Rapid Activation of c-Src Kinase by Dioxin Is Mediated by the Cdc37−HSP90 Complex as Part of Ah Receptor Signaling in MCF10A Cells†

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ABSTRACT: We investigated the mechanism by which activation of the Ah receptor by dioxin (TCDD) was accompanied by rapid activation of c-Src kinase activity. A Western blotting analysis showed that such action of TCDD in MCF10A cells could effectively be suppressed by treatment with a specific inhibitor of Src family kinase, PP-2, as judged by Western blot detection of the active form of Src protein, indicating that Src kinase is directly activated by TCDD. Such an event, occurring within 10−30 min of the addition of TCDD, is also accompanied by simultaneous translocation of both Src and cdc37 proteins from cytosol into the 100000 × *g* membrane fraction containing the plasma membrane. By dissociating the cytosolic Src−cdc37−HSP90 complex with 17 nM geldanamycin, an optimum concentration for affecting this cytosolic cdc37 complex, but not the cytosolic Ah receptor complex, we could show that the action of TCDD in activating c-Src and cdc37 was abolished, but not its action on CYP1A1. The important role of cdc37 in the action of TCDD-induced activation of c-Src was also confirmed by blocking cdc37 gene translation with the antisense oligonucleotide treatment as well as the siRNA preparation designed to silence cdc37 expression. To understand the functional meaning of the disruption of the Src−cdc37−HSP90 complex by 17 nM geldanamycin at the cellular level, we investigated its effect on TCDD-induced anti-apoptotic action. The results showed that geldanamycin at this concentration could also abolish this cellular effect of TCDD. Interestingly, such a role of cdc37 in mediating the action of TCDD appears to be limited to activation of c-Src kinase, but not kinases associated with activation of NFkB, C/EBPα, or ERK. Together, these observations support the hypothesis that there is a specific coordination between the activation of the cytosolic Ah receptor and the c-Src- and cdc37-containing HSP90 complex.

Dioxin is a very toxic environmental pollutant which is known to cause a variety of health problems in humans through activation of a specific cytosolic receptor, Ah receptor (*1*). While the action of the ligand-activated Ah receptor in inducing many detoxification enzymes is well-known, how it generates its toxic signaling is not. One available concrete clue is that the toxic action of TCDD[1] (the most common congener of dioxin) in several types of cells is accompanied by activation of c-Src (*2*). Indeed, it was originally shown by Bombick and Matsumura (*3*) in NIH-3T3 mouse fibroblasts in vitro and in mouse liver in vivo that TCDD caused rapid activation of $pp60^{c\text{-}Src}$. Such an action of TCDD in many types of cells is clearly mediated through the Ah receptor (*4*), considered to be responsible for most of the toxic actions of this compound (*1*). The toxicological importance of c-Src in the action of TCDD has been demonstrated by studying the differential toxicity of TCDD on c-src knockout mice versus its congenic wild-type counterpart, C57BL/6 mice. It was found that TCDD-treated c-src knockout mice exhibited reduced toxic symptoms, particularly in terms of those associated with TCDD-induced "wasting syndrome": hyperlipidemia, liver glycogen depletion, increased hepatic glucose export, downregulation of PECK, and fatty liver (*5*−*7*). Also, in vitro studies have shown that mouse embryonic fibroblasts prepared from these $src^{-/-}$ mice also exhibited reduced sensitivity to TCDD as compared to the $src^{+/+}$ fibroblast counterparts in terms of its action to inhibit adipocyte differentiation (*8*). Thus, there is good reason to further study the mechanism of this Src activation by TCDD. Furthermore, it was reported by Park and Matsumura recently (*9*) that c-Src plays an important role in assisting the action of TCDD in preventing apoptosis of MCF10A cells from taking place as a result of UV irradiation.

Activation of c-Src induced by binding of TCDD to the Ah receptor is known to involve translocation of c-Src protein from cytosol to the plasma membrane as shown in liver WB-F344 cells (*10*) which apparently triggers the growth factor signaling cascade (*11*), including rapid activation of ERK (p42/44 MAPK) (*12*−*15*) in a variety of cells.

The main puzzle has been how TCDD, through its binding to the Ah receptor, could activate c-Src. It is clear now, thanks to the in-depth analysis on the Ah receptor, that the cytosolic form of the Ah receptor complex does not contain

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[1] Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP1A1, cytochrome $P_{450-1A1}$; HSP90, heat shock protein 90; AhR, aryl hydrocarbon receptor; MEBM, mammary epithelial basal medium; DEPC water, diethyl propiocarbonate-purified water; XAP2, X protein-associated protein 2; GA, geldanamycin; FKBP52, FK506 binding protein 52.

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c-Src protein (e.g., ref *16*). The ligand binding subunit of the complex, also sometimes termed AhR, nevertheless appears to be rather promiscuous, being able to respond to many ligands as well as to certain agents capable of activating it through ligand-independent mechanisms (*17*). Despite such excellent advances in the understanding of the Ah receptor activation mechanisms, there is still no clue to relate any of those to the action of the liganded Ah receptor to activate c-Src kinase. Recently, we have found in MCF10A cells in vitro, the same material used in the investigation described here, that TCDD causes a rapid activation of c-Src and ERK kinase activities within 10 min of the addition of TCDD (*18*). Such an observation supports our notion that this action of TCDD is not mediated by direct transactivation of gene transcription, and therefore, this subject deserves a new study approach apart from the traditional gene trans-activation approaches already used.

The *cdc37* gene was first identified in a *Saccharomyces cerevisiae* mutant strain which exhibited a G_1 cell cycle arrest phenotype (*19*). Grammatikakis et al. (*20*) demonstrated that Cdc37 facilitated the association of the protein kinases with Hsp90. The ability of Cdc37 to bind protein kinases and Hsp90 could lead to the hypothesis that Cdc37 acted as a protein kinase targeting cochaperone of Hsp90. The organization of the cytosolic complex containing cdc37 protein ($p50^{cdc37}$) consisting of 2 mol of Hsp90 (heat shock protein 90), p23, and "chaperoned protein" such as Src kinase has been summarized by Pratt et al. (*21*) and Pratt (*22*). In analogy with the well-studied cytosolic complex containing the glucocorticoid receptor (GR), these cytosolic heterocomplex assemblies are thought to represent their resting forms that are ready to be activated by their ligand binding. One clear-cut difference between the steroid receptor complex and those chaperoning protein kinases is that the former contains FKBP52 (FK506 binding protein), but the latter consists of cdc37 protein in place of FKBP52. These two homologous proteins are collectively called "immunophilins". As for the function of these immunophilins, the most accepted view is that they act as transporters of "chaperoned proteins". In the case of the steroid receptor, it is FKBP52 that comigrates into the nucleus, and in the case of protein kinases, cdc37 protein is cotransported to the action site, which is on the plasma membrane in the case of Src kinase. Indeed, the cytosolic Hsp90 complex chaperoning tyrosine kinase was originally discovered to occur for v-Src protein. In contrast, the cytosolic Hsp90 heterocomplex housing the Ah receptor (AhR) is somewhat different from the two classes of complexes mentioned above in that the last one contains a chaperone protein called AIP (also called ARA9 or XAP2), instead of FKBP52 or cdc37. This protein is only 32% homologous to human FKBP12 but is thought to comigrate into the nucleus with AhR (*23*).

Interestingly, there have been reports suggesting that there is cross-talk among these cytosolic complexes. For instance, Widen et al. (*24*) report that GR exhibits cross-talk with Raf-1, which is chaperoned by cdc37 (e.g., ref *25*), just as in the case of Src kinase. AhR has been implicated to cross-talk with the androgen receptor (*26*) in LNCap prostate cancer cells, and furthermore, the cdc37 protein itself appears to fuctionally interact with the androgen receptor (*27*). These observations provide at least the precedents for the current research work, which has been designed to address the question of why and how ligand-induced activation of AhR is associated with subsequent upregulation of Src kinase activities.

The primary objective of this study, therefore, is to test our working hypothesis that TCDD-induced activation of c-Src kinase is mediated by the cross-talk between the ligand-activated Ah receptor in cytosol and the cytosolic c-Src chaperone complex (i.e., Src−cdc37−HSP90 complex) (*18*) and that the cdc37 protein plays an essential role in the process of c-Src kinase activation. To this end, we found that indeed cdc37 plays an indispensable role in the TCDD-induced activation of c-Src and now report the results.

EXPERIMENTAL PROCEDURES

Cell Culture. MCF10A cells were grown in complete growth medium, a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 20 ng/mL epidermal growth factor (EGF), 100 ng/mL cholera toxin (CTX), 5 μg/mL insulin, 500 ng/mL hydrocortisone (H), 5% bovine calf serum, and 1% antibiotic (penicillin and streptomycin). Insulin, hydrocortisone, and CTX are needed to promote MCF10A cells to epithelial cells which are more susceptible to the growth inhibitory action of TCDD (*18*).

Preparation of Cell Extracts. Cells were seeded at a density of 7×10^5 cells/dish on 100 mm tissue culture dishes (Falcon) and incubated in a humidified atmosphere containing 5% (v/v) CO_2 in air at 37 °C. The medium was renewed every 2−3 days. When the cells reached 80% confluency, the medium was changed to MEBM (mammary epithelial basal medium, serum-free, from Clonetics, catalog no. CC3151), supplemented with 100 ng/mL cholera toxin (CTX). Inhibitors were preincubated for 1 h for 1 μM PP-2 or 10 μM PD98059 and for 24 h for 17 nM geldanamycin. After that, 10 nM TCDD in *p*-dioxane or the same volume of *p*-dioxane alone was added directly to the medium.

Whole cell extract, membrane, and NP-40 insoluble fractions were prepared as described previously (*9*, *14*, *17*).

Immunoprecipitation. After cells were treated with 10 nM TCDD for 60 min, they were lysed with NP-40 buffer supplemented with protease inhibitor cocktail (Sigma, 1:100 dilution) on ice for 20 min and centrifuged at $16000 \times g$ for 5 min. Supernatants were incubated with 2 μg of anti-Cdc37 antibody (H-271, Santa Cruz Biotechnology), or in a separate experiment with 2 μg of anti-Src antibody (Src2, Santa Cruz Biotechnology), for 2 h at 4 °C. Afterward, an aliquot of 20 μL of Protein G plus/protein A−agarose conjugate (Oncogene Research Products) was added and further incubated with gentle shaking at 4 °C overnight. The pellet was washed six times via brief centrifugation followed by resuspension into the same volume of fresh NP-40 buffer supplemented with protease inhibitor cocktail. The final pellet was resuspended in 30 μL of "2× SDS sample buffer" and boiled for 5 min to release the proteins bound to the antibody. In each case, the released proteins were probed with the following immunoblotting protocol using both antibodies to confirm the presence of both cdc37 and Src proteins in the immunoprecipitated preparations.

Immunoblotting. SDS−polyacrylamide gel electrophoresis, whole cell extracts, plasma membrane, and NP-40 insoluble fractions were separated by 10% SDS−PAGE at 70−100 V and transferred to PVDF membranes (Bio-Rad) at 70 V

(constant) for 1.5 h with ice block in the Bio-Rad Mini Protean apparatus. The transfer was carried out in a transferring buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 10% methanol]. Membranes were blocked with 0.4% nonfat milk in TBST buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature. Then membranes were incubated with the primary antibody in blocking solution overnight at 4 °C: anti-Src (Src2, Santa Cruz Biotechnology) at a 1:500 dilution, anti-Cdc37 (H-271, Santa Cruz Biotechnology) at a 1:500 dilution, and p-ERK (E-4, Santa Cruz Biotechnology) at a 1:500 dilution. After incubation with the horseradish peroxidase-conjugated secondary antibody in TBST for 3 h at room temperature, blots were developed using the SuperSignal West Pico detection kit (Pierce). All Western blottings were repeated several times for each experiment to confirm the reproducibility of the results being reported herein.

Treatment of Cells with cdc37 Antisense Oligonucleotides. Fifteen-base cdc37 antisense (5′-GCTGTAGTCCACCAT-3′) oligonucleotides were synthesized via the standard procedure.

MCF10A cells were placed in 60 mm tissue culture dishes (corning) at a density of 3.0×10^5 cells/dish in 2 mL of complete growth medium. When the cells reached 50% confluency (in 3 days), cells were washed twice with 2 mL of serum-free MEBM. Transfections were performed using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Antisense oligonucleotides (1 μM) were incubated with oligofectamine in MEBM with a final volume of 2 mL for 24 h at 37 °C in a CO_2 incubator. The same amount of oligofectamine was added directly to mock-transfected dishes without the antisense oligonucleotide. After 24 h in the medium, TCDD was treated without medium change. After 24 h, total RNA was extracted for RT-PCR.

RNA Extraction. MCF10A cells were placed in 60 mm tissue culture dishes (Corning) at a density of 3.0×10^5 cells/dish in 2 mL of complete growth medium. Medium was changed every day until the cells reached 80% confluency. Then the medium was changed to MEBM, supplemented with 10 ng/mL epidermal growth factor (EGF) and 100 ng/mL cholera toxin (CTX). After 24 h, 10 nM TCDD was added. Chemicals were preincubated before TCDD treatment: 20 μM dexamethasone for 40 min and 17 nM geldanamycin for 24 h. When the experiment was finished, cells were washed twice with 2 mL of serum-free MEBM. Trizol (1 mL) was added to the culture dish, and cells were scraped with a cell scraper. DNA was sheared by drawing the solution through a 22 gauge needle four or five times. The solution was transferred to a microcentrifuge tube and centrifuged at 12000 $\times$ *g* for 10 min to eliminate any insolubilized material. Supernatant was transferred to a clean microcentrifuge tube, and 0.2 mL of chloroform was added. The solution was vortexed well and then centrifuged at 12000 $\times$ *g* for 15 min. After the centrifugation was completed, the upper layer was transferred to a new tube. 2-Propanol (0.5 mL) was added, and the solution was mixed and incubated at room temperature for 10 min. The solution was then centrifuged at 12000 $\times$ *g* for 10 min. The supernatant was discarded, and the pellet was dissolved in 300 μL of DEPC water. A 300 μL mixture of phenol and chloroform (1:1) was added, and the mixture was vortexed and centrifuged at 12000 $\times$ *g* for 5 min. The top layer was transferred to a clean tube, and 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added. The mixture was incubated at −20 °C overnight and centrifuged at 12000 $\times$ *g* at 4 °C for 10 min. The supernatant was discarded, and the pellet was rinsed with 70% ethanol. After the pellet had air-dried, it was dissolved in DEPC water.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Reverse transcription was performed with 1 μg of total RNA and RT primer in a final reaction volume of 10 μL. The mixture was run in an annealing program (5 min at 60 °C). RT mixture (30 μL) of the Omiscript kit (Qiagen), dNTPs, 10× RT buffer, and RTO, and RNA-guard (Amersham), was added each tube, and the RT program was run (60 min at 37 °C, 10 min at 70 °C, and cooling to 4 °C). PCR amplification was performed with cDNA, SYBRgreen (Qiagen), RNAase-free water, and each primer in a reaction volume of 20 μL using LightCycler (Roche). The conditions are denaturing for 15 min at 95 °C before the first cycle, 15 s at 94 °C for denaturing, 20 s at 59 °C for annealing, and 20 s at 72 °C for primer extension for 40−50 cycles. The data were normalized to the amount of β-actin signals.

Apoptosis Assay. Apoptosis assays were performed as described by Park and Matsumura (*9*). Briefly, MCF10A cells were grown in complete growth medium for 24 h, and then the medium was changed to serum-free MEBM supplemented with 5 ng/mL EGF, 100 ng/mL CTX, and 1 μg/mL hydrocortisone. After 24 h, TCDD was added and the mixture incubated for 2 h at 37 °C. Chemicals were preincubated before the 10 nM TCDD treatment: 1 μM PP-2 for 1 h, 1 μM FK506 for 12 h, and 17 nM geldanamycin for 24 h. Afterward, the cells were treated with UV irradiation (100 μJ/cm^2) and incubated for 4 h at 37 °C. The cells were incubated with annexin-V-FITC for 10 min at 37 °C in the presence of 200 μM $CaCl_2$. In 10 representative fields, apoptotic cells were counted directly by using the fluorescence microscope.

Statistics. Statistical analyses were performed with ANOVA (LSD) using SYSTAT. All experiments were repeated at least three times, and the significant differences were expressed at a $P < 0.05$ level.

RESULTS

We began this project by first confirming the action of TCDD in rapidly activating c-Src (Figure 1A), as judged by its translocation into the 100000 $\times$ *g* membrane fraction and into the intracellular matrix (i.e., NP-40 insoluble fraction) by 30 min. It must be added that this Src2 antibody does not distinguish the active form of Src from the inactive one, and therefore, its translocation from the cytosol to the 100000 $\times$ *g* membrane fraction has been used as the indication of its activation. We could also ascertain that the above action of TCDD was accompanied by the activation of ERK by 15 min (Figure 1B), as assessed by double phosphorylation on pp42/44, through Western blotting detection with the corresponding specific primary antibodies. According to the preliminary studies, the optimum timing of TCDD incubation for detecting the effect of TCDD on ERK was determined to be 15 min, since its activity declined thereafter. In contrast, the earliest time point for obtaining significant upregulation of c-Src was determined to be 30 min since its level of

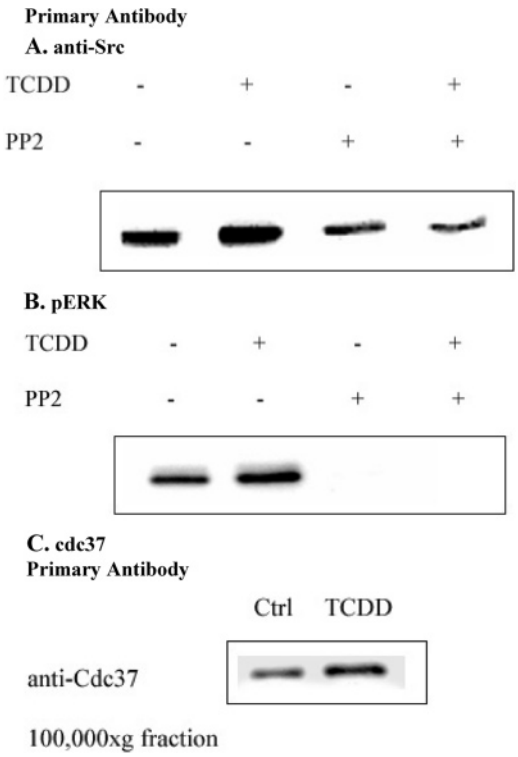

FIGURE 1: Effects of TCDD treatment of MCF10A cells on (A) c-Src, (B) ERK (pp42/44 MAPK), or (C) cdc37 activation. Activation of the function of c-Src (A) and cdc37 (C) was assessed by their translocation from cytosol to the plasma membrane. The cells were grown in the complete growth medium. When the cells reached 80% confluency, the medium was changed to serum-free MEBM containing 10 ng/mL cholera toxin (CTX). After growth factor starvation for 23 h, the cells were preincubated with inhibitor, PP-2 (PP2) or 10 μM PD98059 (PD), for 1 h before TCDD treatment (10 nM). Cells were incubated with 10 nM TCDD for 30 (A and C) or 15 min (B). Control cells were incubated with solvent (*p*-dioxane) of TCDD. Plasma membrane (A) or whole cell extract (B) was prepared as described in Experimental Procedures. All Western blottings were repeated several times to confirm the results, although only one example is shown in each panel.

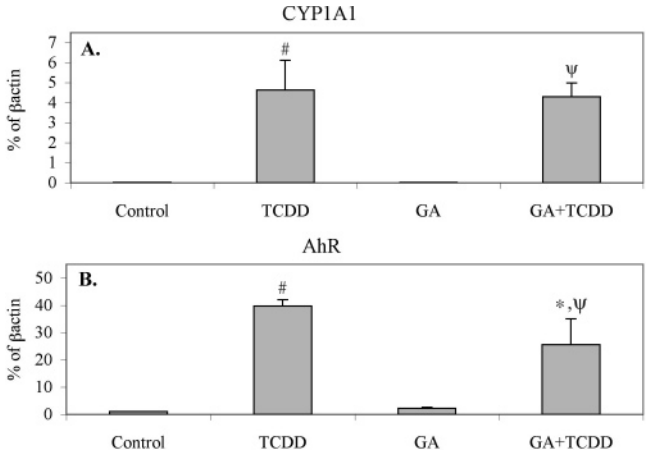

FIGURE 2: Effects of TCDD and geldanamycin (GA) on mRNA expression on CYP1A1 and AhR. MCF10A cells were grown under standard conditions, and experiments were performed as described in Experimental Procedures. TCDD (10 nM) was incubated for 6 h. Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between (#) control and others, (∗) TCDD and others, and (Ψ) GA and others; the statistically significant differences are expressed at the $P < 0.05$ level.

expression steadily increases for the initial period of 5−120 min. The test results showed that as judged by the increase in the titer of doubly phosphorylated forms of p42/44, TCDD was capable of rapidly activating both of these kinases, particularly p42 ERK (the lower band), and furthermore, in the presence of a Src kinase inhibitor PP-2, such an action of TCDD could not be observed in both cases. The possibility that such an action of TCDD is accompanied by translocation of cdc37 protein from the cytosol to the 100000 × *g* membrane fraction as described by Pratt et al. (*21*) was examined next. The result of this Western blot test confirmed the prediction given above (Figure 1C). To detect the Src−cdc37 complex, cells were first treated with TCDD (10 μM) for 60 min, lysed on ice with Nonidate P-40 buffer, and centrifuged at 16000 × *g*, and the supernatant was treated with cdc37 antibody, immunoprecipitated with A/G−agarose, and subsequently probed in Western blots with the anti-Src antibody. The Src content in the cdc37 immunoprecipitate was found to increase by 42.3% (standard deviation ± 28.7%, three replicates compared to control) as the result of TCDD treatment. Probing the same samples with the anti-cdc37 antibody gave a 29.8% increase (standard deviation ± 41.6%) over the control value. While this type of data is not expected to give the quantitative accuracy, they show that these two proteins are found together in those Nonidate P40-lysed cell supernatant fractions. On the other hand, while this observation showed that activation of cdc37 and c-Src appears to take place within 30 min as in the case of c-Src translocation and activation, as judged by their 100000 × *g* membrane fraction translocation, it does not automatically prove that the former is indispensable for the latter movement. Therefore, in the next experiment, we made an attempt to deactivate the Src−cdc37−HSP90 complex by selecting an optimum concentration of geldanamycin, which is a specific inhibitor of HSP90 associating with other chaperone proteins (*22*). After a number of preliminary tests (data not shown), we have selected 17 nM as the optimal dose for this inhibitor to significantly disrupt the c-Src−cdc37−HSP90 complex, but to affect only marginally the Ah receptor, the AhR−XAP2−HSP90 complex in this cell line (Figure 2A). In the data summarized in Figure 2, we could show that TCDD-induced upregulation of both CYP1A1 (Figure 2A) and AhR (Figure 2B) mRNA expressions was not significantly affected by this concentration of geldanamycin (GA) under our experimental condition as judged by quantitative RT-PCR, indicating that the transcriptional functions of AhR were not significantly affected by this treatment. In contrast, at this concentration, geldanamycin unambiguously suppressed the action of TCDD in activating cdc37 (Figure 3A) as well as c-Src (Figure 3C,D), as judged by the extent of their translocation into both plasma membrane and intracellular matrix (i.e., NP-40 insoluble fraction), confirming that geldanamycin is a powerful disruptor of the Src−cdc37−HSP90 complex. Interestingly, this action of geldanamycin does not seem to fully extend to ERK activation (Figure 3B). On the other hand, the pretreatment of cells with antisense cdc37 oligonucleotides could clearly abolish the action of TCDD for 30 min (Figure 3E).

To confirm that the action of GA described above indeed affected the Src−cdc37−HSP90 complex, we elected to study the effect of an antisense oligonucleotide treatment which has been designed specifically to suppress the cdc37

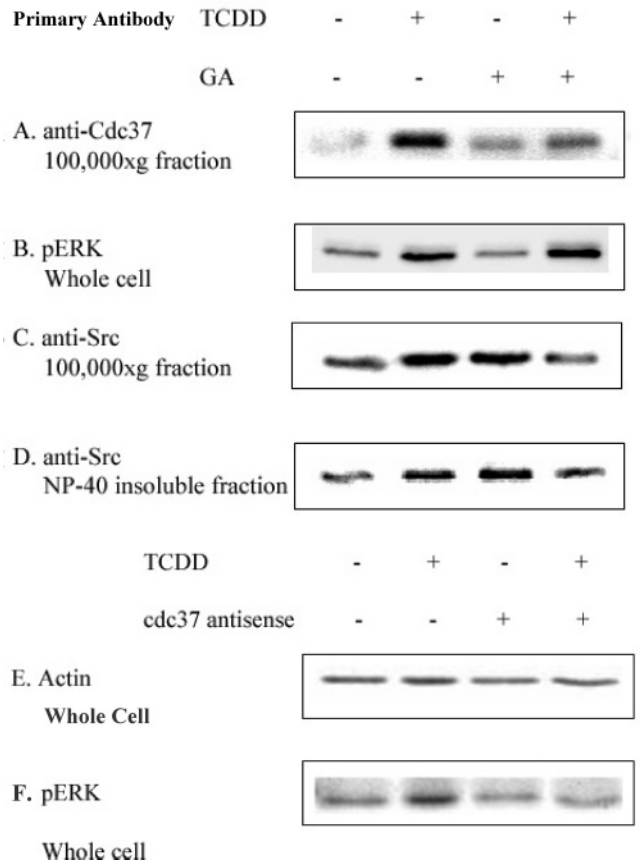

FIGURE 3: Effect of geldanamycin (A−D) and cdc37 antisense treatment (E) on TCDD's action on MCF10A cells. This experiment was performed essentially as described in the legend of Figure 1A, except when the cells reached 80% confluency, the medium was changed to MEBM containing 10 ng/mL CTX and 17 nM geldanamycin (GA) or the antisense treatment agent was added at the same time. After 24 h, cells were incubated with TCDD for 30 (A and C−E) or 15 min (B).

gene translation. The results of this antisense cdc37 treatment showed that the effect of TCDD in inducing translocation of Src protein to the 100000 × *g* membrane fraction was blocked in samples treated with this oligonucleotide blocker, but not in those treated with a mismatch oligonucleotide (Figure 4A). It was found that this antisense treatment also blocked the translocation of Src protein into the nucleus (data not shown). To supplement the studies described above, we have conducted a parallel siRNA silencing experiment on cdc37. This treatment was successful in suppressing the mRNA expression of cdc37 to approximately 50% of "mock" RNA-treated control cells. The result of qRT-PCR analysis of mRNA expressions of COX-2 and IL-1β showed that this treatment reduced the TCDD-stimulated portion of their expression to approximately 50 and 70%, respectively (data not shown). On the other hand, its influence on CYP1A1 expression was statistically insignificant. Additionally, the cdc37 antisense treatment induced a significant suppression of the mRNA expression of the EGF receptor gene, in both control (72.1% inhibition) and 24 h TCDD-treated cells (61.2% inhibition), indicating that significant suppression of cdc37 gene translocation occurred, since cdc37 is known to be needed for the expression of the EGF receptor (ref *28* and data not shown). After screening of additional candidate mRNAs had been carried out, two important nuclear transcription factor (NTF) mRNAs were found to be affected by this antisense treatment (Figure 4B,C). TCDD (24 h exposure) caused a significant increase in the level of mRNA expression of NFkB, a nuclear transcription factor known to mediate inflammatory signaling, particularly that from TNFα, and such an action of TCDD was also abolished by this cdc37 antisense treatment (Figure 4B). In a similar fashion, C/EBPα mRNA was also found to be affected by this treatment (Figure 4C). Exposure of MCF10A cells to TCDD for 24 h increased its level of expression by 24.5% (two replicates) over control (data not shown). The cdc37 antisense treatment suppressed it by 25.5% in control and by 25.0% when cells were treated with TCDD. To make sure that this antisense oligonucleotide treatment itself did not affect the functionality of the Ah receptor after this TCDD incubation period, we checked CYP1A1 (Figure 4D) mRNA induction using quantitative RT-PCR with the same cDNA samples and found that it is not affected at all. The same cDNA was probed for the mRNA expression of cdc37.

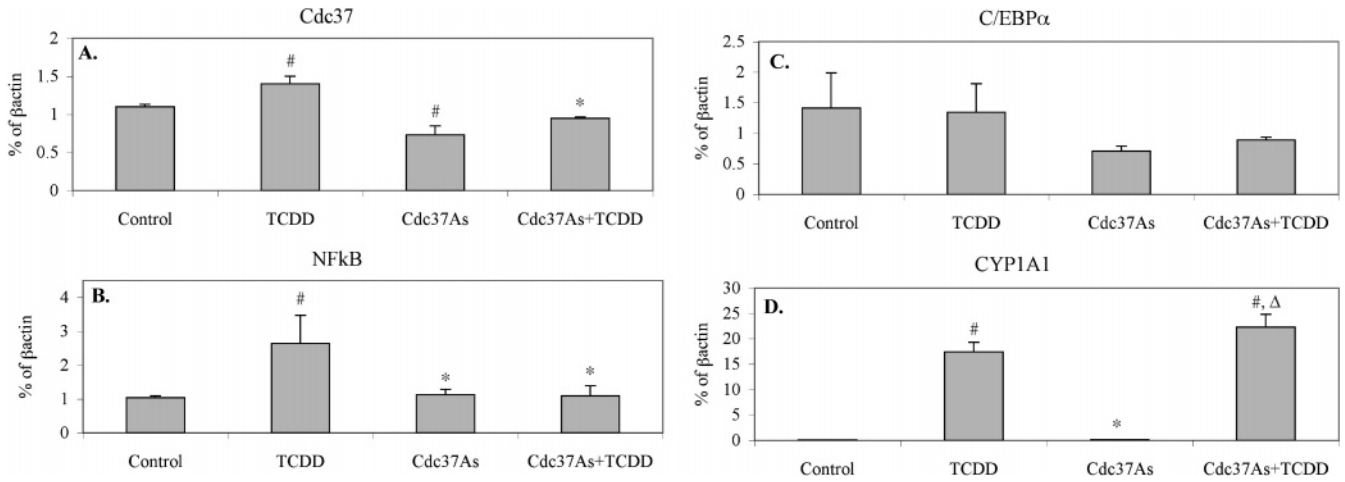

FIGURE 4: Effects of cdc37 antisense (cdc37As) treatment on the effect of the 24 h TCDD exposure on mRNA expression on (A) cdc37 itself, (B) NFkB, (C) C/EBPα, and (D) CYP1A1. In all cases, mismatch antisense oligonucleotides were used for control and TCDD-tested samples. The qRT-PCR experiments were performed as described in Experimental Procedures. Twenty-four hours post-transfection, TCDD or solvent alone was added and the mixture incubated for 24 h. Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between (#) control and others, (∗) TCDD and others, and (Δ) cdc37As and others; the statistically significant differences are expressed at the $P < 0.05$ level.

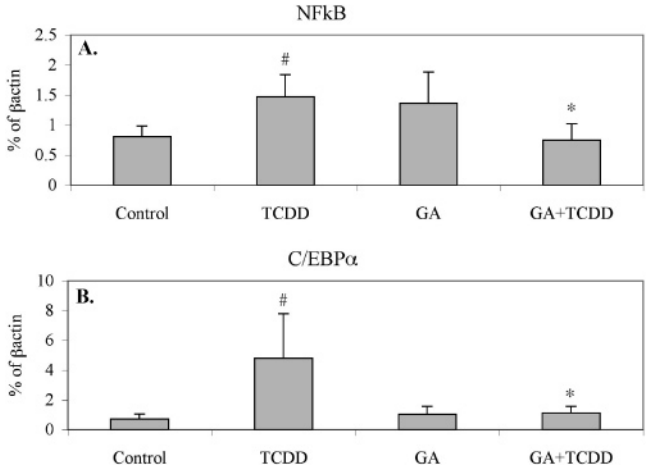

FIGURE 5: Effects of TCDD and geldanamycin (17 nM) on mRNA expression on (A) NFkB and (B) C/EBPα. The experimental protocols were identical to those described in the legend of Figure 3, except that the period of TCDD incubation was 6 h. Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between (#) control and others and (∗) TCDD and others; the statistically significant differences are expressed at the $P < 0.05$ level.

We further confirmed that essentially the same results could be reproduced by replacing the antisense treatment with geldanamycin pretreatment (Figure 5A,B) as the experimental approach to disrupt cdc37 complex formation. In view of these findings, a new question of the possibility of TCDD activating additional kinases through this mechanism of activation of cdc37−HSP90 complexes was raised. This is not a trivial question, since there are many tyrosine kinases packaged with cdc37 and HSP90 (*21*) and since TCDD is known to affect many protein kinases (*2*). This question was addressed by testing the effect of antisense or siRNA blocking of cdc37 mRNA affecting cells treated with TCDD for a very short time of 1 h to assess the immediate to early changes induced by TCDD. The main reason for this approach is that the signal transduction process affected at the early stage of TCDD's action is likely mediated by protein kinases and phosphatases. Since the available quantity of cells treated in this antisense or siRNA approach was small, we relied on the quantitative RT-PCR (qRT-PCR) method to assess the effect of cdc37 antisense treatment.

While the number of genes whose mRNA expressions are significantly affected by TCDD at this early stage is limited, we could show that the expressions of several mRNAs were indeed affected by TCDD. The result of the antisense treatment (Figure 6) showed that although the extent of suppression on cdc37 mRNA was modest, it produced some statistically significant effects (Figure 6A). Under this condition, this antisense treatment reduced statistically significant effects of the 60 min action of TCDD on COX-2 (Figure 6C) and IL-1β (Figure 6D), but not CYP1A1 (Figure 6B). A parallel siRNA experiment produced an approximately 40% reduction in the level of mRNA expression of cdc37 (Figure 7A). This siRNA treatment also produced reduction in the extent of the 60 min action of TCDD on COX-2 (Figure 7C) and IL-1β (Figure 7D) as in the experiment depicted in Figure 6. Additionally, it reduced the effect of TCDD on IL-8 (Figure 7E). However, this treatment

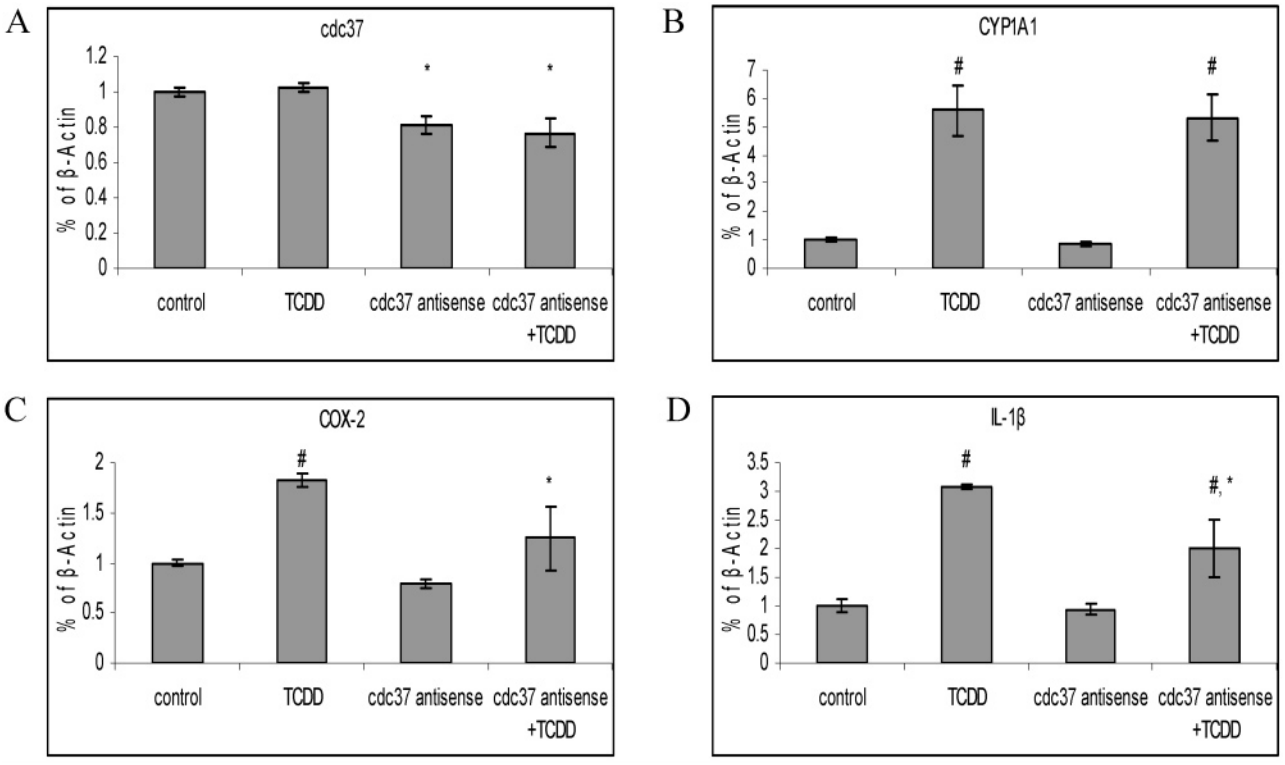

FIGURE 6: Influence of cdc37 antisense treatment on the effect of TCDD in the MCF10A cells. MCF10A cells were treated with or without antisense cdc37 (200 nM) for 48 h and then treated with or without TCDD (10nM) for 1 h. Antisense significantly reduced the level of cdc37 mRNA (A). TCDD strongly induced CYP1A1, and the ability of TCDD to induce CYP1A1 was not affected by the cdc37 antisense treatment (B). TCDD also significantly induced cox-2 and IL-1β. However, the ability to induce cox-2 and IL-1β was significantly abated by the cdc37 antisense treatment (C and D). Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between (#) control and others and (∗) TCDD and others; the statistically significant differences are expressed at the $P < 0.05$ level.

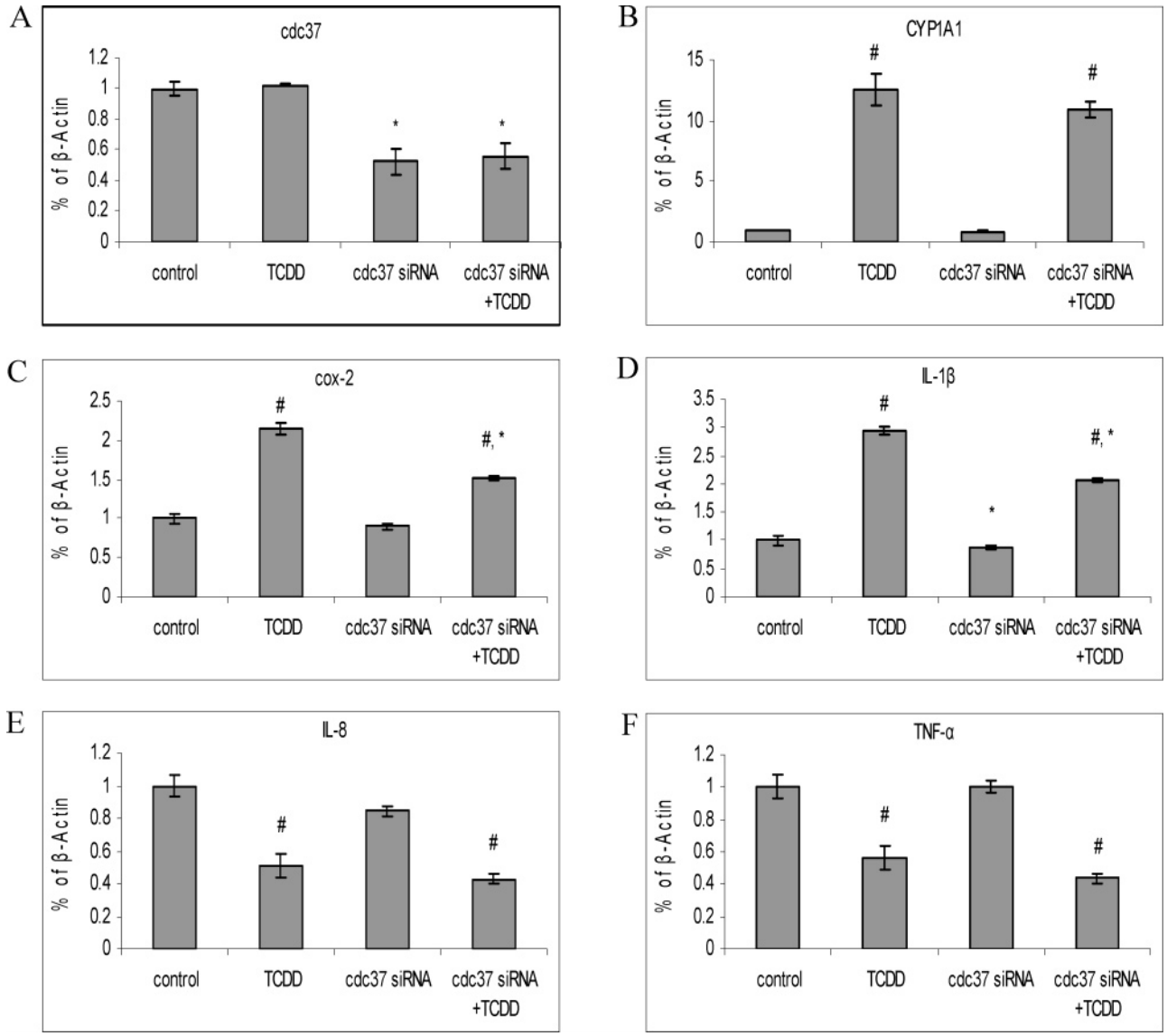

FIGURE 7: Influence of cdc37 siRNA on the effect of TCDD on the MCF10A cells. MCF10A cells were treated with or without cdc37 siRNA (50 nM) for 72 h and then treated with or without TCDD (10 nM) for 1 h. siRNA significantly reduced the level of cdc37 mRNA (A). TCDD strongly induced CYP1A1, and the ability of TCDD to induce CYP1A1 was not affected by the cdc37 siRNA treatment (B). TCDD also significantly induced cox-2 and IL-1β. However, the ability to induce cox-2 and IL-1β was significantly abated by the cdc37 siRNA treatment (C and D). TCDD also significantly reduced IL-8 and TNF-α. However, the ability to inhibit IL-8 and YNF-α was not significantly affected by the cdc37 siRNA treatment (E and F). Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between (#) control and others and (∗) TCDD and others; the statistically significant differences expressed at the $P < 0.05$ level.

did not affect the action of TCDD on CYP1A1 or TNFα (Figure 7B,F).

Next, we asked the question of the importance of this newly found cdc37-mediated pathway with respect to TCDD-induced anti-apoptotic action, used here as an indicator of TCDD-induced changes in whole cell function (*29*). We applied UV radiation to MCF10A cells under a slight growth factor-deprived condition (i.e., 24 h after the last medium/serum change) (*9*) to induce apoptosis even in control cells, to assess the effect of both geldanamycin and TCDD (2 h treatment), even when there was no UV irradiation. The result (Figure 8A) showed that indeed geldanamycin treatment (shown as GA) abolished this anti-apoptotic action of TCDD to prevent UV-irradiated cells from going through apoptosis; i.e., in the presence of geldanamycin (UV + GA), TCDD exhibited no apoptosis rescuing action (UV + GA + TCDD). Just to make sure that c-Src is mediating this action of TCDD, we also repeated the above test with PP-2, a specific Src inhibitor, in place of geldanamycin, and found that they also reversed the action of TCDD (Figure 8B).

DISCUSSION

The action of the Ah receptor in mediating activation of the tyrosine kinase cascade upon its ligand binding is now well recognized (*1*, *2*, *10*, *30*). The main question remaining unanswered is how the TCDD-bound and thereby the activated Ah receptor transmits its signaling to activation of c-Src and on to other tyrosine kinases. This is an important question as pointed by Delescluse et al. (*31*), who showed that the conventional scheme of the Ah receptor activation based on the cytochrome P_{450} induction mechanism does not seem to be applicable to this case. For instance, TCDD-

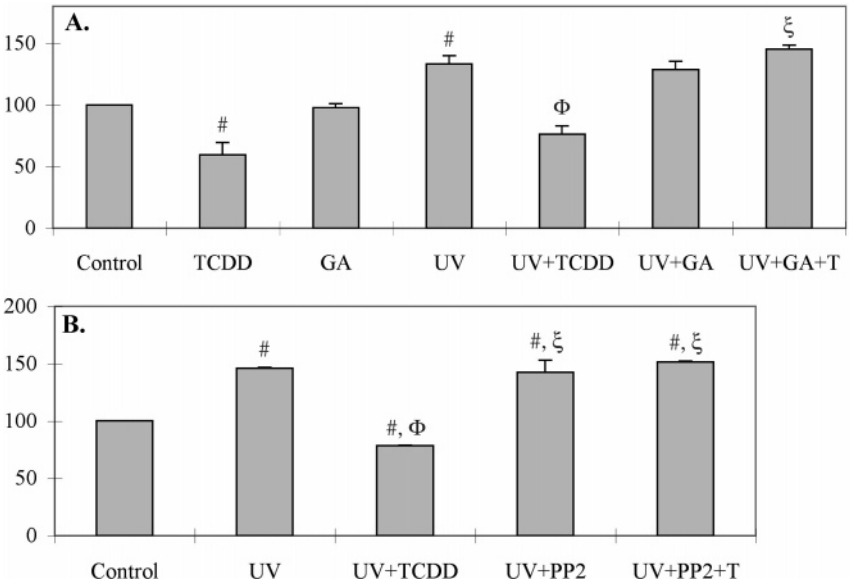

FIGURE 8: Effect of (A) geldanamycin and (B) PP-2 on TCDD's anti-apoptotic effect against UV irradiation-induced apoptosis. The cells were seeded on a 60 mm × 15 mm tissue culture dish at a density of 1.0×10^5 cells/dish in the complete medium. After 24 h, the medium was changed to MEBM serum-free medium, including 5 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, and 1 ng/mL cholera toxin. After being starved for 24 h, the cells were directly treated with 10 nM TCDD for 2 h, exposed to UV, and incubated for 4 h. Geldanamycin (17 nM) was preincubated for 24 h, and PP-2 was preincubated for 1 h before the TCDD treatment. Apoptotic cells were counted as described in Experimental Procedures. (B) The samples treated with TCDD alone (without UV) exhibit the reduced apoptosis level that is indistinguishable from that of UV and TCDD (data not shown). Statistical analyses were conducted with ANOVA, and the results are expressed as paired comparisons between any two of the following: (#) control (=100) and others, (Φ) UV and others, (ξ) UV with TCDD and others. The statistically significant differences are expressed at the $P < 0.05$ level.

induced activation of c-Src kinase in this cell line takes place very rapidly within 10 (*32*) or 15 min (*18*), well before induction of CYP1A1.

The compositional information for the Ah receptor complex in cytosol is now well-known, thanks to the efforts made by many researchers in this field (*33*−*37*). The Ah receptor complex consists of a dimer of 90 kDa heat shock proteins, an immunophilin-like protein XAP-2 (also known as ARA9 or AIP), and a p23 protein in addition to the AhR subunit itself (*36*). A possible role of the Ah receptor in cell signaling through a diverse group of interacting proteins has been identified by Carlson and Perdew (*36*). One of the possibilities of HSP90 mediating cross-talk between the Ah receptor and the estrogen receptor in cytosol has been already shown to operate in MCF-7 and T47-D cells and hER-transfected MDA-MB-231 breast cancer cell lines (*38*).

In contrast to the organization of the Ah receptor, the other two major types of HSP90-chaperoned intracellular heterocomplexes, such as the steroid receptors and protein tyrosine kinase complexes, do not contain XAP2. Rather, in its place, FKBP52 (or CYP-40) for the former or cdc37 (or $p50^{cdc37}$) for the latter complex serves as the cochaperone, binding to both HSP90 through corresponding specific binding domains and the chaperoned protein, in this case, either the steroid receptor subunit or the protein tyrosine kinase protein (*18*). The existence of cross-talk between these two types of intracellular HSP90 complexes has been described by the authors mentioned above, as well as by others. For instance, Boonyaratanakornkit et al. (*39*) have shown that activation of the progesterone receptor by its ligands leads to activation of c-Src kinase through the aid of SH_3 domains of the PR subunit.

The main hypothesis tested in this study has been that the ligand-induced activation of the Ah receptor can lead to direct activation of c-Src kinase through activation of the HSP90- and cdc37-containing intracellular heterocomplex. To this end, we could show that at an optimal concentration, geldanamycin could clearly reduce the level of TCDD-induced activation of c-Src kinase, but not induction of CYP1A1. Such an action of geldanamycin was accompanied by reduction in the extent of action of TCDD in inducing activation of c-Src and translocation of both c-Src and cdc37 proteins into the 100000 × *g* membrane fraction containing plasma membrane (Figure 3A,C).

We selected anti-apoptosis as a marker of toxic signaling of TCDD for the following reasons. (a) Anti-apoptosis involves the whole cell integration and coordination of several signaling pathways, including their cross-talk. (b) In the case of the action of TCDD, it is known to depend on c-Src (*29*). To demonstrate that such an action of TCDD is affected by geldanamycin, we carried out a series of apoptosis tests in the presence and absence of TCDD and/or geldanamycin. The result (Figure 7) showed that in the presence of this concentration of geldanamycin, TCDD exhibited no cell rescuing action from apoptosis induced by UV irradiation (i.e., inhibition of the anti-apoptotic action of TCDD). This anti-apoptotic action of TCDD was also abolished when MCF10A cells were incubated with PP-2, an inhibitor of Src family kinases. While these pieces of evidence of the effectiveness of geldanamycin alone would not be sufficient to prove that the ligand-induced activation of the Ah receptor automatically causes the activation of the HSP90−cdc37−c-Src complex, since we do not know how many more cytosolic HSP90 complexes are affected by geldanamycin at 17 nM, it showed that both HSP90 and cdc37 were needed to carry out this process of c-Src activation that was mediated by the TCDD-activated Ah receptor.

The initial observation that the antisense oligonucleotide-induced reduction in the level of cdc37 gene translation leads to a suppression of the action of TCDD in causing a decline in the level of mRNA expression of EGFR in 24 h had led us to explore the possibility of finding other HSP/cdc37-chaperoned proteins which could play important roles in Ah receptor signaling (*40*). NFkB was selected because of the known role of cytoplasmic HSP90/cdc37 proteins in the activation of IKKα/IKKβ, which acts as the rate-limiting step between TNFα-induced activation of its receptor and the ultimate activation of NFkB (*41*). The finding that TCDD does not activate the cdc37−HSP90−IKKβ complex at this early stage of its action also eliminates the possibility that cross-talk between AhR and cdc37−HSP90 complexes is mediated by casein kinase II, since this kinase is an essential kinase needed for the activation of cdc37−HSP90−IKKβ complexes through phosphorylation of the cdc37 protein. In a similar manner, C/EBPα was selected, since its activation is known to be mediated by the HSP90−cdc37−cdk4 complex, which is also known to be susceptible to GA (*42*). However, it must be pointed out that the TCDD incubation periods chosen for Figures 4 and 5 were relatively long (24 and 6 h, respectively) so the effect of the antisense treatment could be maximized. With such long time periods, TCDD could secondarily affect many biochemical events. However, when we elected to re-examine the effects of suppression of cdc37 expression through both antisense and siRNA approaches on the early action of TCDD at 1 h post-treatment, the result clearly showed that the effect of cdc37 suppression on the action of TCDD is limited to Src and its immediate downstream event, COX-2 and IL-1β activation and down-regulation of IL-8 at this early stage of action of TCDD in this cell line (i.e., there was no effect of cdc37 suppression on the expression of NFκB or C/EBPα). The most logical conclusion is, therefore, that this action of TCDD in activating Src through modulation of the cytosolic cdc37−HSP90 chaperone proteins appears to be very specific to the Src−cdc37−HSP90 complex (and probably those chaperoning its cognate Src family kinases in other types of cells). In this context, the reason why 17 nM geldanamycin did not fully prevent rapid activation of ERK by TCDD (15 min) as assessed by Western blotting on phospho-ERK (Figure 3B) may be the selectivity of geldanamycin in dissociating the HSP90−cdc37−Src complex, but not the HSP90−cdc37−raf-1 complex, which could also be involved in the action of TCDD. It must be pointed out that the direct action target of geldanamycin is HSP90, not cdc37. One interesting observation is that the activation of ERK took place at an earlier time point (15 min) than that of c-Src (30 min). This raises the question of the sequence of events taking place between these two early cell responses. Judging from the finding that PP-2 thoroughly inhibits this early response of ERK to TCDD (Figure 1B), our interpretation is that c-Src is still needed (i.e., upstream component) for the ERK activation. Much more work is needed to verify this interpretation in the future. In comparison, both antisense and siRNA treatments directly target cdc37 expression. Hence, the latter treatments are expected to affect a wider range of cdc37-containing chaperone complexes. Indeed, in the case of ERK activation, cdc37 antisense treatment was effective in blocking the early action of TCDD (30 min) (Figure 3E). This finding also serves as a good example of the need to use appropriate tools in assessing the signaling pathway of the activated AhR. A tool that turned out to be very important in this regard was the duration of the action of TCDD. In the case of shorter TCDD treatment studies, such as that with pERK (Figure 3E) and COX-2 (Figures 6 and 7), the specific blocking action of these treatments on the action of TCDD could be recognized from others that were not affected at that time. Therefore, the consequences of cdc37 blocking observed for the long-term action of TCDD to affect EGFR, NFκB, and C/EBPα are likely due to the secondary results of their blocking action on Src kinase. This conclusion produces strong support for the finding made in our laboratory as well as in others that Src kinase plays an important role in mediating the early action of TCDD. On the other hand, the precise cross-talk mechanism through which the AhR−HSP90−XAP2 complex activates the cdc37−c-Src−HSP90 complex remains as an important question to be addressed in the future.

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