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# Role of HSP90 in Mediating Cross-Talk between the Estrogen Receptor and the Ah Receptor Signal Transduction Pathways

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**ABSTRACT.** Tetrachlorodibenzo-*p*-dioxin (TCDD)-mediated gene transactivation via the Ah receptor (AhR) has been shown to be dependent upon estrogen receptor (ER) expression in human breast cancer cells. We have investigated the 90-kDa heat shock protein (HSP90) as a mediator of cross-talk between the AhR and the ER signal transduction pathways. The effect of HSP90 overexpression on receptor activity was determined by transient transfection assays using a HSP90 expression vector. Ligand-inducible gene expression was inhibited when the HSP90 expression vector was cotransfected with a TCDD-responsive reporter plasmid. However, overexpression of HSP90 did not block induction of an estrogen-responsive reporter plasmid. To determine whether ER facilitates AhR signaling through its ability to squelch HSP90, two vectors expressing protein products that bind HSP90 were transfected into MDA-MB-231 cells. Introduction of (i) He11, an ER deletion mutant that does not bind DNA, and (ii) the ligand-binding domain of human AhR, both led to increased basal and TCDD-inducible *CYP1A1* expression. Finally, the subcellular distribution of HSP90 was investigated in human breast cancer cell lines. These studies showed HSP90 to be primarily cytoplasmic in ER-positive cell lines, whereas in matched ER-negative cell lines HSP90 was distributed equally between the cytoplasm and nucleus. Taken together, these results demonstrate that HSP90 can regulate AhR activity *in vivo*, and that Ah-responsiveness is dependent upon cellular ER content through a mechanism that involves HSP90. BIOCHEM PHARMACOL **58**;9:1395–1403, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** Ah receptor; estrogen receptor; HSP90; breast cancer; 2, 3,7,8-tetrachlorodibenzo-*p*-dioxin; receptor cross-talk

The AhR§ and Arnt are members of the PAS family of transcription factors. Ligand binding to cytosolic AhR initiates nuclear translocation and heterodimerization with Arnt. This complex modulates the transcription of Ah-responsive genes by binding to cognate DNA sequences, known as xenobiotic responsive elements, which are found in the vicinity of target genes. Although TCDD and related AhR agonists do not associate with the ER [1], these compounds exhibit antiestrogenic properties in animal models [2, 3] and in cell culture [4, 5]. Vickers *et al.* [6] demonstrated a second level of cross-talk between AhR and ER when they showed that the Ah-responsiveness (i.e. the ability of TCDD to induce *CYP1A1* expression) of human breast cancer cells is related to their ER content. Moreover, several ER-negative cell lines that express AhR are Ah-nonresponsive, and these include MDA-MB-231 and Adriamycin®-resistant MCF-7 cells, whereas ER-positive wild-type MCF-7 cells are Ah-responsive, along with MDA-MB-231 cells transiently transfected with hER [7, 8]. Presently, mechanisms of interference and cooperation between the AhR and the ER signal transduction pathways are not clear.

AhR and ER can be recovered from untreated target cells extracted in low-salt medium in an 8–9S form that does not bind DNA [9, 10]. The inactive 8–9S form is a complex of the receptor with a set of proteins including a HSP90 dimer. In agonist-treated cells, the receptors can be extracted under high-salt conditions as a smaller 4–5S form, free of any detectable HSP90, and with DNA-binding activity [9, 10]. HSP90 proteins have a critical role in AhR and ER signal transduction. First, receptor activation is defective in a strain of *Saccharomyces cerevisiae* that produces reduced levels (5%) of HSP90 [11, 12]. Second, the HSP90-binding domain colocalizes with the ligand-binding domain in each receptor [13, 14], which suggests that ligand

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§ *Abbreviations*: AhR, aryl hydrocarbon receptor; bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; ER, estrogen receptor; ERE, estrogen responsive element; GR, glucocorticoid receptor; hER, human ER; HSP90, 90kDa heat shock protein; LBD, ligand-binding domain; NLS, nuclear localization signal; PAS, Per-Arnt-Sim homology; PR, progesterone receptor; TAF, transcriptional activation function; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and XRE, xenobiotic responsive element.

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may activate the receptor by displacing HSP90. Third, HSP90 is capable of blocking the binding of either receptor to its respective responsive element in *in vitro* assays [9, 15, 16]. Thus, it generally is believed that HSP90 acts to inhibit the intrinsic DNA binding activity of steroid hormone and Ah receptors either by steric interference and/or by passive interference with the dimerization step.

Here, we propose a model in which ER facilitates AhR signaling through its ability to squelch HSP90. This is based on experiments showing restoration of Ah-responsiveness in MDA-MB-231 cells transfected with receptor deletion mutants that can bind HSP90. Although it has been shown that HSP90 can inhibit the DNA-binding and transactivation activities of AhR and ER in cell-free systems, it has not been demonstrated whether HSP90 can repress AhR and ER transactivation *in vivo*. To address this question, we transfected HSP90 into two ER-positive, Ah-responsive cell lines. These studies indicated that overexpression of HSP90 does not affect ER signaling, but it does repress AhR-mediated gene transactivation. Finally, the subcellular localization of HSP90 and AhR in matched ER-positive and -negative human breast cancer cell lines was investigated.

## MATERIALS AND METHODS

### *Cell Culture*

MCF-7 and T47-D human breast cancer cell lines and Adriamycin-resistant MCF-7 cells [17] were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 IU/mL of penicillin, and 50 μg/mL of streptomycin. MDA-MB-231 cells were maintained in α-Minimum Essential Medium (α-MEM) with 10% fetal bovine serum and penicillin/streptomycin. The S30 cell line was established previously via stable transfection of a hER expression vector into MDA-MB-231 cells [18]. These cells were maintained in phenol red-free α-MEM supplemented with 10% charcoal-stripped fetal bovine serum, penicillin/streptomycin, and 500 μg/mL of the antibiotic G418. For drug treatments, TCDD (Midwest Research Institute) was dissolved in acetone at a concentration of 20 μg/mL. 17β-Estradiol (Sigma) was dissolved in ethanol at a concentration of 1 mg/mL, diluted with medium for a $10^{-5}$ M stock solution, and stored in small aliquots at −20°.

### *Transient Transfection Assays*

The pGL3–1A1 plasmid was constructed by excising a fragment encompassing human *CYP1A1* promoter/exon 1 sequences −1140 to +59 from pRNH241c and subcloning it into the *Sma*I site of the pGL3-Basic luciferase vector (Promega). The HSP90 expression vectors utilized here were described by Kang *et al.* [19]: 90WT contains a copy of chicken HSP90 cDNA ligated into the pSVK3 expression vector; and 90NLS is a similar expression vector but with the addition of the nucleoplasmin NLS inserted into the HSP90 coding sequence. MCF-7 and T47-D cells in 12-well culture dishes were transfected with 250 ng of reporter plasmid (pGL3–1A1), 50 ng of expression vector (pSVK3, 90WT, or 90NLS), 2 ng of the pRL-CMV *Renilla* luciferase control reporter vector (Promega), and 0.6 μL of Lipofectamine reagent by methods provided by the manufacturer (Gibco-BRL). The following day, cells were treated with either vehicle or 10 nM TCDD for 24 hr. Extracts were prepared from transfected cells and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's guidelines. Luciferase activity was calculated as pGL3–1A1 activity/pRL-CMV activity.

For CAT assays, cells seeded in 6-well plates were cotransfected with 2 μg of expression vector (pSVK3, 90WT, or 90NLS), 2 μg of the vit-tk-CAT estrogen-responsive reporter plasmid [20], and 8 μL of Lipofectamine reagent. After a 5-hr incubation, the medium was replaced with phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum. The following day, cells were either left untreated or exposed to $10^{-7}$ M estradiol for 24 hr. Whole cell extracts were obtained to perform CAT enzyme assays. The acetylated and nonacetylated forms of [$^{14}$C]chloramphenicol were separated by thin-layer chromatography, and quantification was performed using a PhosphoImager. CAT activity was standardized to the protein content of the cellular extracts.

### *Stable Transfections*

He11 cDNA was ligated into the *Eco*RI site of pBluescript (Stratagene) and then subcloned into the *Bam*HI and *Kpn*I restriction sites of the pCEP4 episomal eukaryotic expression vector (Invitrogen). The human homolog of the murine AhR ligand-binding domain [21] was polymerase chain reaction (PCR)-amplified from MCF-7 cDNA and subcloned into pCEP4. The upstream primer (CATCTAAGCTTGCCGCCATGAATTTCCAAGGGAAG) was designed to provide a *Hin*dIII restriction site, and the downstream primer (AGTCCCTCGAGGTTAGGGATCCATTATGGCA) to provide a stop codon and *Xho*I site at its terminus. The pCEP4, pCEP4-He11, and pCEP4-AhR/LBD expression vectors were transfected into MDA-MB-231 cells using Lipofectamine reagent. Stable transfectants were selected by culturing cells in 500 μg/mL of the antibiotic Hygromycin B (Boehringer-Mannheim).

### *Northern Blot Analysis*

Total RNA was isolated from cells in log growth phase using the RNAzol B (Tel-Test) method by the manufacturer's guidelines. RNA samples (25 μg/lane) were resolved through 1.1% agarose/formaldehyde/ethidium bromide gels for 3 hr at 100 V and photographed. The RNA was transferred to Zeta-Probe membranes (Bio-Rad) by capillary transfer overnight and hybridized with ultraviolet light. The oligonucleotide probe, consisting of a 1200 bp fragment from a *Pst*I digestion of rat *CYP1A1* cDNA, was

radiolabeled with [α-[$^{32}$P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia). Hybridization was performed in 40% formamide, 4 × standard saline citrate, 4 × Denhardt's reagent, 0.2 mg/mL of salmon sperm DNA, 1.2% SDS, and 10% dextran sulfate at 42° overnight.

### *Immunohistochemistry*

Cells that had been plated the previous day on coverslips were fixed with 4% paraformaldehyde/PBS for 15 min at 37°. Membranes were made permeable with a 0.3% Triton X-100/PBS solution for 5 min followed by blocking for 30 min with 5% BSA/PBS. The specimens were co-incubated with a 1:200 dilution of monoclonal (mouse) anti-HSP90 antibody (SPA-830, StressGen) and a 1:1000 dilution of polyclonal (rabbit) anti-AhR antibody in 5% BSA/PBS for 2 hr at 37°. The secondary antibodies consisted of rhodamine-conjugated rat anti-mouse IgG (1:100 dilution) and fluorescein-conjugated rat anti-rabbit IgG (1:100 dilution) and were applied for 1 hr at 37°. HSP90 and AhR cellular localization was determined using a Zeiss LSM410 confocal microscope.

To confirm the correct localization of 90WT and 90NLS, transfected cells prepared as above were incubated with BF4 monoclonal (rat) IgG followed by fluorescein-conjugated goat anti-rat IgG. The BF4 antibody is specific for avian HSP90 [19] and did not recognize human HSP90.

### *Immunoblot Analysis*

Cells in exponential phase growth on 100-mm plates were washed with PBS, detached with a cell scraper, and sedimented. The cells were resuspended in 0.5 mL lysis buffer [10 mM Tris–HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.01 mg/mL of leupeptin, 0.01 mg/mL of pepstatin, 0.01 mg/mL of aprotinin] per 2 × $10^7$ cells and incubated on ice for 10 min. Samples were spun down at high speed, and the protein content of the supernatant was quantitated by the Bradford assay (Bio-Rad). Protein samples (25 μg/lane) were resolved through 10% polyacrylamide-SDS gels and electrophoretically transferred to nitrocellulose (BioBlot-NC; Costar) by standard techniques. The blots were stained with Ponceau S to verify the efficiency of transfer and the gel loading. If areas of the gel did not transfer properly or if differences in gel loading were apparent, the blot was not used. Membranes were blocked overnight in BLOTTO buffer [50 mM Tris–HCl (pH 7.5), 0.2% Tween-20, 150 mM NaCl, 5% dry milk powder] at 4° and then washed four times in TTBS+ buffer [50 mM Tris–HCl (pH 7.5), 0.5% Tween-20, 300 mM NaCl] for a total of 40 min. Immunoreactive staining was carried out with a 1:1000 dilution of anti-HSP90 antibody or 1:1000 dilution of anti-β-actin antibody (Boehringer-Mannheim) in BLOTTO buffer for 2 hr at room temperature. Membranes were washed as previously and incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody in BLOTTO buffer for 1 hr at room temperature. Following another wash cycle, specific proteins were detected by the enhanced chemiluminescence system from Amersham as described by the manufacturer. Bovine HSP90 (SPP-780, StressGen) was used a positive control.

### *Densitometry*

Radiographic and enhanced chemiluminescence exposures were scanned into a Power Macintosh computer with a UMAX VistaS-12 scanner and Adobe Photoshop 3.0.5 software. Then images were quantified with the use of National Institutes of Health Image 1.61b7 software. Quantification was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a specific set of samples. Then the mean value of the intensity within the tool was determined for (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest.

## RESULTS AND DISCUSSION

### In Vivo *Inhibition of AhR Signaling by HSP90*

TCDD has been proposed to act as an antiestrogen by enhancing estradiol metabolism [22–27] or by decreasing transcription of the ER gene [28]. Alternatively, Kharat and Saatcioglu [29] have suggested that the antiestrogenic nature of TCDD results from the ability of liganded AhR to block ER transactivation of gene expression. In DNA binding experiments, these researchers showed that co-treatment of MCF-7 cells with estradiol and TCDD completely blocked ERE-specific DNA binding by the ER, and, reciprocally, blocked the xenobiotic responsive element-specific DNA binding activity of the AhR [29]. The validity of these three models is uncertain, as Safe and coworkers have reported contradictory data for each of them [1, 30–33]. It has been shown that ER protein levels are decreased by up to 50–75% in TCDD-treated animals [3, 34] and cells in culture [1, 31, 35]. However, this finding does not appear to account for interference between the AhR and ER signal transduction pathways, since TCDD remains antiestrogenic in cells where ER levels cannot be down-regulated due to constitutive expression from an ER expression vector [29, 36].

HSP90 is a potential mediator of cross-talk, since it interacts with both the AhR and the ER and is known to repress receptor signaling *in vitro*. The working hypothesis for the studies presented here was that drug treatment could lead to an increase of unbound HSP90, which would be inhibitory to receptor signaling. To determine whether overexpression of HSP90 interferes with the transactivation functions of the AhR and ER *in vivo*, we performed transient transfection assays with ER-positive, Ah-responsive MCF-7 and T47-D cells.

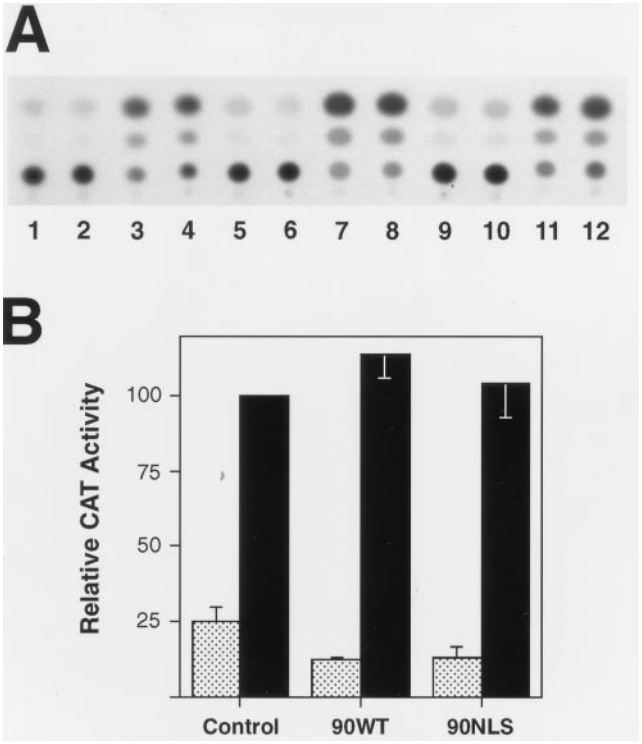


**FIG. 1. Effect of HSP90 overexpression on ER-mediated gene transactivation. (A) Representative assay. MCF-7 cells were transiently transfected with an expression vector (pSVK-3, lanes 1–4; 90WT, lanes 5–8; 90NLS, lanes 9–12) and an estrogen-responsive reporter plasmid (vit-tk-CAT). The following day, transfected cells were either left untreated (lanes 1, 2, 5, 6, 9, 10) or exposed to $10^{-7}$ M estradiol (lanes 3, 4, 7, 8, 11, 12) for 24 hr. CAT enzyme assays were performed as described in Materials and Methods. (B) Lack of inhibition of ER signaling *in vivo* by overexpression of HSP90. Untreated, stippled bars; estradiol-treated, solid bars. Results shown are the average of four separate experiments in MCF-7 cells, each performed in duplicate, and are expressed as percentages of acetylated substrate ± SEM.**

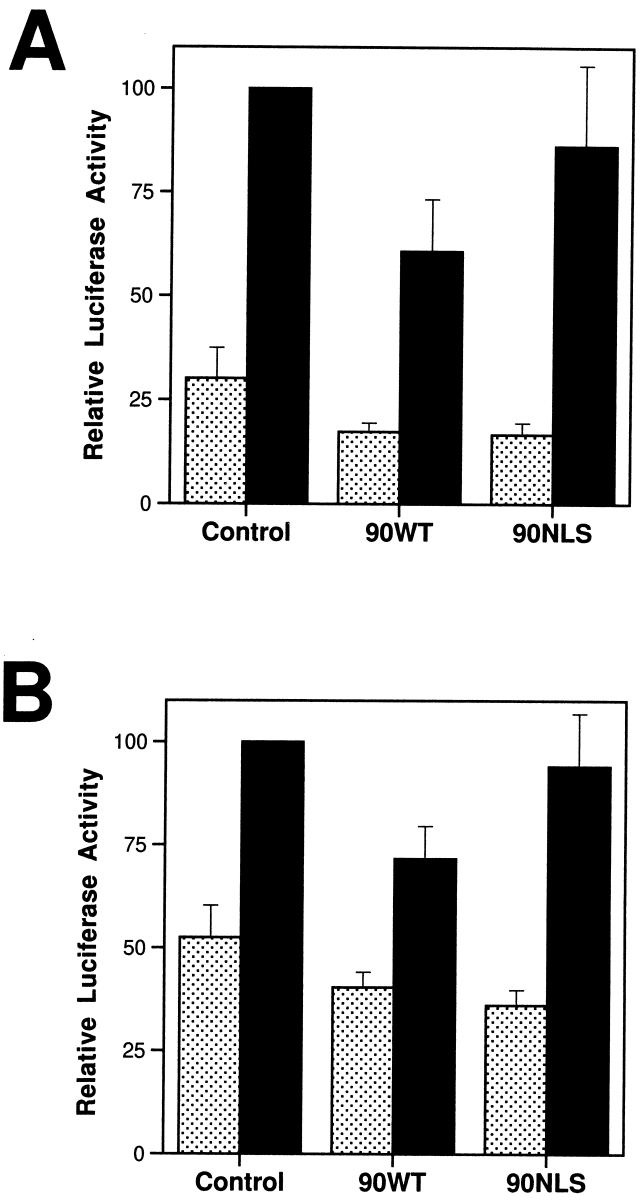


**FIG. 2. Effect of targeted HSP90 overexpression on Ah receptor signaling. Assays were performed with (A) MCF-7 cells, and (B) T47-D cells. Cells plated in 12-well dishes were cotransfected with an expression plasmid (pSVK3, 90WT, or 90NLS), a TCDD-responsive reporter plasmid (pGL3–1A1), and pRL-CMV to control for differences in transfection efficiencies and cell number from sample to sample. The next day, transfectants were treated with vehicle alone (stippled bars) or 10 nM TCDD (solid bars) for 24 hr. Luciferase activity was detected in whole cell extracts using a luminometer equipped with dual automatic injectors. Results shown are the average of three separate experiments each performed in triplicate and are expressed as a function of pGL3–1A1 activity/pRL-CMV activity ± SEM.**

For the first experiment, an expression vector, either the control plasmid (pSVK3) or a vector containing HSP90 cDNA (90WT or 90NLS) was cotransfected into cells along with an estrogen-responsive reporter construct. The following day, transfected cells were treated with estradiol for 24 hr. Results from these studies showed that there was no significant difference between estradiol-induced CAT activity in HSP90-transfected cells and CAT activity in cells carrying the empty expression vector (Fig. 1). In the second experiment, cells were cotransfected with an expression vector (pSVK3, 90WT, or 90NLS), a TCDD-responsive reporter plasmid (pGL3–1A1), and a control reporter vector (pRL-CMV). Following a 24-hr exposure to 10 nM TCDD, cells were lysed on the culture dish, and the samples were collected for luciferase activity determination. Overexpression of wild-type HSP90 resulted in a significant decrease of pGL3–1A1 luciferase expression. As shown in Fig. 2, TCDD-induced luciferase activity was limited to about 70 and 60% of control levels in MCF-7 and T47-D cells, respectively. Conversely, HSP90 that was targeted to the nucleus (90NLS) failed to decrease induction of pGL3–1A1 significantly (Fig. 2).

Although all steroid hormone receptors bind to HSP90, there appear to be differences among them with respect to the outcome of this association. The AhR is more similar to the GR in that it must be bound to HSP90 in order to bind ligand [15, 37]. Deletions of amino acids within the LBD of

the GR yield derivatives that constitutively activate transcription [38]. Thus, the LBD of the GR is solely capable of repressing receptor action, presumably in an indirect manner via interaction with HSP90. Interestingly, this motif retains its repressive function both in rearranged receptor derivatives and when fused to the unrelated adenovirus E1A [39] and c-Myc transcription factors [40]. Similar evidence exists for the AhR: Pongratz *et al.* [15] were able to show that a HSP90-free form of the AhR did not form a stable complex with TCDD but bound DNA constitutively. In contrast, the role of HSP90 in ER signaling is not as apparent. It has been shown that HSP90 assists the ER in assuming a proper DNA binding conformation [41], although it is not required for ligand binding [13]. In addition, White and coworkers [42] have shown that ER chimeras that do not associate with HSP90 are functional hormone-dependent transcriptional activators *in vivo*, and that an amino acid substitution of one residue in the LBD of the ER leads to a high level of constitutive activity, in spite of a stable interaction with HSP90 [43]. These observations can be related to the fact that the GR (and presumably the AhR) form stable associations with HSP90, whereas the ER forms a relatively weak association detectable only at non-physiological low-salt concentrations [41, 44].

The 90NLS expression vector was utilized in these experiments to determine whether the subcellular localization of HSP90 impacted upon receptor signaling. Differential localization of HSP90 was confirmed by transient transfection of 90WT and 90NLS plasmids into MCF-7 and T47-D cells followed by immunofluorescent staining with an avian HSP90-specific antibody as described in Materials and Methods (data not shown). In electrophoretic mobility shift assays using purified HSP90 and ER, Sabbah *et al.* [16] have reported that binding of ER to the ERE is inversely dependent upon the concentration of HSP90 present in the reaction, and that HSP90 is capable of dissociating preformed ER–ERE complexes. Based on these results, it was postulated that during heat shock ER-dependent transcription may be repressed due to increased HSP90 transcription and translocation of HSP90 into the nucleus [16]. However, our results indicated that under *in vivo* conditions overexpression of HSP90 in either the cytoplasm or nucleus does not inhibit ER signaling (Fig. 1). It is likely that HSP90 mediates ER folding as a molecular chaperone, but has less of a role in repressing the intrinsic DNA binding activity of the ER. For the AhR, only 90WT was found to inhibit receptor signaling (Fig. 2). These data suggest that HSP90 can block the initial binding of TCDD to the AhR in the cytoplasm, but fail to compete for preformed TCDD–AhR complexes in the nucleus.

### *Restoration of Ah-Responsiveness by Expression of HSP90-Binding Proteins*

ER-negative MDA-MB-231 cells can be converted to an Ah-responsive phenotype through transient transfection of a hER expression vector [8]. We have introduced two expression vectors into MDA-MB-231 cells to determine whether the ER facilitates AhR signaling through its association with HSP90. The vectors were constructed to overexpress HSP90-binding proteins in the form of receptor deletion mutants. It has been reported previously that the LBD of the AhR constitutes a minimal structural requirement for HSP90 binding [21], whereas the LBD and an additional sequence element at the C-terminus of the DBD appear to be necessary for HSP90 binding by the ER [13, 45]. The pCEP4-He11 expression vector contains a hER cDNA insert with deletion of the core DBD region (Δ185–251) [20]. As shown in Fig. 3, basal and TCDD-induced expression of *CYP1A1* were increased ~7.5- and 24.7-fold, respectively, in pCEP4-He11-transfected cells relative to TCDD-induced *CYP1A1* expression in cells harboring the empty expression vector.

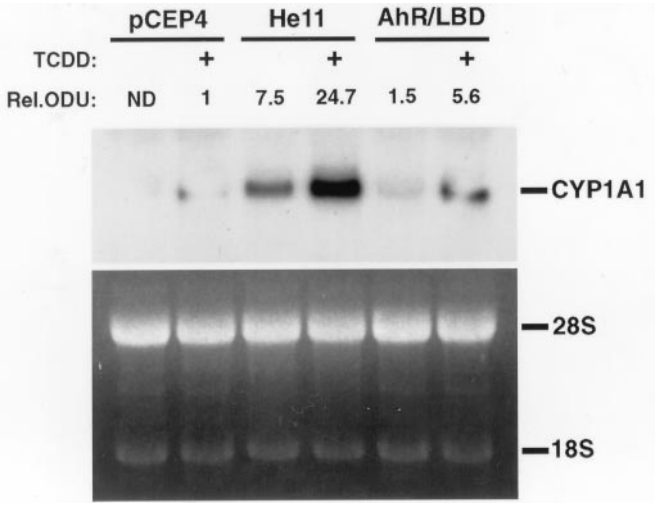


**FIG. 3. Determination of Ah-responsiveness in cells overexpressing HSP90-binding proteins. Upper panel: Analysis of *CYP1A1* expression in MDA-MB-231 cells. Stable transfectants were selected for retaining the pCEP4 episomal eukaryotic expression vector, pCEP4-He11, or pCEP4-AhR/LBD. Cells were treated either with vehicle alone or 10 nM TCDD for 24 hr prior to RNA isolation. Northern blot analysis was performed as described in Materials and Methods. Exogenous mRNA expression was verified by reprobing the membrane with radiolabeled He11 and AhR/LBD oligonucleotides. Lower panel: Photograph of total RNA run on an agarose/ethidium bromide gel to assess RNA integrity and quantity. 18S and 28S indicate the location of ribosomal RNA bands. These data are representative of two experiments using different RNA preparations. Similar results were also achieved by cotransfecting the above expression vectors with the TCDD-responsive pGL3–1A1 reporter plasmid in transient transfection assays. ND, not determined; Rel. ODU, relative optical density units.**

These data indicated that the ER is not transactivating a critical gene in the AhR signal transduction pathway. To exclude the possibility that the TAF1 or TAF2 transactivation regions within the ER are squelching an inhibitory factor that also is associated with the AhR, we constructed an expression vector containing a short region of human AhR spanning the overlapping ligand- and HSP90-binding

domains. The design of this vector was based on work by Whitelaw *et al.* [21], who delineated a region within the murine AhR, located between amino acids 230 and 421, that co-immunoprecipitates with HSP90. Stable transfection of MDA-MB-231 cells with pCEP4-AhR/LBD was found to increase TCDD-induced *CYP1A1* transcript levels ~5.6-fold relative to control cells (Fig. 3). A probable explanation for lower levels of *CYP1A1* expression in pCEP4-AhR/LBD- versus pCEP4-He11-transfected cells is that the former protein product has less affinity for HSP90: although the LBD of AhR fulfills the minimal requirement for HSP90 binding, it has been shown that involvement of the bHLH domain is necessary for high affinity binding [46]. However, by restricting our experiments to the LBD, we have minimized the likelihood that factors apart from HSP90 are responsible for increasing Ah-responsiveness in this model. Therefore, these data strongly suggest that Ah-responsiveness can be restored in MDA-MB-231 cells by squelching HSP90.

Previously, Wang *et al.* [47] cotransfected mutant ER expression plasmids and TCDD-responsive reporter constructs into ER-negative Hs578T cells. These studies showed that expression of C-terminal-deleted ER (He15, Δ282–595) did not restore Ah-responsiveness, whereas TCDD-induced CAT activity was increased 23-fold in cells expressing N-terminal-deleted ER (He19, Δ1–178). Significantly, the He15 and He19 protein products possess differential affinities for HSP90, since only He19 forms 8–9S complexes when expressed in COS-7 cells [13]. Therefore, these data support the hypothesis that AhR signaling can be enhanced via expression of HSP90-binding proteins.

Thomsen *et al.* [8] showed that *CYP1A1* promoter-CAT activity was not increased significantly by TCDD in MDA-MB-231 cells transfected with a human progesterone receptor expression plasmid, indicating that not all HSP90-binding proteins have the ability to restore Ah-responsiveness. This finding may be related to the localization of the PR relative to ER, He11, He15, and AhR/LBD. Steroid receptors actively shuttle between the cytoplasm and nucleus through the accessibility of nuclear localization signals on their surfaces [48]. However, these motifs vary structurally and in number between the various family members. For the cytoplasmic GR, two NLSs have been characterized, one between the DBD and LBD regions and the other within the LBD [49]. It is thought that binding of ligand to the GR causes release of HSP90, unmasking of its NLS(s), and subsequent translocation of the receptor to the nucleus [50]. In the case of the PR, three putative NLSs have been identified in the second zinc finger of the DBD, the hinge region, and a weaker site in the LBD [51, 52]. These different signals cooperate to elicit a strong nuclear localization. In contrast, only one NLS has been identified in the ER, which is located between the LBD and DBD regions [53]. Although the ER is found primarily in the nucleus, a portion also is found in the cytoplasm, as evidenced by the finding that about 15% of the ER content of MCF-7 cells resides in the cytosolic fraction [54]. We have shown that expression of 90NLS does not affect Ah-responsiveness in MCF-7 and T47-D cells. In a similar fashion, it may be that nuclear squelching of HSP90 by the PR does not modify AhR signaling appreciably. It remains to be determined, however, whether a greater amount of ER is cytoplasmic relative to PR, or if these receptors possess different affinities for HSP90.

### *Correlation of HSP90 Subcellular Localization with ER Status*

Two HSP90 homologues are expressed in higher eukaryotes, which are termed α and β in humans (reviewed in Ref. 55). Only a minor proportion of the total cellular content of HSP90 normally is situated in the nucleus. During heat shock, a reversible time-dependent nuclear translocation of HSP90 occurs [56]. It has been shown that estrogen treatment increases cellular HSP90α mRNA and protein levels [57, 58], although it is not known whether estradiol or TCDD alters the subcellular localization of HSP90. To address this question, MCF-7, Adriamycin-resistant MCF-7, MDA-MB-231, and S30 cells were incubated with either $10^{-7}$ M estradiol or 10 nM TCDD for 0, 1, and 2.5 hr on coverslips, fixed, and then probed with an anti-HSP90 antibody that recognizes HSP90α and β proteins. In each instance, drug exposure did not cause a change in HSP90 subcellular distribution as determined by confocal microscopy (data not shown).

These results indicated that estradiol and TCDD did not invoke a nuclear translocation of HSP90, which has been shown to coincide with a transient paralysis of estradiol-mediated gene transactivation [59]. However, it was discovered that the subcellular localization of HSP90 correlated with the ER status of the cell line (Fig. 4). HSP90 is primarily cytoplasmic in ER-expressing cell lines MCF-7 and S30 (panels 1A and 4A), whereas ER-negative Adriamycin-resistant MCF-7 and MDA-MB-231 cells have an equal distribution of HSP90 between the cytoplasm and nucleus (panels 2A and 3A). No differences in HSP90 expression were found between the matched $ER^+/ER^-$ cell lines by immunoblot analysis (data not shown).

To determine whether ER expression had an effect on AhR distribution, cells were co-incubated with anti-AhR antibody. As shown in Fig. 4, AhR is visualized throughout the cytoplasm in MCF-7 cells (panel 1B), in contrast to Adriamycin-resistant MCF-7 cells (panel 2B), where there is an uneven distribution of AhR that is more associated with the cell periphery. MDA-MB-231 cells have a similar pattern of expression as seen in Adriamycin-resistant MCF-7 cells (panel 3B vs 2B), and transfection of hER into this cell line appears to restore the phenotype observed in MCF-7 wild-type cells (panel 4B vs 1B). It will be interesting to determine whether the subcellular localization of AhR has an effect on its transactivation function.

In conclusion, data presented here provide evidence for the mechanism of cooperation between the AhR and ER

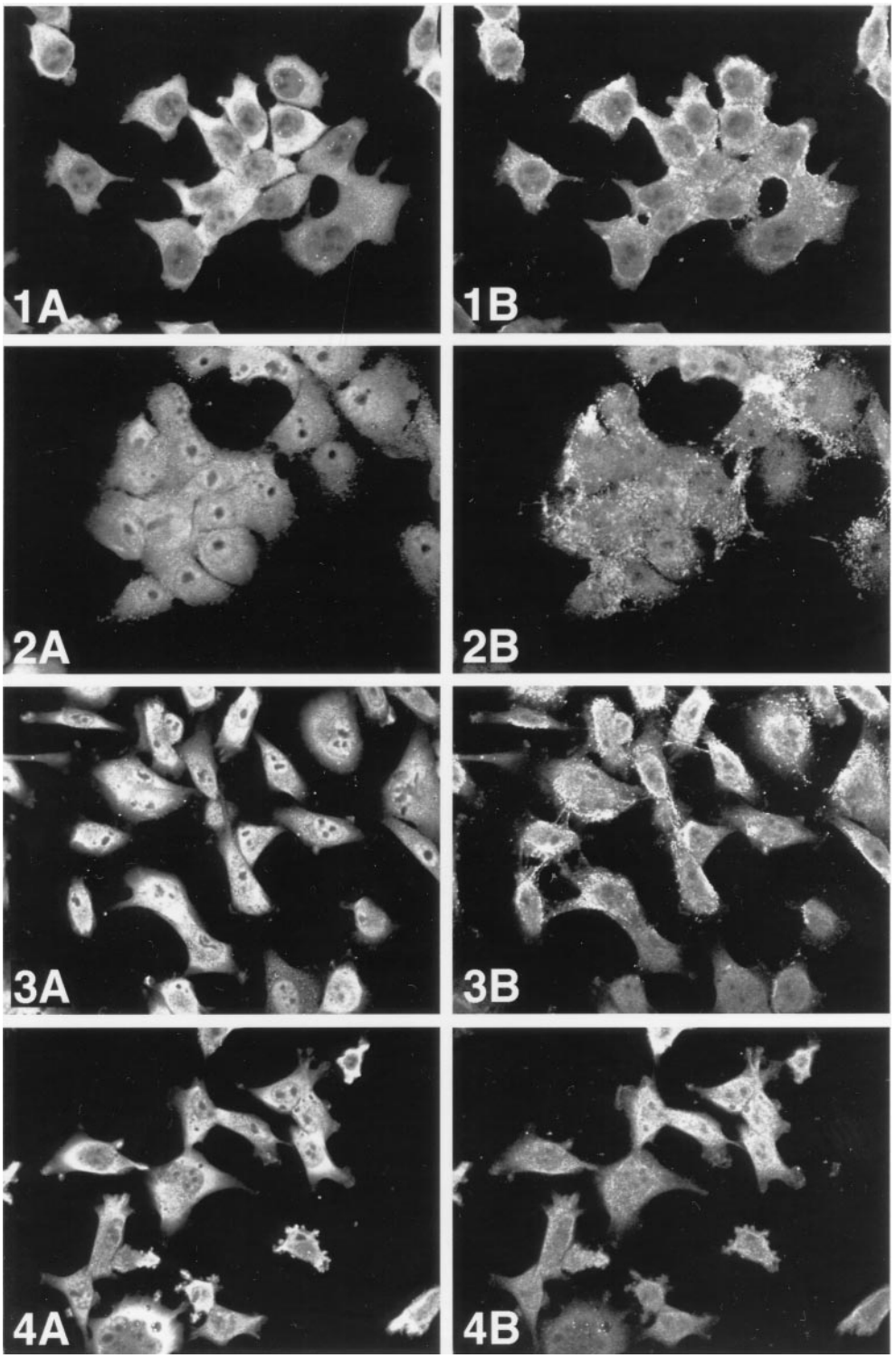


**FIG. 4. Association of HSP90 localization with ER status in matched ER-negative and -positive human breast cancer cells. Cells plated the previous day on coverslips were fixed and co-incubated with anti-HSP90 and anti-Ah receptor antibodies as described in Materials and Methods. Subcellular localization of (A) HSP90 and (B) Ah receptor were detected by indirect immunofluorescence staining and laser confocal microscopy. (1) $ER^+$ wild-type MCF-7 cells; (2) $ER^-$ Adriamycin-resistant MCF-7 cells; (3) $ER^-$ MDA-MB-231 cells; and (4) $ER^+$ S30 cells.**

signaling pathways. We have utilized receptor deletion mutants to squelch HSP90, resulting in restoration of Ah-responsiveness in ER-negative human breast cancer cells. In addition, overexpression of HSP90 was found to modulate AhR signaling *in vivo*. Transfection of HSP90 did not have a similar effect on ER activation. It is possible that these results reflect a greater stability of the AhR-HSP90 association relative to ER-HSP90. Finally, we have exam-

ined the subcellular localization of HSP90 in human breast cancer cell lines. HSP90 was found to be primarily cytoplasmic in ER-positive cells, whereas in ER-negative cells HSP90 was distributed equally between the cytoplasm and the nucleus.

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