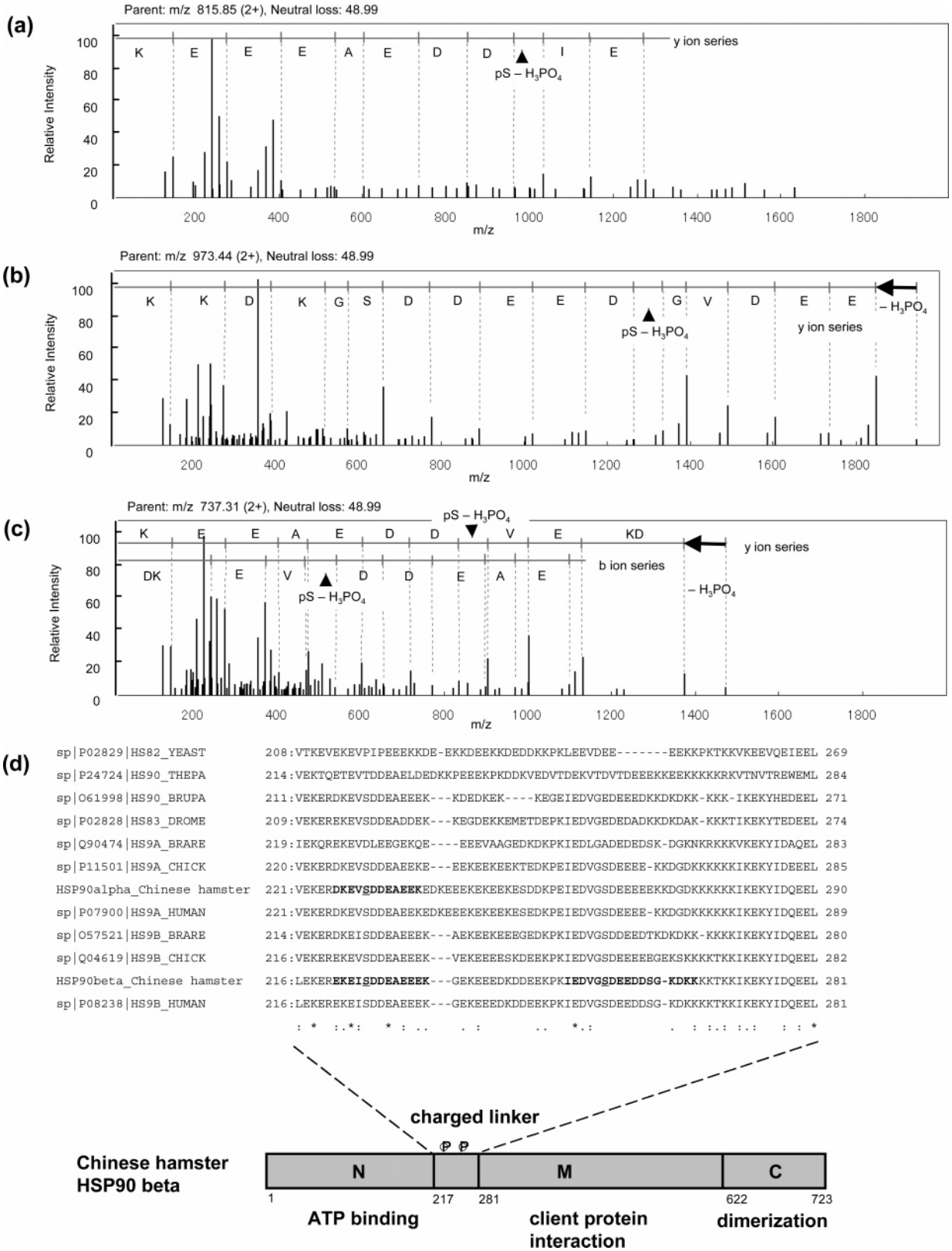

FIGURE 3: HSP90s in complex with AhR are phosphorylated at Ser225 and Ser254 of HSP90β and Ser230 of HSP90α. Tryptic peptides of HSP90s constituting the AhR complex were fractionated by IMAC and analyzed by LC-MS/MS. ESI-MS/MS spectra of several phosphopeptides were obtained by scanning for the neutral loss of 98 Da to detect phosphopeptides. The product ion spectrum of the precursor at *m/z* 815.85 (2+) identified Ser225 of HSP90β as being phosphorylated (a). The product ion spectrum of the precursor at *m/z* 973.44 (2+) identified Ser254 of HSP90β as being phosphorylated (b). The product ion spectrum of the precursor at *m/z* 737.31 (2+) identified Ser230 of HSP90α as being phosphorylated (c). Amino acid sequence alignment of the charged linker regions (d). The sequence alignment was performed using Clustal W(1.7). The observed fragments are shown in boldface type. Underlined residues indicate the phosphorylation sites revealed in this study. A diagram of the domain structure of Chinese hamster HSP90β is also shown.

tag or anti-HA tag antibodies covalently linked to agarose beads. In the immunoprecipitation assay combination with HA-HSP90β adsorption and AhR-FLAG detection, the replacement of both Ser225 and Ser254 by Glu, S225/254E,

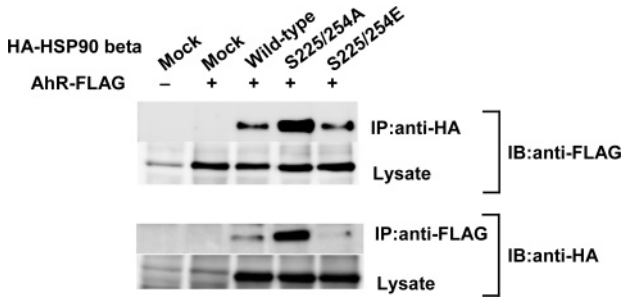

FIGURE 4: Mutations at phosphorylation sites of HSP90β affect their affinity for AhR. Wild-type or mutant HA-HSP90β (0.5 μg of expression vector) was transiently coexpressed in an aliquot of COS7 cells with AhR-FLAG (0.5 μg of expression vector). Equal amounts of the cell lysate and the complex bound to the anti-HA or anti-FLAG agarose were analyzed by immunoblotting with the anti-FLAG or anti-HA antibodies.

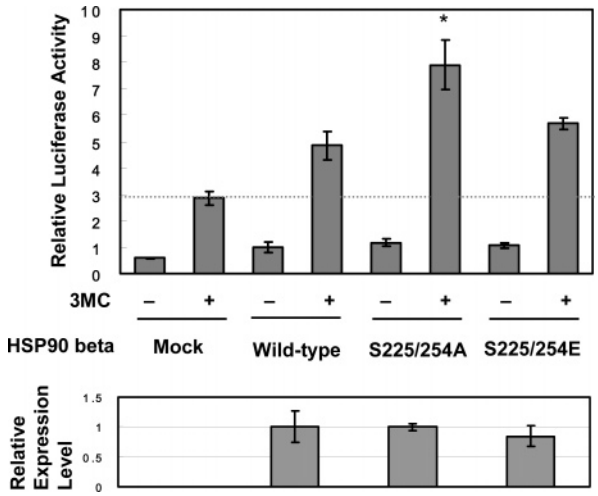

FIGURE 5: The replacement of Ser225 and Ser254 by Ala, S225/254A, increased the AhR transcription activity. Wild-type or mutant HA-HSP90β was transiently coexpressed in an aliquot of Hepa1 cells with the XRE-driven luciferase reporter gene. These cells were pretreated with 1 μM 3MC, and the luciferase activity and the protein concentration of the cell lysates were measured. The dotted line indicates the basal level of the AhR activity in Hepa1 cells. Each transfection was performed in triplicate. Error bars denote standard deviations. The Student two-tailed *t* test was used to determine statistical significance ($\alpha = 0.05$). A value accompanied by an asterisk (*) is significantly different from that of the wild-type HSP90β. Equal amounts of cell lysates, without the 3MC stimulation for the luciferase assay, were subjected to an immunoblotting analysis with anti-HA antibodies to confirm the equivalent levels of HSP90β mutant expression.

had no effect on the interaction with AhR, as compared with the wild-type HSP90β (Figure 4). Notably, the replacement of both Ser225 and Ser254 by Ala, S225/254A, increased the binding affinity for AhR. The assay that combined AhR-FLAG adsorption and HA-HSP90β detection revealed that the Glu substitution reduced the binding affinity of HSP90β for AhR (Figure 4). These observations indicate that the mutations at the phosphorylation sites of HSP90β modulate the interaction with AhR and suggest that the negative charges at the Ser225 and Ser254 phosphorylation sites may weaken the association with AhR.

To further explore the issue of whether the mutations at the phosphorylation sites of HSP90β affect the AhR function, wild-type or mutant HA-HSP90β was transiently coexpressed in Hepa1 cells with the XRE-driven luciferase reporter gene, and their transcriptional activities were compared. The transfected Hepa1 cells that transiently expressed the wild-type human HSP90β enhanced the basal AhR transcription activity, as compared to the level of the control Hepa1 cells (Figure 5). This observation suggests that the augmented expression of human HSP90β in Hepa1 cells caused the formation of an additional functional complex with AhR, which was nonfunctional by itself in vivo. Although the S225/254E mutant showed almost the same level of luciferase expression as the wild-type HSP90β, the S225/254A mutant exhibited more potent ligand-induced transcriptional activity than the wild-type HSP90β (Figure 5). This apparent enhancement of the reporter gene activity is not due to a different expression level of the HA-HSP90β mutants. It is likely that the apparent enhancement of the ligand-induced AhR transcription activity resulted from the Ala substitution at the HSP90β phosphorylation sites. Together with the immunoprecipitation result, in which the S225/254A mutation strengthened its association with AhR, our mutagenesis study suggests that the phosphorylations of HSP90β at Ser225 and Ser254 in the charged linker may negatively regulate the formation of the functional AhR complex in the steady-state cytosol.

Some putative roles of the charged linker in HSP90 function have been previously discussed. Binert et al. predicted that the charged linker of HSP90 may play a role in the interaction of HSP90 with steroid hormone receptors (*37*). Scheibel et al. proposed that the charged linker regulates the chaperone function of the N-terminal domain (*36*). On the other hand, a previous study by Meyer et al., using a GST-HSP90 fusion protein generated in *Escherichia coli*, revealed that the middle and C-terminal domains of HSP90 were involved in binding to AhR and XAP2, respectively (*16*). The present study suggested that the charged linker phosphorylations of HSP90 weaken the interaction of HSP90 with AhR. Therefore, the charged linker phosphorylations may alter the domain orientation, so that the middle domain partly restricts the interaction with AhR. The present results on the HSP90 phosphorylations within the cytosolic AhR complex are a further step toward understanding the complicated HSP90 molecule, which seems to regulate the diverse functions of the different HSP90 client proteins.

Concluding Remarks. Posttranslational modifications are a common regulatory mechanism for eukaryotic cells to control protein function. In particular, protein phosphorylation modulates the DNA-binding activity, translocation, and protein−protein interactions of many protein factors. In this context, the electrostatic heterogeneity due to partial phosphorylation is a particular nuisance in preparing phosphoprotein crystals for three-dimensional structure determinations, which will reveal the details of the AhR inactivation and activation mechanisms. The information presented here provides a basis for further posttranslational modification analyses of the AhR complex and will be useful in designing new constructs of the cytosolic AhR complex that are suitable for X-ray crystallography.

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