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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Induced Activation of a Protein Tyrosine Kinase, pp60^{src}, in Murine Hepatic Cytosol Using a Cell-Free System

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SUMMARY

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was found to activate protein kinases under cell- and nucleus-free conditions in isolated C57 mouse liver cytosol (100,000 × *g* supernatant). This action of TCDD was found to be aryl hydrocarbon receptor (AHR) dependent, concentration dependent, and inhibited by genistein, a tyrosine kinase inhibitor. The lowest concentration of TCDD to produce a statistically significant increase in protein phosphorylation was 10 pM. We also investigated the possibility that a protein kinase is physically associated with the cytosolic AHR complex. Kinase renaturation tests designed to detect reactivated protein kinases after electrophoresis in sodium dodecyl sulfate-polyacrylamide gels revealed the presence of a 60-kDa kinase in the washed immunoprecipitate obtained from liver cytosol using anti-AHR antibody (IgG) and protein A/G/agarose beads but not when a nonspecific IgG was used instead of anti-AHR antibody. The same 60-kDa band was present in an immunoprecipitate prepared in a similar manner from the same cytosol but with anti-heat shock protein 90 antibody (IgM). This 60-kDa kinase was found to be activated by TCDD treatment of whole cytosol from untreated mice. Moreover, pp60^{src} immunoprecipitated from cytosol that had been previously treated with TCDD under cell-free conditions exhibited 2-fold more kinase activity than the equivalent preparation treated with a solvent control. Again, such an effect of TCDD could not be detected when a nonspecific IgG was used in place of an anti-pp60^{src} antibody. Increased protein phosphorylation was observed after direct TCDD treatment of immunoprecipitates obtained using antibodies to AHR and pp60^{src}, respectively, but not when a nonspecific IgG was used for immunoprecipitation in either case. This observation is consistent with the idea that in cytosol, the AHR and pp60^{src} coexist as part of a multimeric protein complex that can be specifically coimmunoprecipitated. These results provide evidence that (i) TCDD activates protein kinases in murine hepatic cytosol, (ii) a 60-kDa protein kinase is associated with the cytosolic form of the AHR complex, (iii) ligand binding directly activates this kinase because TCDD treatment of immunoprecipitated AHR complex results in increased protein kinase activity, and (iv) the AHR-associated protein kinase seems to be pp60^{src} kinase. The current findings provide a clue to a potentially important mechanism by which TCDD can exert rapid, pleiotropic effects through the AHR-associated kinase to alter functions of many proteins through a cascade of protein phosphorylations.

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TCDD is the most toxic member of a large class of structurally related chemicals called halogenated aromatic hydrocarbons, which includes polychlorinated dibenzo-*p*-dioxins and dibenzofurans, polychlorinated biphenyls, and polybrominated biphenyls (1). Found as an environmental contaminant, TCDD causes a variety of species-specific toxic effects in domestic animals, wildlife, and humans (2, 3). Some of the toxic effects of TCDD include cancer, birth defects, immune suppression, and death (for a review, see Ref. 3). In addition, numerous studies have shown that many of TCDDs effects result from changes in cell growth and differentiation (2, 4, 5). Because PKs, particularly PTKs, are known to play such an important role in regulating cell growth and differentiation, several groups of scientists have been studying the potential role of PKs in the mechanism of action of TCDD (for a review, see Ref. 6).

PK activation is one of the most consistently observed biochemical effects of TCDD; it occurs in many species (7, 8), many tissues (7, 9, 10), and numerous cell culture systems

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHR, aryl hydrocarbon receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PK, protein kinase; PTK, protein tyrosine kinase; HSP, heat shock protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ST, sucrose-Tris; MENG, 3-(*N*-morpholino)propanesulfonic acid/EDTA/NaCl/glycerol.

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(11–13). The activation of PKs has been clearly shown to be dependent on TCDD binding to the AHR, a cytosolic, ligand-activated transcription factor of the basic helix-loop-helix type (14, 15). Evidence of AHR dependence for TCDD-induced PK activation includes dose-response relationships, structure-activity relationships, the use of responsive and nonresponsive strains of animals, and the use of AHR blockers (7–15).

To date, most of the effects of TCDD have been attributed to a mechanism by which TCDD binds to the AHR, which then translocates to the nucleus, in which it forms a complex with another basic helix-loop-helix protein, AHR nuclear translocator. The AHR/AHR nuclear translocator heterodimer then binds to dioxin-responsive elements on the DNA, positively or negatively regulating transcription. This mechanism has been clearly shown to be responsible for the induction by TCDD of metabolizing enzymes, such as cytochrome P4501AI and rat glutathione-*S*-transferase Ya subunit (16, 17). However, this mechanism seems insufficient to account for some effects of TCDD that occur rapidly, such as increased Ca^{2+} uptake (18), increased PTK activity (10), and nontranscriptional regulation of proteins (19).

The purposes of this study were to test the hypotheses that (i) TCDD could activate PKs from murine hepatic cytosol in a cell- and nucleus-free system (hereafter referred to as cell-free) and (ii) a PK is physically associated with the AHR complex in cytosol. We found that TCDD treatment of cytosol increased PTK activity in a concentration- and AHR-dependent manner. In addition, we determined that the 60-kDa PK associated with the AHR and activated on treatment with TCDD is $pp60^{src}$.

Materials and Methods

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Genistein, α-naphthoflavone, 4,7-phenanthroline, and all other biochemicals were procured from Sigma Chemical (St. Louis, MO). TCDD was a gift from Dow Chemical (Midland, MI) and was >99% pure (by gas-liquid chromatography). Stock solutions of chemicals used in this study were prepared in *p*-dioxane. A Hamilton syringe was used to add chemicals directly to cytosol preparations. All control samples received an equal volume of *p*-dioxane.

Preparation of liver cytosol. Eight-week-old male C57Bl/6N and DBA/2J mice were obtained from Simonsen Laboratories (Gilroy, CA). Animals were housed in stainless steel cages in groups of four and were provided with standard mouse pellet diet and water *ad libitum*. Animals were killed by cervical dislocation, and the livers were excised immediately, rinsed in phosphate-buffered saline, and chilled on ice. Each gram of liver tissue was minced and then homogenized in 3 ml of ST buffer (0.32 M sucrose, 30 mM Tris·HCl, pH 7.4). Liver tissues were homogenized in ST buffer with 20 strokes by a mechanically driven Teflon/glass homogenizer. The crude homogenate was first centrifuged at 800 × *g* for 10 min at 4° in a microfuge; then, the resulting supernatant was centrifuged at 100,000 × *g* for 1 hr at 4°. The supernatant was set aside as cytosol and frozen in 100-μl aliquots at −80° until needed. Liver cytosol preparation from AHR knockout mice ($Ahr^{-/-}$) was the generous gift of Drs. P. Fernandez-Salguero, J. Peters and F. Gonzalez (National Institutes of Health, Bethesda, MD; Ref. 20), and liver cytosol preparation from $pp60^{src}$ knockout mice ($src^{-/-}$; Ref. 21) was the kind gift of Dr. E. Enan (Department of Environmental Toxicology and the Center for Environmental Health Sciences, University of California, Davis). The genetic backgrounds are C57Bl/6N for $Ahr^{+/+}$ and $Ahr^{-/-}$ and B6,129 for $src^{-/-}$.

Cell-free treatment and conditions for the measurement of PK activity. To minimize variations within an experiment, all of the cytosolic protein and buffers needed for an entire experiment were mixed together; then, aliquots containing equal amounts of protein were added to fresh tubes ready for treatment with test agents. Kinase activity was measured in a buffer designed to partially select for tyrosine kinases using conditions similar to those of Kobayashi *et al.* (22), with some modifications. In brief, 100 μg of cytosolic protein in a volume of 44 μl of ST buffer was added to 50 μl of 2× PK buffer (containing 20 mM HEPES, pH 7.4, 20 mM $MnCl_2$, and 2 mM EGTA, with 2 mM phenylmethylsulfonyl fluoride, 0.4% aprotinin, 40 μg/ml leupeptin, 1.4 μg/ml pepstatin A, and 200 μM Na_3VO_4 added immediately before use for each experiment), 1 μl of test agent, and 5 μl of ATP (final concentration, 3 μM unlabeled ATP mixed with 1 μCi of [γ-^{32}P]ATP) and then incubated for 5 min at 25°. In each cell-free assay, 1 μl of TCDD (final concentrations, 10 nM to 1 pM TCDD) or 1 μl of *p*-dioxane, serving as a control, was added to the reaction mixture in a glass test tube. The concentration of *p*-dioxane used in each of these assays produced no discernible differences from samples receiving no solvent.[2]

Immunoprecipitation. Immunoprecipitation conditions were similar to those of Whitelaw *et al.* (23), with some modifications. Cytosolic proteins were reacted with antibodies in a volume of 1 ml of MENG buffer (containing 25 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.4, 1 mM EDTA, 0.02% sodium azide, 10% glycerol, and 50 mM NaCl), with 1 mM phenylmethylsulfonyl fluoride, 0.02% aprotinin, and 1 mM dithiothreitol for 2.5 hr at 4° with end-over-end rotation. The antibodies used in this study were polyclonal rabbit IgG anti-AHR (a generous gift of Dr. K. Tullis, Department of Environmental Toxicology and the Center for Environmental Health Sciences, University of California, Davis), monoclonal mouse IgM anti-HSP90 clone 3G3 from Affinity Bioreagents (Golden, CO) (24), monoclonal mouse IgG anti-src 327 from Oncogene Science (Uniondale, NY), and polyclonal rabbit IgG anti-$pp60^{c\text{-}src}$ from Santa Cruz Biochemicals (Santa Cruz, CA). All immunoprecipitation reactions were run in parallel with rabbit IgG or mouse IgG (Sigma Chemical) as controls for nonspecific binding of proteins in immune complexes (referred to as nonspecific IgG). Protein A/G agarose beads (Santa Cruz Biochemicals) or goat anti-mouse IgM conjugated to agarose beads (Sigma) were added to each sample and centrifuged at 2000 × *g* at 4° to collect immune pellets. After washing of the immune pellet five times with MENG buffer, immunoprecipitated proteins were subjected to a PK assay or electrophoresis in SDS-polyacrylamide gels.

SDS-PAGE of native ^{32}P-phosphoproteins. C57 liver cytosolic protein (100 μg in 100 μl of 1× PK buffer) was pretreated for 3 min with 1 μM α-naphthoflavone, 1 μM 4,7-phenanthroline, 50 μM genistein, or no addition before the addition of *p*-dioxane or 10 nM TCDD and [γ-^{32}P]ATP for a 5-min incubation at 25°. The reaction was stopped by the addition of 33 μl of 4× Laemmli treatment buffer (7). Samples were heated to 95° for 5 min and then electrophoresed using SDS-PAGE. After staining, destaining, and drying, the gels were exposed to x-ray film for autoradiography.

Phosphorylation assay using exogenous substrates and the phosphocellulose paper method. To measure exogenous substrate phosphorylation after cell-free treatment with TCDD, the same PK conditions were used as described above with a few modifications. In brief, 1 μl of test agent was added to 25 μg of protein in a volume of 89 μl 1× PK buffer. A range of TCDD concentrations from 10^{-8} to 10^{-12} M were compared with *p*-dioxane treatments (solvent control). Four replicates were analyzed per treatment. After incubation for 10 min at 30° with the test agent, 10 μl of 1× PK buffer containing 2 μCi [γ-^{32}P]ATP (final concentration, 3 μM) and one of the following exogenous substrates [final concentrations, 0.4 mg/ml histone type III S (Sigma), 0.5 mg/ml enolase (Sigma), or 0.2 mM RR-SRC peptide (RRLIEDAEYAARG; Sigma)] was added and

[2] A. Blankenship and F. Matsumura, unpublished observations.

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then incubated for 1 min at 30°. To terminate the reaction, 20-μl aliquots were spotted onto 1 × 2-cm-diameter phosphocellulose papers (Whatman, Clifton, NJ) as described by Akers *et al.* (25). After drying, each disc was washed four times in a large volume of 85 mM phosphoric acid, dried, and added to 4 ml of scintillation cocktail for liquid scintillation counting. To obtain the net ^{32}P-phosphorylation on RR-SRC and enolase, background was determined by running a parallel set of treatments in the absence of added substrate.

Immune complex kinase assay. Immune complexes from untreated C57 liver cytosol were incubated for 10 min with 10 nM TCDD or *p*-dioxane at 25° in 100 μl of PK buffer consisting of 10 mM Tris·HCl, pH 7.5, 5 mM $MnCl_2$, 8 μg of histone/reaction. Then, 2 μCi of [γ-^{32}P]ATP (3 μM) was added for 1 additional min at 25°. The reaction was terminated by the addition of 34 μl of 4× Laemmli treatment buffer, heated to 95° for 5 min, and then electrophoresed in SDS-polyacrylamide gels. After staining, destaining, and drying, the gels were exposed to x-ray film for autoradiography.

Immune complexes from cell-free treated C57 mouse liver cytosol (10 nM TCDD or *p*-dioxane for 10 min at 25° in a total volume of 100 μl) were (i) mixed with 1× Laemmli treatment buffer, heated to 50° for 10 min, and then electrophoresed in SDS-polyacrylamide gels for subsequent analysis by kinase renaturation or (ii) resuspended in 50 μl of PK buffer and 3 μM ATP with 2 μCi of [γ-^{32}P]ATP. Three replicates were analyzed per treatment. To terminate the reaction, 20-μl aliquots were spotted onto 2-cm-diameter phosphocellulose papers. After drying, each disc was washed four times in a large volume of 85 mM phosphoric acid, dried, and added to 4 ml of scintillation cocktail for liquid scintillation counting.

PK renaturation after electrophoresis in SDS-polyacrylamide gels. The conditions were the same as those described by Hutchcroft *et al.* (26), with some modifications. In brief, 400 μg of protein from C57 mouse liver cytosol was treated with 10 nM TCDD or *p*-dioxane for 10 min at 25° in a total volume of 100 μl. Some of the samples were then mixed with 33 μl of 4× Laemmli treatment buffer and heated to 50° for 10 min before electrophoresis on SDS-polyacrylamide gels. Other samples were immunoprecipitated with 4 μg of antibody in 900 μl of MENG buffer for 2.5 hr at 4° with end-over-end rotation and then mixed with 1× Laemmli treatment buffer and heated to 50° for 10 min.

SDS-polyacrylamide gels were electrophoresed at 10 mA overnight. To remove the SDS, the gels were washed two times with 40 mM HEPES, pH 7.4, with 25% 2-propanol and then four times with 40 mM HEPES, pH 7.4 (200 ml/wash and 1 hr/wash). Gels were preincubated with phosphorylation buffer consisting of 25 mM HEPES, pH 7.4, 10 mM $MnCl_2$, and 100 μM Na_3VO_4 for 1 hr and then incubated with 50 ml of phosphorylation buffer with 250 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) for 3 hr with shaking. To remove excess [γ-^{32}P]ATP and fix the proteins in the gel, each gel was rinsed with 40 mM HEPES, pH 7.4, and then incubated overnight with 600 ml of 5% trichloroacetic acid and 1% sodium pyrophosphate with 20 g of Dowex 2X-8 resin in dialysis tubing. After extensive washes with 5% trichloroacetic acid and 1% sodium pyrophosphate, gels were stained, destained, dried, and exposed to x-ray film to detect labeled bands.

Results

SDS-PAGE Analysis of ^{32}P-Phosphorylated Proteins after Cell-Free Treatment with TCDD

C57 mouse liver cytosol treated with 10 nM TCDD for 5 min resulted in an increase in protein phosphorylation on endogenous (native) substrate proteins compared with a control treated with only the vehicle (Fig. 1). This effect of TCDD was replicated eight times and found to be statistically significant (control, 100 ± 9%; TCDD, 140 ± 13%; $p \leq 0.001$, using a two-tailed Student's *t* test). Cotreatment with 1 μM 4,7-phenanthroline and 1 μM α-naphthoflavone, two well known

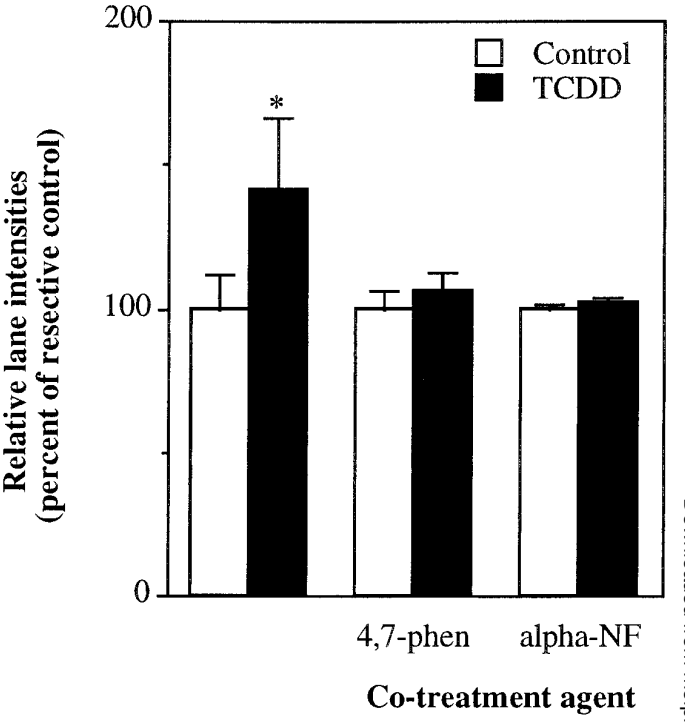

Fig. 1. Cell-free kinase activation of C57 mouse liver cytosol (100,000 × *g*) treated with TCDD or *p*-dioxane in the presence and absence of AHR blockers. Cytosol (100 μg) was prepared and treated with test agents as described (see Materials and Methods). The results are shown as relative lane intensities from autoradiograms of SDS-PAGE electrophoretograms of ^{32}P-labeled proteins as determined by an image acquisition and analysis system (AMBIS). *, Significantly different from respective control values at $p \leq 0.001$ (eight experiment; two-tailed Student's *t* test). *4,7-phen*, 4,7-Phenanthroline (1 μM). *alpha-NF*, α-naphthoflavone (1 μM).

AHR blockers which work specifically at this concentration range, blocked the action of TCDD. Note that no specific exogenously added substrate proteins were used, and therefore, no hint of the nature of the activated kinase(s) could be expected on the basis of this experiment.

Concentration-Dependent Effects of TCDD on PK Activity Using Three Different Exogenous Substrates

Treatment of C57 mouse liver cytosol for 10 min with TCDD in the presence of RR-SRC peptide (known to be relatively specific for Src-type kinases), enolase, or histone and [γ-^{32}P]ATP produced more ^{32}P-phosphorylation than a solvent-treated control in a concentration-dependent manner (Fig. 2). For RR-SRC and enolase, which are specific substrates for tyrosine kinases, maximal response was observed at 10^{-9} M TCDD, and half-maximal response was observed between ~10^{-10} and ~10^{-11} M. Using histone as a general PK substrate, maximal response was observed at 10^{-8} M, and half-maximal response was observed between ~10^{-9} and ~10^{-10} M.

Dependence on the AHR for PK Activation by TCDD

Structure-activity relationship. Congeners with a wide-range of binding affinities for the AHR were tested for their ability to activate PK or PKs under cell-free conditions using histone phosphorylation (Fig. 3). 1,2,4,7,8-Pentachlorodibenzo-*p*-dioxin (10^{-8} M) and 3,4,3′,4′-tetrachlorobiphenyl (10^{-7} M), which can bind to and activate the AHR, produced

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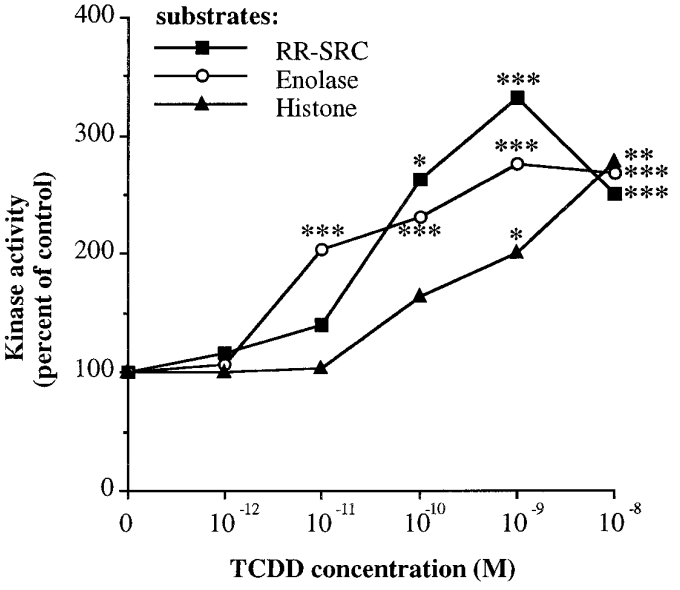

Fig. 2. Dose-response of the ability of TCDD to increase RR-SRC peptide, enolase, and histone phosphorylation after cell-free treatment of C57 mouse liver cytosol (100,000 × *g* supernatant). Cytosol (100 μg) was prepared and treated, and PK activity was measured as described (see Materials and Methods). Values are mean of four replicates per treatment (standard deviations were within 15% of the mean values). *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$, significantly different from respective control values (two-tailed Student's *t* test).

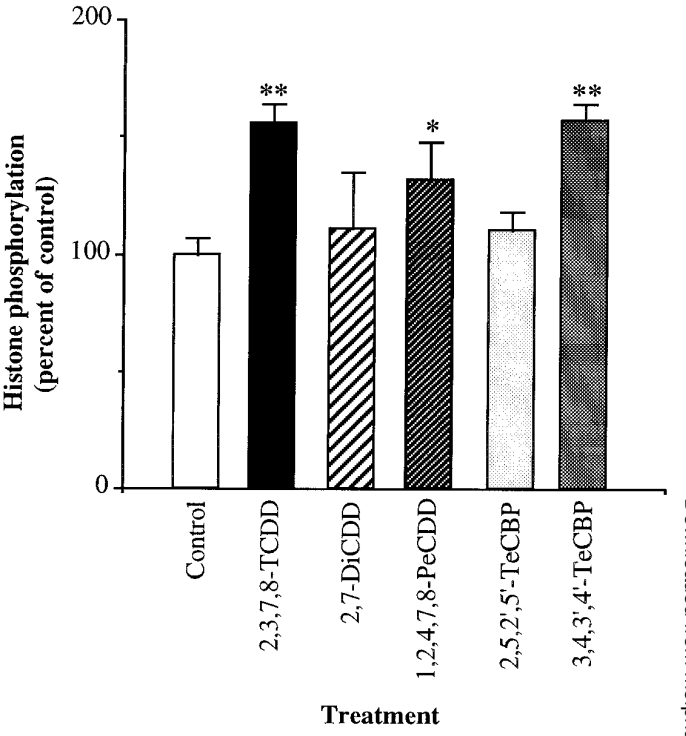

Fig. 3. Structure-activity relationship of dioxin and polychlorinated biphenyl congeners with varying AHR binding affinities to increase PK activity under cell-free conditions. Cytosol (100 μg) was prepared and treated, and PK was activity measured as described using histone as an exogenous substrate (see Materials and Methods). Values are mean of four replicates per treatment ± 1 standard deviation. *, $p \leq 0.05$; and **, $p \leq 0.01$, significantly different from respective control values (two-tailed Student's *t* test). *DiCDD*, dichlorodibenzo-*p*-dioxin (10^{-7} M). *PeCDD*, pentachlorodibenzo-*p*-dioxin (10^{-8} M). *TeCBP*, tetrachlorobiphenyl (10^{-7} M). TCDD, 10^{-9} M.

statistically significant increases in histone phosphorylation. In contrast, 2,7-dichlorodibenzo-*p*-dioxin (10^{-7} M) and 2,5,2′,5′-tetrachlorobiphenyl (10^{-7} M), which bind rather poorly to the AHR, did not significantly affect histone phosphorylation. Their abilities to activate PK or PKs under cell-free conditions correlate well with their affinities to AHR as previously reported (1).

PK activities in four different mouse liver cytosol preparations treated with 1 nM TCDD under cell-free conditions in the presence and absence of α-naphthoflavone and genistein. PK activities were measured in four different mouse liver cytosol preparations that had been treated with 1 nM TCDD under cell-free conditions (Table 1). In C57Bl/6N, TCDD produced a statistically significant increase in kinase activity in the presence of all three exogenous substrates (RR-SRC peptide, enolase, and histone) and on endogenous (native) proteins in the absence of an exogenous substrate. This effect of TCDD was prevented when measured in the presence of α-naphthoflavone, a partial AHR antagonist, and genistein, a tyrosine kinase inhibitor. Furthermore, 1 nM TCDD did not stimulate PK activation in liver cytosol from DBA/2J mice, a TCDD-nonresponsive strain; AHR knockout mice ($Ahr^{-/-}$); or src knockout mice ($src^{-/-}$).

PK activation in $Ahr^{+/+}$ but not in $Ahr^{-/-}$ mouse liver cytosol after *in vivo* treatment with TCDD. Liver cytosol from mice treated with 40 μg/kg TCDD (single intraperitoneal injection) or control for 24 hr were kindly provided by Drs. P. Fernandez-Salguero and F. Gonzalez. Similar to results with cell-free treatment (Table 1), TCDD treatment *in vivo* significantly increased the overall PK activity in $Ahr^{+/+}$ but not $Ahr^{-/-}$ mice (Fig. 4). Interestingly, one protein band at ~36 kDa was more phosphorylated in the control samples in the $Ahr^{+/+}$ strain compared with TCDD. However, this effect was also observed in the preparation from the $Ahr^{-/-}$ strain, indicating that it is not an AHR-dependent phenomenon.

TCDD Treatment of Immunoprecipitated AHR Leads to Increased PK Activity

When the AHR was first isolated through immunoprecipitation and then treated with 10 nM TCDD and [γ-^{32}P]ATP, more ^{32}P-phosphorylation of native proteins took place than with control treatment ($p \leq 0.001$, a two-tailed Student's *t* test; Fig. 5). When $pp60^{src}$ was first isolated through immunoprecipitation and then treated under the identical test conditions as the AHR, TCDD similarly increased ^{32}P-phosphorylation of native proteins. However, when nonspecifically bound proteins were immunoprecipitated with a nonspecific IgG under the identical test conditions, TCDD did not stimulate kinase activity.

Association of a PK with the Immunoprecipitated AHR Complex

On renaturation of kinases after SDS-PAGE from cell-free treated C57 mouse liver cytosol, a 60-kDa PK was identified. TCDD activated this kinase 55% over control (Fig. 6, *lanes 1 and 2*). This kinase seems to be the same that coimmunoprecipitates with the AHR (Fig. 6, *lanes 3 and 4*; Fig. 7, *lane 1*) and is present when HSP90 is immunoprecipitated with 3G3 antibody (Fig. 6, *lanes 5 and 6*). No kinase activity was

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TABLE 1

Effect of TCDD on the cell-free activation of protein kinases in four different mouse liver cytosol preparations

Twenty-five milligrams of liver cytosol (100,000 × *g* supernatant) was used in each assay for each mouse strain tested. AHR and src knockout mouse strains are designated by $Ahr^{-/-}$ and $src^{-/-}$, respectively. Samples were treated using cell-free conditions with TCDD or *p*-dioxane (solvent control) for 10 min at 30° before addition of [γ-^{32}P]ATP and substrate for 1 min at 30° as described in Materials and Methods. For each treatment group, there were at least three or four replicates. Kinase activities in the presence of RR-SRC and enolase represent net phosphorylation of substrate, whereas background was not subtracted when histone was used as a substrate. Background was determined by running a parallel set of treatments in the absence of substrate. Kinase activities, which were determined using the phosphocellulose paper method, are expressed in terms of pmol of phosphate incorporation/20 nmol of RR-SRC/25 of mg protein/min, pmol of phosphate incorporation/50 mg of enolase/25 mg of protein/min, and pmol of phosphate incorporation/40 mg of histone/25 mg of protein/min. Kinase activity using native substrates were determined by densitometric scanning of SDS-PAGE gels as described in Materials and Methods; results are expressed as a percentage of respective control.

Source of liver cytosol	Treatment	Kinase activity			
		Exogenous substrates			Native substrates
		RR-SRC	Enolase	Histone	
C57Bl/6N	Control	0.77 ± 0.36	1.44 ± 0.04	3.90 ± 0.74	100 ± 9
	1 nM TCDD	3.59 ± 0.47[a]	3.45 ± 0.32[b]	7.79 ± 2.96[c]	140 ± 13[a]
	Control + α-NF	1.13 ± 0.38	1.27 ± 0.39	4.15 ± 0.03	100 ± 2
	1 nM TCDD + α-NF	1.47 ± 0.77	1.57 ± 0.30	3.63 ± 0.37	102 ± 2
	Control + genistein	0.90 ± 0.41	1.45 ± 0.06	N.D.	100 ± 22
	1 nM TCDD + genistein	1.15 ± 0.24	1.04 ± 0.23	N.D.	87 ± 17
DBA/2J	Control	1.26 ± 0.32	1.19 ± 0.47	3.06 ± 0.24	100 ± 10
	1 nM TCDD	1.08 ± 0.85	1.26 ± 0.30	3.08 ± 0.27	95 ± 9
$Ahr^{-/-}$	Control	1.00 ± 0.40	1.30 ± 0.47	N.D.	100 ± 19
	1 nM TCDD	1.09 ± 0.38	1.25 ± 0.07	N.D.	73 ± 12
$src^{-/-}$	Control	1.28 ± 0.13	1.37 ± 0.10	4.70 ± 0.34	N.D.
	1 nM TCDD	1.27 ± 0.07	1.19 ± 0.42	4.74 ± 0.06	N.D.

[a] $p \leq 0.001$ between TCDD and respective control treatments (using a two-tailed Student's *t* test).
[b] $p \leq 0.005$ between TCDD and respective control treatments (using a two-tailed Student's *t* test).
[c] $p \leq 0.05$ between TCDD and respective control treatments (using a two-tailed Student's *t* test).
N.D., not determined; α-NF, α-naphthoflavone.

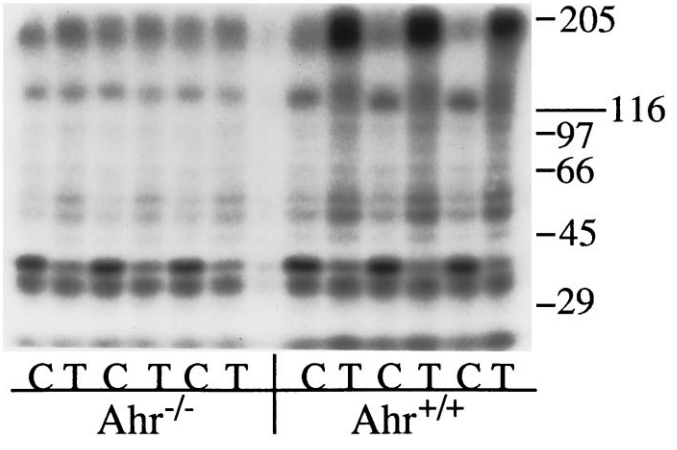

Fig. 4. PK activities of liver cytosol from $Ahr^{-/-}$ and $Ahr^{+/+}$ mice that had been previously treated with 40 μg/kg TCDD (*T*) for 20 hr (kindly provided by Dr. P. Fernandez-Salguero). Cytosol was incubated for 5 min in PK buffer in the presence of [γ-^{32}P]ATP (1 μCi; 3 μM) as described in Materials and Methods. Results are shown as an autoradiogram of a SDS-PAGE electrophoretogram of ^{32}P-labeled proteins. *C*, control.

observed with nonspecifically bound proteins immunoprecipitated with a nonspecific IgG (Fig. 7, *lane 2*). A 50-kDa band with strong intensity was observed in Fig. 6 (*lanes 1 and 2*, total cytosol) and was also present at a much lower intensity (*lanes 5 and 6*, HSP90 immune complex). This 50-kDa band represents an unidentified PK that is apparently abundant or highly active or represents more than one protein on the basis of the strong intensity of the signal (at least for total cytosol). Despite the intensity of this signal, TCDD does not affect its intensity (*lane 1*, control; *lane 2*, TCDD treated), and it does not appear in the AHR immune complex (Fig. 6, *lanes 3 and 4*; Fig. 7, *lane 1*) or in the nonspecific immune complex (Fig. 7, *lane 2*).

TCDD Increases pp60^{src} Activity After Cell-Free Treatment of C57 Liver Cytosol

After treatment of C57 mouse liver cytosol (100,000 × *g* supernatant) with 10 nM TCDD or *p*-dioxane (solvent control) for 10 min at 25°, pp60^{src} was immunoprecipitated. The immune pellets were washed extensively with wash buffer and then subjected to two different assays to determine PK activity. In one assay, immune pellets were incubated with [γ-^{32}P]ATP and kinase buffer containing histone as a substrate. Immunoprecipitated pp60^{src} from TCDD-treated cytosol exhibited >2-fold greater kinase activity than pp60^{src} immunoprecipitated from solvent-treated cytosol (Table 2). This result represents the average of three independent experiments and was statistically significant at $p \leq 0.01$(two-tailed Student's *t* test). When a nonspecific IgG was used in place of the anti-pp60^{src} antibody, no difference was detected between control and TCDD treatments.

In another assay, proteins in the immune pellets were resolved by SDS-polyacrylamide gel electrophoresis, renatured, and incubated in kinase buffer containing [γ-^{32}P]ATP. A single 60-kDa kinase was detected, which exhibited greater activity from samples immunoprecipitated after TCDD treatment than from samples immunoprecipitated after treatment with solvent only (Fig. 8). This experiment was repeated ,and the results (expressed in terms of percentage of control as determined by densitometric analysis) are presented in Table 2. This effect of TCDD was statistically significant at $p \leq 0.005$ (two-tailed Student's *t* test). Because phosphorylation occurred in the absence of other substrates included in the gel matrix, this kinase seem to be capable of autophosphorylation, as would be expected with pp60^{src}. No

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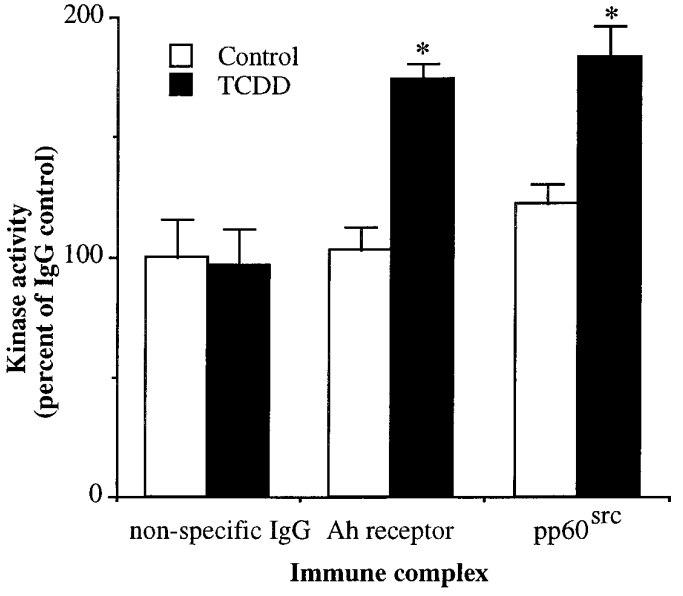

Fig. 5. Kinase activity after TCDD treatment of immunoprecipitates using antibodies to AHR, pp60^{src}, or a nonspecific IgG. Immunoprecipitation reactions (from 100 μg of C57 mouse liver cytosol; 100,000 $\times$ *g* supernatant) used an anti-AHR antibody, anti-pp60^{src} antibody, or a nonspecific IgG and protein A/G agarose as described (see Materials and Methods). The immune pellets were extensively washed and then treated with either 10 nM TCDD or *p*-dioxane (solvent control) for 10 min at 25°. Immune pellets were then incubated with 2 μCi of [γ-^{32}P]ATP in 100 μl of phosphorylation buffer consisting of 10 mM HEPES, pH 7.4, 10 mM $MnCl_2$, and 1 mM EGTA for 1 min at 25°. The reaction was terminated by the addition of 34 μl of 4$\times$ SDS sample treatment buffer. The results are shown as relative quantitative scanning of an autoradiogram of an SDS-PAGE electrophoretogram of ^{32}P-labeled proteins (using the image acquisition and analysis system AMBIS; scanning results are expressed as arbitrary units) of an autoradiogram of an SDS-PAGE electrophoretogram of ^{32}P-labeled proteins. $*$, $p \leq 0.05$, significantly different from respective control values (three experiments; two-tailed Student's *t* test).

kinase activity was detected when a nonspecific IgG was used in place of the anti-pp60^{src} antibody (Fig. 7, *lane 2*).

Discussion

The ability of TCDD to affect PK activity, particularly PTKs, including pp60^{src}, has been clearly shown to be an important step in the mechanism of action of TCDD (9–15). There were two main lines of evidence that prompted us to investigate the possibility that TCDD might activate PKs in a cell-free system. First, the very rapid nature of the effects of TCDD effects on cellular phosphorylation (10) suggested that TCDD might be activating PKs through a dioxin-responsive element-independent mechanism; these phosphorylation events occurred so rapidly (within minutes) that altered transcription and translation of PKs represent an unlikely mechanism to explain these very early events.

Second, the results of the current investigation have clearly shown that TCDD has the property to activate PTK or PTKs when directly added to isolated liver cytosol under a cell-free condition (Figs. 1–5). Such a phenomenon cannot be explained on the basis of TCDD-induced activation of gene expression because it occurs very rapidly as well as in the absence of nuclei and microsomes, two organelles needed for transcription and *de novo* protein synthesis. These organelles could not have contaminated the cytosol preparations because the centrifugal condition that was applied was stringent.

We also obtained evidence that this cell-free activation

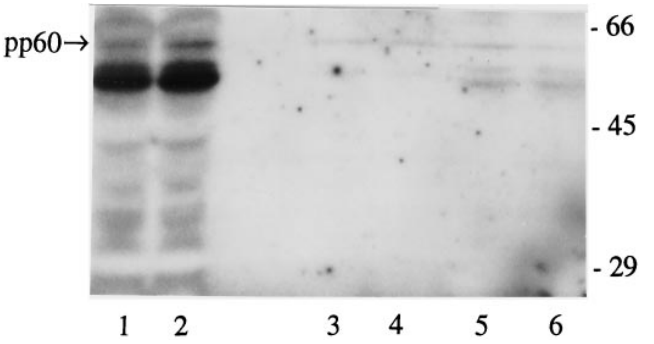

Fig. 6. Kinase renaturation of both cell-free treated cytosol and AHR and HSP90 immune complexes immunoprecipitated from C57 mouse liver cytosol. After cell-free treatment of 400 μg of protein from C57 liver cytosol (100,000 $\times$ *g* supernatant) for 10 min with 10 nM TCDD or *p*-dioxane, some of the samples were mixed with an equal volume of 2$\times$ SDS sample treatment buffer and then electrophoresed in regular-size 10% SDS-polyacylamide gels. Samples to be immunoprecipitated were adsorbed to protein A/G agarose with anti-AHR antibody or goat anti-mouse IgM agarose with anti-HSP90 antibody (clone 3G3). The immune pellets were washed extensively in MENG buffer, and then the proteins in the immune pellets were electrophoresed in SDS-polyacylamide gels. After removal of SDS and renaturing of PKs, gels were incubated with phosphorylation buffer as described in Materials and Methods. *Lane 1*, whole cytosol (solvent control treatment). *Lane 2*, whole cytosol (10 nM TCDD treatment). *Lanes 3 and 4*, immunoprecipitated with anti-AHR antibody. *Lanes 5 and 6*, immunoprecipitated with anti-HSP90 antibody. Relative quantitative scanning using the image acquisition and analysis system AMBIS; scanning results are expressed as arbitrary units. Lane designations and the area quantified (in arbitrary units) were *lane 1*, 60-kDa protein, 48,292; and *lane 2*, 60-kDa protein, 74,717.

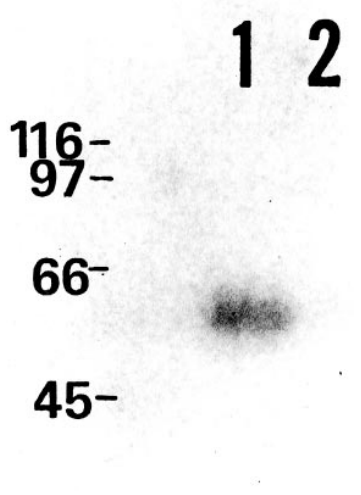

Fig. 7. Kinase renaturation of AHR and nonspecific immune complexes immunoprecipitated from C57 mouse liver cytosol. Samples were immunoprecipitated as described in Materials and Methods with either an anti-AHR antibody or a nonspecific IgG. The immune pellets were washed extensively, electrophoresed in SDS-polyacylamide minigels, and subjected to kinase renaturation as described in the legend for Fig. 1. *Lane 1*, AHR immune complex. *Lane 2*, nonspecific immune complex. Note that only one kinase (60 kDa) was detected in the AHR immune complex and that no kinases were detected in the nonspecific immune complex. [Although the data represented in Fig. 6 (*lanes 3 and 4*) and Fig. 7 (*lane 1*) are consistent, the difference in appearance of the bands is due to a slightly overloaded minigel (in Fig. 7) versus a normally loaded regular-sized gel (in Fig. 6).]

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TABLE 2

Phosphorylation of histone by pp60^{src} kinase immunoprecipitated after *cell-free* TCDD treatment of C57 mouse liver cytosol

Identical aliquots of C57 liver cytosol were incubated with 10 nM TCDD or *p*-dioxane only (control) for 10 min at 25° as described in Materials and Methods. After treatment, pp60^{src} was immunoprecipitated as described in Materials and Methods. The immune complex was washed extensively and then resuspended in 50 ml of kinase buffer containing 10 mM Tris, pH 7.5, 5 mM $MnCl_2$, 3 mM unlabeled ATP, 2 mCi [γ-^{32}P]ATP, and 8 mg of histone/reaction. After a 15 min reaction at 25°, two 20-ml aliquots of the reaction mix was spotted onto two phosphocellulose discs. After extensive washing of the phosphocellulose discs, the remaining radioactivity was counted in a liquid scintillation counter. The control values for the pp60^{src} immune complexes were ~3-fold greater than the control values for the nonspecific immune complexes (nonspecific IgG); values are mean ± standard deviation from three experiments. Relative quantitative scanning using AMBIS (refer to Fig. 6 for a representative gel); values are mean ± standard deviation of four experiments.

Antibody used for immunoprecipitation	Treatment	Histone phosphorylation	Kinase renaturation
		% of respective control	
Anti-pp60^{src}	Control	100 ± 18	100 ± 21
	10 nM TCDD	220 ± 38[a]	238 ± 59[b]
Nonspecific IgG	Control	100 ± 16	No detectable activity
	10 nM TCDD	98 ± 16	No detectable activity

[a] Significantly different from control values at $p \leq 0.01$ (two-tailed Student's *t* test).
[b] Significantly different from control values at $p < 0.005$ (two-tailed Student's *t* test).
SD, standard deviation

phenomenon is mediated by the AHR, as is the case with many other TCDD-induced parameters. The evidence supporting AHR-dependency are the blocking actions of α-naphthoflavone and 4,7-phenanthrolene, the lack of kinase activation by TCDD in liver cytosol preparations from DBA/2J and Ahr$^{-/-}$ mice, and the rank order among dioxin-related chemicals in activating kinases being similar to that of their affinity to AHR.

That the type of TCDD-activated kinase(s) is likely to be a tyrosine kinase has been shown by the fact that RR-SRC and enolase served as better substrates than histone in detecting the effect of TCDD at low concentrations (Fig. 2). RR-SRC has only tyrosine residues as phosphorylatable sites, with no serine or threonine residues. Furthermore, enolase is a well-established specific substrate for Src-type tyrosine kinases. In addition, genistein, a specific PTK inhibitor, was able to block the actions of TCDD. Therefore, it is likely that TCDD is activating a PTK within this cell-free system. TCDD has been shown to activate PTKs in a number of *in vivo* and *in vitro* cell culture models, including activation of pp60^{src} (12) and the epidermal growth factor receptor-associated kinase (11, 27). Other groups have reported very early activation of PKs by TCDD and TCDD-related chemicals, such as increased pp56^{lck} and pp59^{fyn} activity in T cells after 10 min with dimethylbenz(a)anthracene (28), increased PTK activity in B cells after 5 min with TCDD, increased pp60^{src} levels and activity within 30 min with TCDD (28a), increased PKC activity in a cell-free system in thymocytes (29), and increased protein phosphorylation in a cell-free system with guinea pig adipose tissue (7).

Having established that TCDD-induced cell-free activation of kinases at least involves PTK or PTKs, we must consider the identity of the trigger kinase that is directly affected by binding of TCDD to the AHR. Certainly, it is known that many kinases phosphorylate other kinases (i.e., phosphorylation cascades); even in well-washed immunoprecipitation preparations, contamination could be a problem. Therefore, great care must be taken in designing the experiment and interpreting the results. The first piece of evidence indicating the presence of a single kinase is the existence of only one 60-kDa band in AHR immune complexes detected by a kinase renaturation assay (Fig. 6). In addition to the tendency of this renaturation assay to detect only PKs, the appearance of ^{32}P-phosphorylating action in the absence of exogenously added substrate proteins indicates that it detected only the type of kinases that can autophosphorylate (i.e., Src-like kinases are well known to autophosphorylate). Furthermore, RR-SRC, which was used experiments represented in Table 1 and Fig. 2 to detect the action of TCDD, is a specifically designed artificial substrate preferentially phosphorylated by Src-like kinases. The main proof of the identity of this kinase indeed being pp60^{src} has been provided by the immunoreactivity of the anti-pp60$^{c\text{-}src}$ antibody (Santa Cruz Biochemicals), which is a very specific antibody directed at the eight-amino acid sequence of its amino-terminal epitope. It has been shown to react only with pp60^{src} and not with any other type of Src-family or Src-like kinases. The facts that this antibody coimmunprecipitated AHR and that TCDD specifically activated pp60^{src} by its binding cannot be ascribed to mere coincidence or contamination, particularly when one considers the lack of TCDD responsiveness in all other nonspecific immune complexes tested as blanks.

Finally, the lack of the effectiveness of TCDD to activate the kinase in cytosol preparations from src knockout mice (Src$^{-/-}$) must be viewed as solid confirmation of our diagnosis. In this strain, originally prepared by Boyce *et al.* (21), other Src-family kinases have been shown to increase, such as pp62^{yes}, pp56^{lck}, and p59^{fyn}, probably to compensate for the lack of pp60^{src} itself. Yet, in the specific absence of pp60^{src}, cytosolic preparations did not respond to TCDD, even when a universal substrate, such as histone, was used to detect kinase activity (Table 1). More definitive experiments were attempted, such as immunoprecipitation with one antibody, electrophoresis, and then a Western blot analysis with an antibody to another protein in the complex. However, such an approach failed to detect either pp60^{src} in an anti-AHR immune complex or AHR in an anti-pp60^{src} immune complex. Unfortunately, there were interferences (possibly from the IgGs) near where both pp60^{src} and AHR would be detected using conventional Western techniques. Attempts were also made but were unsuccessful at using ^{125}I-radiolabeled anti-pp60^{src} antibodies in a Western analysis of an anti-AHR immune complex. However, using this technique, we were unable to detect pp60^{src} even in membrane fractions in which we could detect pp60^{src} with nonradiolabeled anti-pp60^{src} antibodies (using the same clone), which suggests that the radiolabeling procedure may have disrupted the ability of the antibody to recognize antigen. Therefore, the results from these experiments were inconclusive.

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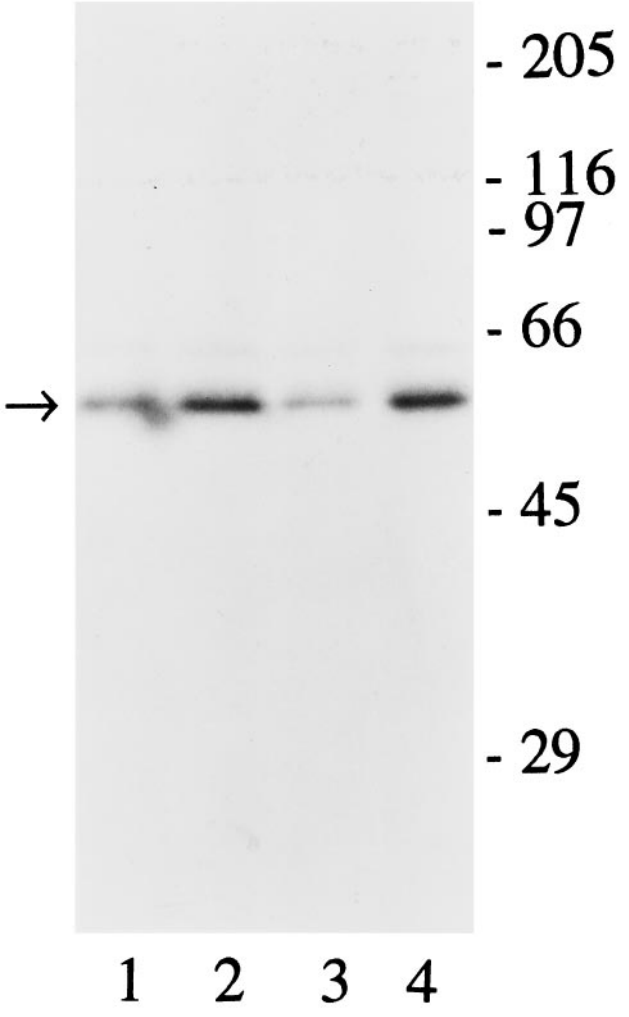

Fig. 8. Kinase renaturation of pp60^{src} immunoprecipitated after cell-free treatment of C57 mouse liver cytosol. After cell-free treatment for 10 min with 10 nM TCDD or *p*-dioxane, aliquots of 400 μg of protein from C57 liver cytosol (100,000 $\times$ *g*) were adsorbed to protein A/G agarose with anti-Src 327 or anti-pp60$^{c\text{-}src}$ antibody (Santa Cruz Bichemicals). The immune pellets were analyzed as described in the legend for Fig. 1. Lanes 1 and 2, immunoprecipitated with anti-pp60$^{c\text{-}src}$ antibody (Santa Cruz Biochemicals). *Lanes 3 and 4*, immunoprecipitated with anti-Src 327 antibody (Oncogene Science). *Lanes 1 and 3*, solvent control treatments. *Lanes 2 and 4*, 10 nM TCDD treatments. Relative quantitative scanning was performed with the image acquisition and analysis system AMBIS/Scanalytics; scanning results are expressed as arbitrary units. Lane designations and the area quantified were *lane 1*, 60-kDa protein, 11,234; *lane 2*, 60-kDa protein, 23,368; *lane 3*, 60-kDa protein, 5,893; and *lane 4*, 60-kDa protein, 25,039.

It must be added, however, that a related study from this laboratory recently confirmed the presence of pp60^{src} in a complex with AHR using two-dimensional PAGE in cytosol preparations from adipose tissue of male guinea pigs (30).[3] Furthermore, the association of pp60^{src} with the cytosolic AHR complex was also confirmed in MCF-7 human breast cancer cells).[3] Thus, this arrangement to contain pp60^{src} as a built-in kinase within the cytosolic form of the AHR complex is likely to be a common feature among many TCDD-sensitive animals.

Are there any precedents for such an arrangement of cytosolic receptors being associated with PKs? Actually, it is not so uncommon to find kinases associated with hormone receptors in cytosol. Hutchinson *et al.* (31) have shown that the glucocorticoid receptor accepts pp60$^{v\text{-}src}$, and possibly pp60$^{c\text{-}src}$, along with HSPs hsp90, hsp70, and hsp56 (see also review in Ref. 32). Prolactin receptor is known to be associated with pp60^{src} (33). Estradiol treatment of MCF-7 cells leads to an immediate and transient stimulation of PTK activity, possibly pp60^{src}, within 10 sec (34). In addition, the progesterone receptor has been shown to be associated with several PKs (35, 36). In the current study, it is still unclear whether pp60^{src} is associated with the AHR, HSP90, or both. There are data to show an association of HSP90 and pp60^{src} (23, 37), and HSP90 may even play a regulatory role in the activity of pp60^{src} (38), but whether pp60^{src} can also directly associate with the AHR remains to be tested. Interestingly, HSP90 has been shown to associate with several other PKs, including Raf (39), casein kinase II (40), and eIF-2α kinase (41). Therefore, sufficient precedents are available to indicate that such an arrangement of cytosolic receptors being associated with HSP90 and PKs is not really unusual. These kinases are likely to be playing the role of facilitating and amplifying the function of the receptor to transduce the message of these hormones.

It is likely that the role of pp60^{src} is also to facilitate and amplify the functions of AHR. Viewed in this way, the results of this current work do not present a conflicting viewpoint of the existing theory of the role of the AHR in the action mechanism of TCDD but instead add a new dimension to the role of the AHR.

Acknowledgments

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[3] H. Kakeya, E. Enan, and F. Matsumura, unpublished observations.

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