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Identification of c-Src as the Integral Component of the Cytosolic Ah Receptor Complex, Transducing the Signal of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Through the Protein Phosphorylation Pathway

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ABSTRACT. We have shown previously that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) under cell-free conditions causes a significant rise in protein tyrosine kinase activity of cytosol from male guinea pig adipose tissue, and that such an effect of TCDD is Ah-receptor dependent. In the present study, we obtained evidence indicating that c-Src protein kinase is the protein kinase activated by TCDD and that this kinase is associated specifically with the Ah-receptor-complex proteins in cytosol from adipose tissue and liver of guinea pig and liver of C57Bl/6J mouse, and in NIH 3T3 mouse fibroblast cells. Here, we present evidence that c-Src protein is functionally attached to the Ah-receptor (AhR) and is specifically activated upon ligand binding. This conclusion is based on several lines of evidence: (a) TCDD caused activation of protein tyrosine kinase activity when administered directly to purified Ah-receptor immunoprecipitate; (b) this stimulatory effect of TCDD was abolished when the cytosol was immunodepleted of c-Src protein or Ah-receptor protein by preincubating with anti-c-Src or anti-Ah-receptor antibody, followed by the addition of TCDD to the remaining portions of cytosol; (c) when Ah-receptor immunoprecipitate was incubated with TCDD, and the kinase(s) released to the supernatant was analyzed on autoradiography of two-dimensional (2D) electrophoresis, ^{32}P-labeled c-Src protein was recognized; (d) the same ^{32}P-labeled-phosphoprotein with M_r = 60 kDa and pI = 6.1 was found in the immunoprecipitate with anti-c-Src antibody on 2D autoradiograms; (e) this same phosphoprotein disappeared when the supernatant of the Ah-receptor immunoprecipitate was immunodepleted of c-Src protein by anti-c-Src antibody; and (f) a structure–activity relationship study with TCDD and three dioxin-congeners revealed a rank order for their potency in activation of c-Src kinase activity to be identical to that of previously determined toxicity indices: i.e. TCDD > 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PCDD) > 1,2,4,7,8-pentachlorodibenzo-*p*-dioxin (1,2,4,7,8-PCDD) > 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD). Consistent with these results, TCDD-induced c-Src kinase activity was abolished when c-Src immunoprecipitate's suspension was preincubated with 0.1 or 1 μM α-naphthoflavone (AhR blocker) for 10 min prior to the addition of TCDD. In addition, pretreatment of 3T3 fibroblast cells with 3-methylcholanthrene abolished TCDD-induced c-Src kinase activity in AhR-immunoprecipitate. We conclude that c-Src protein kinase is associated specifically with the AhR complex along with hsp90 in the cytosol of these cells and that upon ligand binding to the Ah-receptor subunit, c-Src is activated and released from the complex. BIOCHEM PHARMACOL 52;10:1599–1612, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. c-Src; Ah receptor; TCDD; protein phosphorylation; guinea pig; mice

Dioxin-type chemicals are a class of toxic chemicals that are known to act mainly through the AhR,† which is present in cytosol and nucleus of mammalian cells. This class of chemicals includes very toxic and carcinogenic environmental pollutants such as polychlorinated dioxins, benzofurans, biphenyls, and naphthalenes [1–3]. Because of the toxicological importance of these chemicals, much research effort has been focused on the role of the AhR [4–7] particularly in relation to the mechanism of its ligand binding

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† *Abbreviations:* AhR, Ah receptor; CYP, cytochrome P450; SDS–PAGE 2D, sodium dodecyl sulfate–polyacrylamide two-dimensional gel electrophoresis; hsp, heat shock protein; HAP, hydroxylapatite; PMSF, phenylmethylsulfonyl fluoride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 1,2,3,7,8-PCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin; 1,2,4,7,8-PCDD, 1,2,4,7,8-pentachlorodibenzo-*p*-dioxin; 2,7-DCDD, 2,7-dichlorodibenzo-*p*-dioxin; AhRE, Ah-responsive elements; DMEM, Dulbecco's Modified Eagle's Medium; MC, 3-methylcholanthrene.

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activities, subsequent translocation into nucleus, and activation of the *CYP 1A1* gene [8, 9]. Briefly, upon ligand binding, the bound AhR subunit dissociates from the original cytosolic complex containing hsp90 and 50 [10] travels into the nucleus where it forms a heterodimer with another helix-loop-helix type nuclear transcription factor called "Arnt," and finally binds with the enhancer DNA region of genes possessing a specific sequence "GCGTG-3'," dioxin- or xenobiotics response element (DRE or XRE, respectively), and thereby activates their gene expression [11, 12]. While this basic pathway in activating *CYP 1A1* gene is well recognized and its activation mechanism is becoming clear, there are many unanswered questions with regard to the function and significance of the AhR itself. For example, the AhR is already expressed in 2–8 cell stages of the preimplantation mouse embryo [13], which does not express any cytochrome P450 genes. Yet, these embryos respond to TCDD, showing accelerated cavitation particularly in the presence of exogenously added EGF.

One of the main questions that we have been pursuing is the meaning of the TCDD-induced rise in protein tyrosine kinase activity [14, 15]. Previously we have shown that 10 nM TCDD added directly (cell-free condition) to isolated cytosol (100,000 *g* supernatant) from male guinea pig adipose tissue caused an immediate rise (within 2 min) of the overall protein kinase activities. We determined that such a rise is due to an increase in protein tyrosine phosphorylation as opposed to serine and threonine phosphorylation, based upon the inhibitory action of genistein, a specific tyrosine kinase inhibitor, and specific phosphorylation on an artificial peptide substrate, RR-SRC, which has only tyrosine residues as the phosphorylation site(s). Furthermore, we determined that such a cell-free activation process is clearly mediated by the AhR, and not due to the direct action of TCDD on protein kinases themselves. The role of phosphorylation on AhR itself has been studied by different laboratories (for examples, see Refs. 16–18). Their results suggested that phosphorylation may play an essential role in the dioxin signaling pathway. For instance, Carrier *et al.* [16] found that phosphorylation is required for the formation of AhR complexes, since *in vitro* dephosphorylation of nuclear extracts from TCDD-treated Hepa 1 cells abolishes the capacity of the AhR to form specific complexes with its cognate AhRE sequences. They concluded that the phosphorylation site(s) exists on at least one of the proteins constituting the transcriptional complex, possibly the AhR itself. Consistent with these findings, Mahon and Gasiewicz [19] have localized phosphorylation sites of the AhR in Hepa 1 cells to two regions located in the C-terminal half of the protein.

Many researchers have provided evidence that AhR is associated with hsp90 (for examples, see Refs. 20–22). As for Src association with heat shock proteins, Whitelaw *et al.* [10] reported that a 50-kDa cytosolic protein complexed with hsp90 is the same protein complexed with $pp^{60v\text{-}src}$/hsp90 in cells transformed by the Rous sarcoma virus. It has been suggested that the hsp90-p^{50} complex is involved in the transportation or docking of tyrosine kinases that reach the plasma membrane via routes other than the endoplasmic reticulum, Golgi, or cytoskeletal apparatus [23]. Along with hsp90, proteins of molecular weight = 63,000, 56,000, 50,000, and 188,000 were co-precipitated with hsp90 by mouse monoclonal antibodies 8D3 or 3M/3G3p^{90} (3G3) [24]. Immunoprecipitation of ^{32}P-orthophosphate-labeled Hepa-1 cytosol with mAb 8D3 showed that both hsp90 and p^{50} are phosphorylated [24]. The pI of the 50 kDa protein (5.35) was similar to that reported for the phosphorylated p^{50} complex with $pp^{60v\text{-}src}$/hsp90 in cells infected with Rous sarcoma virus [25]. However, to the best of our knowledge nobody has shown previously that AhR is directly associated with c-Src. In the current study, we present evidence indicating that such association is specific and likely to be functionally meaningful with respect to the action mechanism of the AhR.

Materials and Methods

Chemicals

γ-^{32}P ATP (3000 Ci/mmol) and [^{35}S]methionine (1228 Ci/mmol) were purchased from the Amersham Co. (Arlington, IL). c-Src (SRC 2) rabbit polyclonal (IgG) antibody, carboxy terminal epitope (Cat. No. sc-18), was purchased from the Santa Cruz Biotechnology Co. (Santa Cruz, CA). Mouse monoclonal v-Src antibody was purchased from Oncogene Science (Manhasset, NY). Cellex CM (Cation Exchange Cellulose, with exchange capacity 0.71 mEq/g) was purchased from Bio-Rad Laboratories (Richmond, CA). Substrate peptide, RR-SRC (RRLIEDAEYAARG) for Src-type tyrosine kinase assay, protein A-Sepharose CL-4B, and all other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). α-Naphthoflavone was purchased from the Aldrich Chemical Co. (Milwaukee, WI). We synthesized a peptide corresponding to the published sequence of AhR N-terminal 15 amino acids and raised antibodies against the corresponding peptide according to the general protocol developed by Poland and coworkers [26–28]. Briefly, the peptide was coupled to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) and injected into a male New Zealand rabbit. The serum was checked for antigenicity toward the peptide and purified for IgG using an affinity column containing protein-A Sepharose CL-4B. Unlabeled TCDD and [^{3}H]TCDD (34 Ci/mmol) were gifts from the Dow Chemical Co. (Midland, MI) with more than 99.99% purity.

AhR Immunoprecipitation Protocol and In Vitro *Kinase Activity Assay*

Male guinea pigs (*Cavia porcellus*) 6- to 8-weeks-old (250–300 g) were used throughout the study unless otherwise cited. Abdominal adipose tissue of untreated animals was removed and homogenized in 3 vol of MENG buffer [10]

(25 mM 4-morpholinepropanesulfonic acid, pH 7.5, 2 mM EDTA, 0.02% NaN_3 and 10% glycerol) containing 1 mM PMSF, 1 μg/mL of aprotinin, and leupeptin. The 100,000 *g* cytosol was prepared as described by Enan and Matsumura [7]. The adipose tissues were homogenized in 3 vol. of MENG buffer containing protease inhibitors. The homogenates were centrifuged at 5000 *g* for 15 min at 4° using a Sorvall centrifuge. The supernatant was carefully collected, avoiding any contamination with the top lipid layer, and recentrifuged at 100,000 *g* for 60 min at 4° using a Beckman ultracentrifuge. The 100,000 *g* cytosol was collected, and the protein concentration was adjusted to 9 mg/mL. An aliquot (18 mg protein) of this cytosol was passed through a CM-cellulose column (1 cm length × 0.5 cm diameter) that had been equilibrated with 5 mL of 10 mM 1,4-piperazinediethanesulfonic acid buffer, pH 6.8, containing 1 mM EDTA, 0.5 mM dithiothreitol and 1 mM NaN_3 at 4° to first eliminate positively charged proteins [29]. The eluate was pooled and then divided into three aliquots in Eppendorf tubes. The first portion (10 mg cytosolic protein) was incubated with AhR antibody (2 μg antibody protein/1 mg cytosolic protein); the second portion (2 mg cytosolic protein) was incubated with non-specific antibody, as a negative control (for this purpose the same rabbit pre-immune serum was purified for IgG under exactly the same conditions as the serum from AhR immunized rabbits [7]; and the third portion (2 mg cytosolic protein) was used without any treatment or addition (i.e. blank test). All three fractions were incubated for 2 hr on ice in a final volume of 5 mg protein in 1.0 mL MENG buffer, followed by the addition of 30 μL protein-A Sepharose CL-4B (100 mg protein-A Sepharose CL-4B/1 mL 50 mM HEPES, pH 7.4) per 5 mg cytosolic protein and mixed end-over-end for 1 hr at 4°. The immunocomplexes were precipitated using an Eppendorf microfuge at the maximum speed (14,000 rpm) for 10 min at 4°. After four washes with 1 mL of a 1:2 mixture of MENG:RIPA buffer (v/v) (RIPA buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EGTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin), the final pellets were washed once more with MENG buffer containing 50 mM NaCl and resuspended in 1.2 mL of 50 mM HEPES buffer, pH 7.4. In the case of non-specific antibody and without antibody experiments, the final precipitate was resuspended in 250 μL of 50 mM HEPES buffer, pH 7.4. TCDD (10 nM) or the same volume of the vehicle only (dioxane, 1%) was added to 40 μL of the pellet suspension. After 10 min at 30°, the assay buffer (containing 10 mM $MnCl_2$, 500 μM substrate peptide, RR-SRC, in 40 μL final volume of 50 mM HEPES buffer, pH 7.4) was added with 1 μCi[γ-^{32}P]ATP (3000 Ci/mmol, 1 μM final concentration). The reaction was stopped after 60 sec by the addition of 10 μL trichloroacetic acid (20%) followed by rapid centrifugation using an Eppendorf microfuge (10,000 rpm for 3 min at 4°). An aliquot of 20 μL of the supernatant was spotted onto phosphocellulose paper followed by three washes with 85 mM H_3PO_4, and the radioactivity was counted in 4 mL of liquid scintillation solution [7, 15]. Samples were tested in triplicate in the presence and the absence of the exogenously added substrate peptide. Each set of tests was confirmed by at least three independent experiments (experiments shown in Table 1). Data are expressed as means ± SD.

[^{35}S]Methionine Incorporation

NIH 3T3 fibroblast cells were plated at a density of 2×10^6 cells per 100-mm plastic culture dish in 5 mL DMEM supplemented with 10% calf serum. After 24 hr, the cells were washed three times with methionine-deficient DMEM prior to the addition of 0.2 mCi [^{35}S]methionine in 5 mL of methionine-deficient DMEM with 10% calf serum. After 18 hr at 37° in 5% CO_2, the medium was withdrawn and the cells were washed four times with ice-cold PBS. The cells were then scraped in 1 mL MENG buffer plus 1% SDS, homogenized in a glass/glass homogenizer, and 100,000 *g* cytosol was isolated. Aliquots of 10 mg cytosolic protein were incubated with AhR antibody (2 μg antibody/mg cytosolic protein) and the AhR antibody:AhR protein complex was precipitated with protein A Sepharose as described above. The immunoprecipitate was treated with either 10 nM TCDD or the same volume of vehicle only for 60 min. The final pellets were mixed with 4× SDS-loading buffer [14], boiled for 5 min, and subjected to 10% SDS–PAGE. The gel was stained and destained, and then an autoradiograph was developed as described before [14].

[^{3}H]-TCDD Binding Assay

An aliquot of cytosol from guinea pig adipose tissue (10 mg protein) was passed through a CM-cellulose column as described above. An aliquot of the eluate (300 μg protein) was incubated with 0.4 nM [^{3}H]TCDD (13.64 nCi/mL eluate) for 30 min at 25° prior to the addition of AhR-antibody or non-specific antibody (negative control) and kept on ice. After 2 hr, aliquots (15 μL) of protein-A Sepharose CL-4B were added and mixed end-over-end at 4°. After 60 min, the labeled AhR/protein-A Sepharose complex was precipitated using an Eppendorf microfuge, and the pellets were washed four times with MENG:RIPA (1:2, v/v) as before. The pellets were resuspended in 500 μL MENG buffer and divided into two aliquots. The first aliquot was counted directly for radioactivity (data shown in Table 2, Expt. I). The second aliquot was incubated with 500 mM NaCl for 60 min on ice followed by centrifugation in an Eppendorf microfuge at 14,000 rpm for 10 min. The supernatant was carefully collected and transferred into a tube contains 250 μL of HAP to quantify AhR [30]. Briefly, the HAP suspension was mixed end-over-end at 4° for 30 min, followed by the addition of 1 mL of 0.5% Triton X-100 in HEDG buffer [25 mM HEPES, 1.5 mM EDTA, 1.0 mM DTT, and 10% (v/v) glycerol, pH adjusted to 7.4 by 1 M Tris] to each tube, then mixed and centrifuged to precipitate the [^{3}H]AhR-HAP complex. The precipitate

TABLE 1. TCDD effect on kinase activity of AhR-immunoprecipitate from the cytosol of guinea pig adipose tissue assessed under cell-free conditions using RR-SRC as an artificial substrate

	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/ mg equivalent of cytosolic protein)*		Fold stimulation
	Control	TCDD	(TCDD/Control)
Experiment I			
With AhR-Ab	670 ± 90	1670 ± 100†	2.5
Without AhR-Ab	600 ± 80	640 ± 110	1.1
With non-specific IgG	520 ± 40	580 ± 70	1.1
With AhR-Ab, Without RR-SRC	140 ± 10	150 ± 10	1.1
Experiment II			
With AhR-Ab	750 ± 40	2860 ± 100†	3.8
With AhR/Ab + VO_4	790 ± 90	3020 ± 140†	3.8

* Protein content of the original cytosol was measured. The quantity of immunoprecipitate protein derived from the equivalent of the milligram quantity of original cytosol aliquot was calculated. The background values (without RR-SRC) were 140 ± 10 and 150 ± 10 dpm for control and TCDD-treated samples, respectively. These values were measured with AhR-Ab. Data are means ± SD for three independent experiments; each was run in triplicate (n = 9).

† Significantly different from the corresponding control value at $P \leq 0.01$ (Cochran t-test).

was washed twice with HEDG buffer containing 0.5% Triton X-100, resuspended in 0.7 mL ethanol, and transferred into 4 mL liquid scintillation solution for counting (data shown in Table 2, Expt. II). The effect of α-naphthoflavone (AhR blocker) on [^{3}H]TCDD binding in the presence and absence of 10 nM unlabeled TCDD was performed with AhR-immunoprecipitate as described in Expt. II, Table 2, except that unlabeled TCDD was used instead of NaCl (data shown in Table 2, Expt. III). The AhR blocker was added 10 min prior to the addition of unlabeled TCDD.

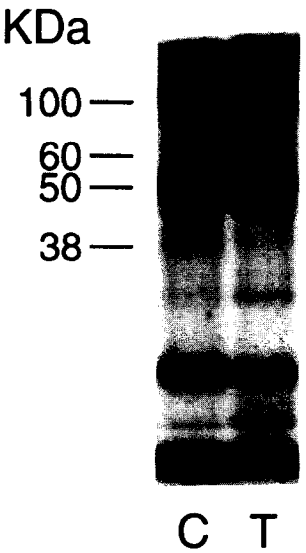

FIG. 1. Autoradiograph representing [^{35}S]methionine-labeled AhR-immunocomplex immunoprecipitates with AhR antibody from cytosol of prelabeled [^{35}S]methionine NIH 3T3 fibroblast cells. The suspension of AhR-immunoprecipitate was treated with 10 nM TCDD or the same volume of vehicle only for 60 min and analyzed with SDS–PAGE followed by development of autoradiograph as described in Materials and Methods. Samples were treated with SDS-loading buffer and directly transferred to the wells of 10% SDS–PAGE. C = control, T = TCDD-treated.

Each determination was done in triplicate. The data are expressed as mean/mg equivalent of cytosolic protein ± SD of two different experiments (N = 6). A concentration–response of unlabeled TCDD (0.01, 0.1, 1.0, and 10 nM) on [^{3}H]TCDD binding to the AhR-immunoprecipitate was performed. In a parallel experiment a concentration–response effect of unlabeled TCDD on AhR-associated kinase activity of guinea pig adipose tissue was performed using RR-SRC, [γ-^{32}P]ATP and the phosphocellulose paper method as described above (data shown in Fig. 2).

Dissociation of AhR Immunocomplex by TCDD and NaCl

The time–course of the effect of TCDD on dissociation of AhR from the AhR-immunocomplex-protein A Sepharose was investigated as follows. First, immunoprecipitation with AhR antibody or the non-specific antibody was carried out exactly as described above except that the final immunoprecipitate suspensions (100-μL aliquots) were incubated with TCDD (10 nM) or the same volume of vehicle only for either 10 or 60 min at 30°, which was followed by centrifugation using an Eppendorf microfuge. The supernatants were carefully decanted and saved, and the pellets were washed one time with HEPES buffer, pH 7.4, and resuspended in 100 μL HEPES buffer, pH 7.4. Aliquots of 30 μL from each fraction were used to measure kinase activity using the phosphocellulose papers as described above (experiment shown in Table 3). Second, the AhR/protein-A Sepharose precipitate was incubated with 250 or 500 mM NaCl [10] in 100 μL of 50 mM HEPES buffer for 10, 30, and 60 min on ice. The suspensions were centrifuged as before, and the supernatants and pellets were carefully isolated and each was incubated separately with 10 nM TCDD or the same volume of vehicle only (p-dioxane) for 60 min followed by measuring kinase activity using RR-SRC and the phosphocellulose paper method (experiment shown in Table 4).

TABLE 2. Studies on the specific presence of AhR in the immunoprecipitate of guinea pig cytosol obtained by using anti-AhR antibody*

Treatment	[^{3}H]TCDD binding activity (dpm/mg equivalent of cytosolic protein)			
			Expt. III	
	Expt. I	Expt. II	Control	TCDD
With AhR antibody	5390 ± 380	2560 ± 250	2590 ± 260	500 ± 30†
With non-specific IgG	2390 ± 200	1590 ± 80	300 ± 30	300 ± 20
With AhR antibody plus α-NF			710 ± 40	510 ± 30

* Expt. I: Total [^{3}H]TCDD/cytosolic AhR immunoprecipitate was counted directly after washing four times with MENG:RIPA as described in Materials and Methods. Expt. II: the [^{3}H]TCDD/cytosolic AhR immunoprecipitate was incubated with 500 mM NaCl for 60 min (see Table 4) on ice, followed by centrifugation to collect the supernatant and transfer into a tube containing 250 μL HAP to quantify AhR [7, 30], as described in Materials and Methods. Expt. III: the [^{3}H]TCDD/cytosolic AhR immunoprecipitate was incubated with and without 10 nM TCDD for 60 min at 30° followed by isolation of the supernatant to quantify [^{3}H]TCDD binding as described in Expt. II. α-NF = 1 μM α-naphthoflavone (10 μM 4,7-phenanthroline gave an identical result) was added 10 min prior to TCDD addition. Values are means ± SD of two different experiments; each was run in triplicate (N = 6).

† Significantly different from control values, $P \leq 0.01$ (Cochran *t*-test). Specific activity of [^{3}H]TCDD was 34 Ci/mmol (i.e. 1000 dpm = 13.37 fmol).

Assay Method for TCDD-Induced Kinase Activity of c-Src/Ah-Receptor Complex

To investigate whether c-Src protein kinase is an associated protein and a key enzyme in the AhR-complex, the following experiment was carried out. First, aliquots of AhR/protein-A Sepharose immunoprecipitate suspension (100 μL) in 50 mM HEPES buffer, pH 7.4, was incubated with 10 nM TCDD or the same volume of vehicle only for 60 min on ice. The supernatant was carefully collected after centrifugation as before and divided into two portions. The first portion was used directly to measure kinase activity as described above (experiment shown in Table 5, Expt. I). The second portion was immunoreacted with c-Src antibody or v-Src antibody. A non-specific antibody was used along with Src antibodies as a negative control. The immunoprecipitates were collected, washed, resuspended in 50 mM HEPES buffer, pH 7.4, and incubated with 10 nM TCDD at 30°. After 60 min the supernatant was collected as before, and kinase activity was determined exactly as described in the case of AhR (experiment shown in Table 5, Expt. II). Kinase activity was measured using RR-SRC as a substrate and the phosphocellulose paper method to measure the incorporation of ^{32}P into the substrate as described above.

Immunodepletion of Src-Proteins with Src Antibodies

Next we studied whether TCDD is still capable of inducing cytosolic kinase activity after the immunodepletion of c-Src from cytosol. In this experiment, the AhR-immunoprecipitate was incubated with 10 nM TCDD or the same volume of vehicle only for 60 min at 30°. The resulting supernatants were collected and additionally treated with two Src antibodies, c-Src and v-Src, along with non-specific antibody. After the Src-immunoprecipitates were collected, kinase activity was measured in the remaining cytosol using RR-SRC as a substrate and the phosphocellulose paper method as described above (experiment shown in Table 5, Expt. III). Briefly, an aliquot of the remaining cytosol (40 μL) was incubated with 10 nM TCDD or the same volume of vehicle only (*p*-dioxane). After 60 min at 30°, the protein was phosphorylated with [γ-^{32}P]ATP in the same assay buffer as described above. In another experiment, we studied the potency of the AhR blocker, α-naphthoflavone [15, 31] to inhibit the TCDD-induced kinase activity of c-Src-protein. In this experiment, c-Src-immunoprecipitate was incubated with 0.1 and 1.0 μM α-naphthoflavone in 100 μL final volume of 50 mM HEPES buffer, pH 7.4, for 10 min prior to the addition of 10 nM TCDD or the same volume of vehicle only. After 60 min at 30° the supernatants were isolated, and the kinase activity was measured using RR-SRC and the phosphocellulose paper method as before. A non-specific rabbit antibody was used along with c-Src antibody as a negative control (experiment shown in Table 6).

SDS–PAGE 2D

AhR-associated protein complex phosphorylation was also studied by runing 2D PAGE using 5 μCi[γ-^{32}P]ATP [15] without exogenously added substrate peptide (RR-SRC). Briefly, 5 mg protein equivalent of CM-cellulose eluate of cytosol was immunoreacted with AhR antibody under the same conditions as before. The AhR immunoprecipitate was washed and resuspended in 50 μL of 50 mM HEPES buffer, pH 7.4, and incubated with 10 nM TCDD or the same volume of the vehicle only at 30°. After 60 min the supernatants were centrifugally isolated and phosphorylated with 5 μCi [γ-^{32}P]ATP in the presence of 10 mM $MnCl_2$. The reaction was stopped after 1 min by the addition of the

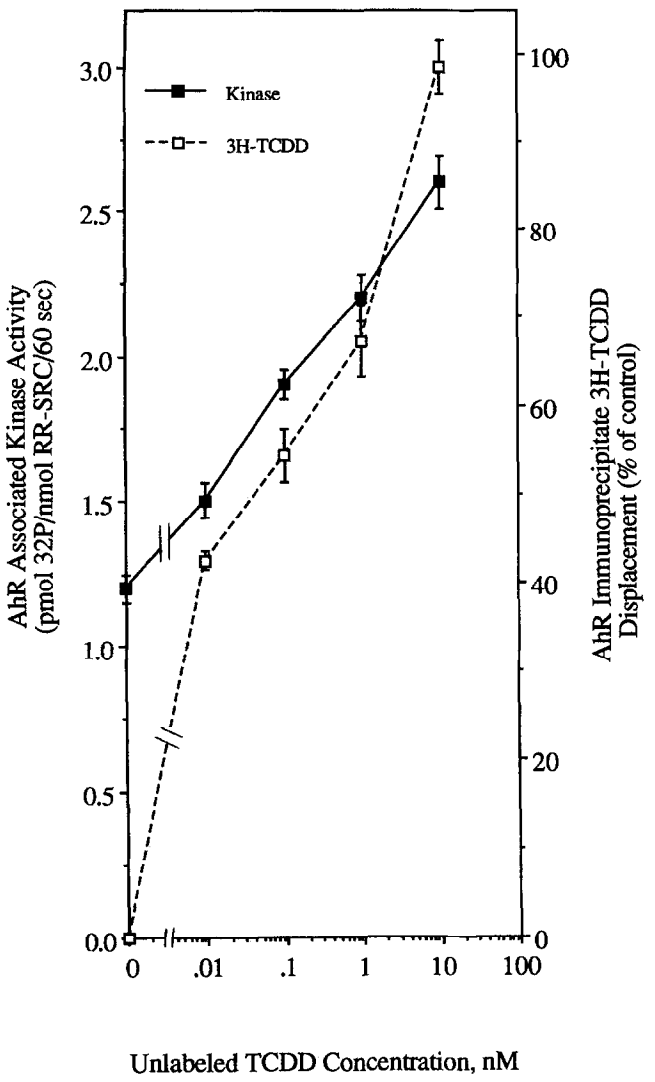

FIG. 2. Concentration–response relationship between added unlabeled TCDD and displaced [^{3}H]TCDD binding or increase in protein tyrosine kinase activity occurring in AhR-immunoprecipitate. Aliquots of 100,000 *g* adipose tissue cytosolic protein of guinea pig were incubated with [^{3}H]TCDD (0.4 nM) for 30 min at 25° prior to the addition of AhR antibody to immunoprecipitate [^{3}H]TCDD:AhR-complex. The immunoprecipitate was washed as described in Materials and Methods and incubated with different concentrations (0.01, 0.1, 1.0, and 10 nM) of unlabeled TCDD. After 60 min the supernatant was collected to quantify the displaced [^{3}H]TCDD from AhR-complex as described in Materials and Methods. In a parallel experiment, but without [^{3}H]TCDD, kinase activity was measured in the supernatant of the TCDD-treated AhR-immunoprecipitate using RR-SRC and the phosphocellulose paper method as described in Materials and Methods.

same volume of sample buffer [15], and the [^{32}P]phosphoproteins were analyzed first through gel tubes at 400 V for 13 hr, and thereafter the voltage was increased to 800 V for 60 min. The gel was removed from the tube and placed along the top edge of a 10% SDS–PAGE gel and electrophoresed for the second dimension [15]. After gel drying, autoradiographs were developed.

Structure-Activity Relationship of Dioxin Congeners on c-Src Kinase Activity

TCDD and three other dioxin-congeners: 1,2,3,7,8- and 1,2,4,7,8-PCDD and 2,7-DCDD with varying binding affinities to AhR [1, 2] were used in this experiment. The eluate from a CM-column (18 mg protein) was incubated with c-Src antibody (4 μg protein) for 2 hr on ice followed by immunoprecipitation with protein-A Sepharose, and washed as described before. The immunoprecipitate was resuspended in 4 mL of 50 mM HEPES buffer, pH 7.4, and aliquots (100 μL each) were incubated with 1 and 10 nM TCDD or 10 and 100 nM concentrations of other test dioxin-congeners. Control samples received the same volume of vehicle only. After 60 min at 30° the supernatants were isolated at 14,000 rpm using an Eppendorf microfuge for 10 min at 4° and used for the kinase activity assay using RR-SRC as a substrate peptide and the phosphocellulose paper method as described above (experiment shown in Table 7).

Cell-Free Effect of TCDD on AhR-Associated Kinase(s) Activity in Liver of Guinea Pig and C57Bl/6J Mouse, and in NIH 3T3 Fibroblast Cells

Livers perfused with ice-cold MENG buffer plus 1.15% KCl containing protease inhibitors (1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin) were removed from guinea pig and C57Bl/6J mouse. The livers were homogenized in 3 vol. of MENG buffer containing protease inhibitors. The homogenates were centrifuged at 5000 *g* for 15 min at 4° using a Sorvall centrifuge. The supernatant was carefully collected, avoiding any contamination with the top lipid layer, and recentrifuged at 100,000 *g* for 60 min at 4° using a Beckman ultracentrifuge. The 100,000 *g* cytosol was collected, and the protein concentration was adjusted to 15 mg/mL. In the case of NIH 3T3 fibroblast cells, the cells were plated at a density of 1×10^6 cells per 100-mm plastic culture plate in 10 mL of DMEM supplemented with 10% calf serum. At 80–85% confluence, the cells were washed three times with 37° PBS followed by collection of the cells in 1 mL of MENG buffer plus protease inhibitors, and the 100,000 *g* cytosol was prepared as described above. Protein concentration was adjusted to 10 mg/mL. Aliquots (15 mg protein) of the cytosol were either used directly for the assay or frozen immediately in liquid nitrogen and stored at −80° until later use. AhR immunoprecipitation was performed, and its associated kinase(s) activity was measured in the presence and absence of 10 nM TCDD with tyrosine kinase assay buffer containing RR-SRC and [γ-^{32}P]ATP as before (data shown in Table 8).

Effect of MC on TCDD-Induced AhR Associated c-Src Kinase Activity in NIH 3T3 Fibroblast Cells

Cells were cultured and maintained as described above. The cells were treated with 0.1 mM MC for 3 hr followed by preparation of 100,000 *g* cytosol as described above. AhR

TABLE 3. Time-course effect of TCDD on AhR-immunoprecipitated protein complex to dissociate kinase activity from pellets and release to supernatant under cell-free conditions*

TCDD-incubation time (min)	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein)	
	Control	TCDD
With AhR antibody		
Experiment I (10 min)		
Total (sup + pellets)	1230 ± 100	2140 ± 100†
Supernatant	1380 ± 80	1470 ± 60
Pellets	1330 ± 60	1550 ± 100
Experiment II (60 min)		
Total (sup + pellets)	1200 ± 80	2390 ± 170†
Supernatant	750 ± 40	1870 ± 130†
Pellets	1310 ± 20	1070 ± 60
With non-specific IgG	390 ± 20	420 ± 20

* A suspension of AhR immunoprecipitate still attached to protein-A Sepharose was incubated with 10 nM TCDD for 10 or 60 min, then centrifuged, and the supernatant and pellets were assessed separately for RR-SRC phosphorylating activity as in Table 1. All values were subtracted from background values (in the absence of RR-SRC). Data are means ± SD for three independent experiments; each was run in triplicate.

† Significantly different from the corresponding control value at $P \leq 0.01$ (Cochran t-test).

immunoprecipitation was performed, and its associated kinase(s) activity was measured in the presence and absence of 10 nM TCDD with tyrosine kinase assay buffer containing RR-SRC and [γ-^{32}P]ATP as before (data shown in Table 9). The data are the means (± SD) of three experiments with three replicates per experiment (N = 9).

Results

In the experiment shown in Table 1, we first collected the AhR complex from isolated cytosol of male guinea pig adipose tissue using a specific rabbit polyclonal antibody (IgG) against the *N*-terminal (15 amino acids) of the AhR through immunoprecipitation with protein-A Sepharose. The precipitate was washed thoroughly, suspended in 50 mM HEPES buffer, pH 7.4, incubated with 10 nM TCDD, and protein tyrosine kinase activity was assayed in the presence of RR-SRC, [γ-^{32}P]ATP, and $MnCl_2$ using the phosphocellulose paper method [7, 14, 15]. The results (Table 1) clearly showed that TCDD stimulates ^{32}P-phosphorylation on RR-SRC under these experimental conditions. A non-specific antibody (IgG from preimmune serum from the same rabbit) did not immunoprecipitate proteins whose kinases could be activated by TCDD. In the absence of RR-SRC, the overall phosphorylation on endogenous proteins was low. The addition of Na_3VO_4, a known protein tyro-

TABLE 4. Effect of different concentrations of NaCl on the dissociation of AhR-immunoprecipitate proteins from protein A-Sepharose*

NaCl-treatment time (min)	Kinase activity (dpm^{32}p incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein)			
	With 250 mM NaCl		With 500 mM NaCl	
	Supernatant	Pellets	Supernatant	Pellets
10 min				
Control	890 ± 80	1200 ± 110	680 ± 50	580 ± 50
TCDD	960 ± 80	1560 ± 130†	1800 ± 80‡	440 ± 30
30 min				
Control	950 ± 100	1190 ± 80	ND§	ND
TCDD	1120 ± 90	2370 ± 140‡	ND	ND
60 min				
Control	960 ± 60	1130 ± 70	720 ± 60	490 ± 30
TCDD	1210 ± 110*	2400 ± 170‡	2010 ± 100‡	400 ± 60

* AhR immunoprecipitate was incubated with either 250 or 500 mM NaCl on ice for 10, 30, and 60 min followed by centrifugation to isolate the supernatant and pellets. Both fractions were incubated with 10 nM TCDD or the same volume of vehicle only for 60 min prior to the addition of kinase assay buffer to measure kinase activity using the phosphocellulose paper method as shown in Table 1. All data were subtracted from background values. Values are means ± SD of two independent experiments; each was run in triplicate (N = 6).

†,‡ Significantly different from the corresponding control value at $\dagger P \leq 0.05$ and $\ddagger P \leq 0.01$, respectively (Cochran t-test).

§ ND = not determined.

TABLE 5. TCDD effect on kinase activity of AhR-immunoprecipitate and the effect of immunodepletion of c-Src kinase from supernatant using two Src antibodies

	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein)	
	Control	TCDD
Experiment I*		
AhR-antibody	800 ± 80	1990 ± 50†
Experiment II*		
Immunoprecipitates with the following antibodies:		
AhR-antibody	850 ± 20	2660 ± 140†
c-Src-antibody	670 ± 70	1740 ± 70†
v-Src-antibody	600 ± 80	1140 ± 60†
Non-specific IgG	290 ± 30	310 ± 30
Experiment III§		
Supernatant immunodepleted with the following antibodies:		
c-Src-antibody	470 ± 40	420 ± 10
v-Src-antibody	600 ± 70	200 ± 20
Non-specific IgG	850 ± 30	1300 ± 70‡

Values are means ± SD for three independent experiments, each was run in triplicate (N = 9). All values were subtracted from background values (without RR-SRC).

* The immunoprecipitated AhR complex and Src complexes were prepared and treated with TCDD for 60 min prior to the isolation of the supernatants, which were used to measure kinase activity.

†,‡ Significantly different from corresponding control values at $\dagger P \leq 0.01$ or $\ddagger P \leq 0.05$, respectively (Cochran *t*-test).

§ The remaining supernatant fractions after Src-protein immunoprecipitation with Src antibodies were used in this experiment.

sine phosphatase inhibitor, did not change the outcome, suggesting that there was no significant protein tyrosine phosphatase(s) associated with AhR (under the current conditions) and that this action of TCDD to stimulate protein phosphorylation was not due to inhibition of protein tyrosine phosphatase(s). To confirm the specificity of AhR antibody, we prepared [^{35}S]methionine-labeled cytosolic fractions from NIH 3T3 cells, and ran immunoprecipitation with the AhR antibody, which was analyzed with SDS–PAGE. The data showed the presence of proteins with molecular weights of approximately 100,000 (AhR),

TABLE 6. Blocking effect of α-naphthoflavone on TCDD-induced kinase activity of c-Src immunoprecipitate complex under cell-free conditions

	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein	
	Control	TCDD
c Src immunoprecipitate	590 ± 50	1260 ± 100†
With α-naphthoflavone*		
0.1 μM	600 ± 70	740 ± 50‡
1.0 μM	580 ± 50	580 ± 40
With non-specific IgG	160 ± 10	150 ± 20

Data are means ± SD for three independent experiments; each was run in triplicate (n = 9). All values were subtracted from background values (in the absence of RR-SRC).

* α-Naphthoflavone (AhR blocker) was added to the c-Src immunoprecipitate suspension for 10 min prior to the addition of TCDD followed by kinase activity assay as described in Materials and Methods.

†,‡ Significantly different from the corresponding control values at $\dagger P \leq 0.01$ or $\ddagger P \leq 0.05$, respectively (Cochran *t*-test).

TABLE 7. Structure–activity relationship studies of TCDD and three other dioxin congeners on c-Src kinase activity in AhR-immunoprecipitate from cytosol of guinea pig adipose tissue under cell-free conditions

Treatment	Kinase activity (dpm ^{32}P incorporated into RR-SRC/60 sec/mg equivalent of cytosolic protein)
Control (vehicle only)	520 ± 40
2,3,7,8,-TCDD	
1 nM	820 ± 40*
10 nM	1930 ± 110†
1,2,3,7,8-PCDD	
10 nM	530 ± 20
100 nM	1200 ± 80†
1,2,4,7,8-PCDD	
10 nM	500 ± 30
100 nM	640 ± 40
2,7-DCDD	
10 nM	500 ± 40
100 nM	510 ± 60

Data are the means ± SD of three independent experiments, each of which was run in triplicate (N = 9). c-Src immunoprecipitate was suspended in 50 mM HEPES buffer and incubated with TCDD or other test dioxin congeners for 60 min at 30° followed by centrifugation to collect the supernatant. The kinase assay was run using RR-SRC substrate peptide, 10 mM $MnCl_2$, and the phosphocellulose paper method.

*,† Significantly different from control value at $*P \leq 0.05$ or $\dagger P \leq 0.01$, respectively (Cochran *t*-test).

60,000, 50,000 and 38,000 that were immunoprecipitated with anti-AhR antibody only (Fig. 1), but not with non-specific antibody (data not shown).

To ascertain that under these experimental conditions, this particular antibody was specifically reacting with the AhR, we used specific [^{3}H]TCDD (0.4 nM) binding as a marker for the presence of cytosolic AhR protein. Immunoprecipitation with anti-AhR antibody clearly gave higher levels of [^{3}H]TCDD binding that were found with non-specific IgG precipitate (Table 2). In addition, the data showed that 10 nM unlabeled TCDD gave approximately 100% displacement of [^{3}H]TCDD binding to AhR-immunoprecipitate. A similar displacement effect was observed when 1 μM α-naphthoflavone was added to [^{3}H]TCDD:AhR-immunoprecipitate complex (Table 2, Expt. III). This displacement effect of unlabeled TCDD on [^{3}H]TCDD-bound immunocomplex was accompanied by the stimulatory action of TCDD on AhR-associated kinase activity in a concentration-dependent manner. A 45% displacement of [^{3}H]TCDD binding and a 20% increase in AhR-associated kinase activity was found when 0.01 nM unlabeled TCDD was added to the reaction (Fig. 2). Again 10 nM unlabeled TCDD induced approximately 100% [^{3}H]TCDD displacement, and this effect was associated with a 2-fold increase in AhR-associated kinase(s) activity as compared with the control values.

In the next experiment, we asked the question whether this kinase was still attached to the AhR after its activation by ligand binding. While the AhR immunocomplex was still attached to protein-A Sepharose, it was incubated with 10 nM TCDD or the same volume of vehicle only. After 10 min at 30° the reaction product was centrifuged, and the resulting pellet and supernatant were analyzed separately for protein kinase activities. These short-term incubation results were somewhat equivocal (Table 3, Expt. I), and therefore, we extended the TCDD incubation period from 10 to 60 min (Table 3, Expt. II). The results clearly showed that a portion of TCDD-induced kinase activity was now found in the supernatant instead of the pellets. Such a result suggests that the kinase protein must have physically dissociated from AhR and moved to the supernatant, as AhR itself was expected to be still attached to the AhR antibody/protein-A Sepharose pellets.

Next, we studied the effect of high concentrations of NaCl on c-Src-AhR association, as such treatments have been shown by another research group to dissociate the AhR-hsp90 complex [20]. The AhR immunoprecipitate-containing AhR/protein-A Sepharose complex was incubated with two different concentrations (250 and 500 mM) of NaCl for 10, 30, and 60 min on ice, and centrifuged to separate the pellets and supernatant. Both of these fractions were incubated separately with 10 nM TCDD, and ^{32}P-

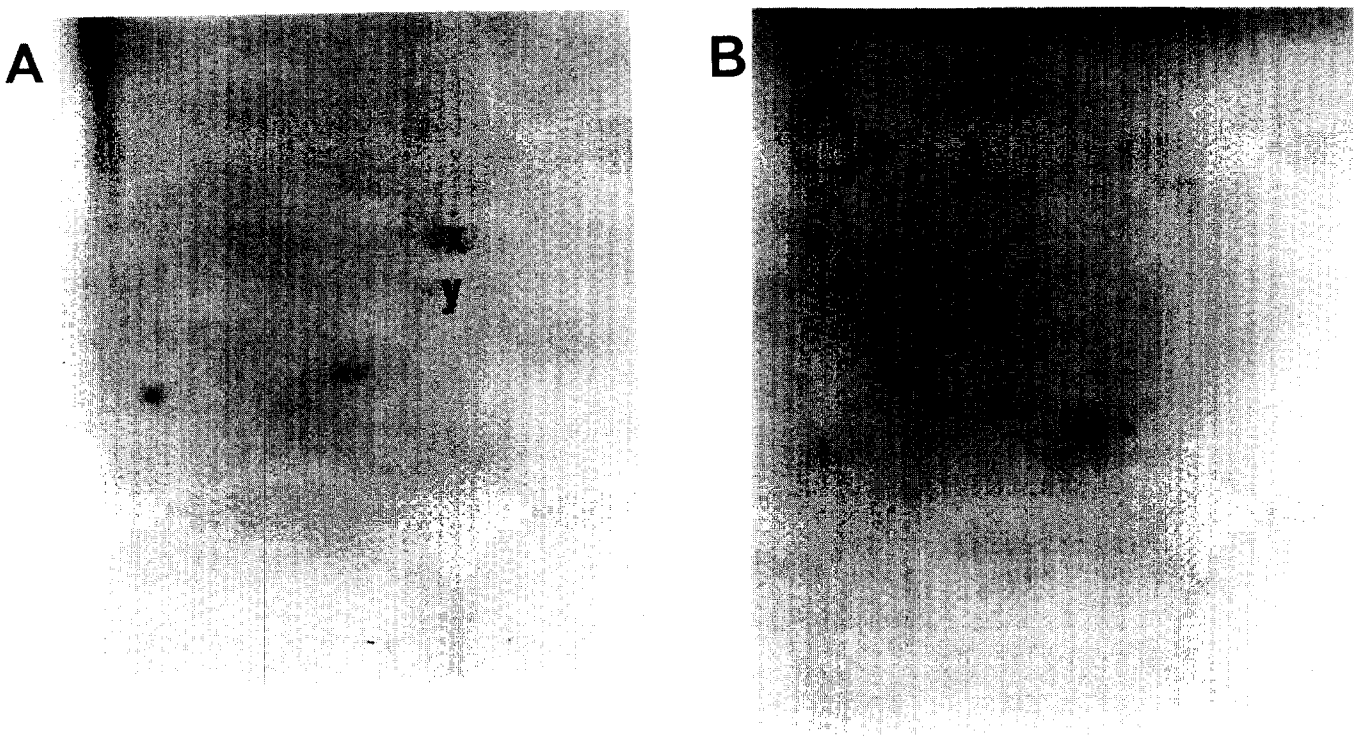

FIG. 3. Autoradiograph representing 2D gel electrophoresis. (A) AhR immunoprecipitate was incubated with 10 nM TCDD at 30° for 60 min followed by centrifugation and the supernatant was collected. The resulting supernatant was phosphorylated with [γ-^{32}P]ATP and 10 mM $MnCl_2$ and the ^{32}P-labeled phosphoproteins were analyzed using 2D gel electrophoresis. (B) Immunoprecipitate was prepared exactly as in Panel A except that c-Src antibody was used in the initial immunoprecipitation step instead of AhR antibody. Two other tests, one matched control to (A) (solvent added in place of TCDD) and another (A) secondarily treated with c-Src antibody, showed no recognizable radiolabeled spots and therefore their autoradiograms are not shown. The position of ^{32}P-autophosphorylated c-Src is shown by arrows; "x" and "y" in (A) indicate two phosphoproteins associated with the AhR.

phosphorylation on RR-SRC was studied as before. The results (Table 4) showed that at the high NaCl concentration (500 mM), the phenomenon of TCDD-induced increase in phosphorylation in pellets disappeared as compared with those treated with a low NaCl concentration (250 mM). Instead, the same phenomenon of TCDD-induced activation of kinase shifted to the supernatants. In addition, it was noticed that even at the lower concentration of NaCl, TCDD increased the kinase activity in supernatant provided the incubation time was long enough (Table 4).

To address whether AhR would co-immunoprecipitate with c-Src or not, c-Src antibody was used as the immunoprecipitation agent, instead of AhR antibody, in the initial step of immunoprecipitation from CM-cellulose eluate. After four washes, the final immunoprecipitate was used to investigate the action of TCDD to activate kinase activity. The results showed that the immunoprecipitate with c-Src antibody also responded to 10 nM TCDD in a similar manner as that precipitated with the AhR antibody (Table 5, Expt. II). In the next experiment (Table 5, Expt. III), the AhR immunoprecipitate suspension was first incubated with 10 nM TCDD or the same volume of vehicle only at 30° for 60 min, followed by centrifugation to collect the supernatant. To study whether such a treatment released c-Src, the resulting supernatants were additionally immunodepleted with two Src antibodies, anti-c-Src or anti-v-Src along with non-specific IgG. The results showed that this second treatment with Src antibodies (i.e. immunodepletion) completely removed TCDD's stimulation of kinase from the supernatant, while the same treatment with non-specific IgG did not. This c-Src antibody is a very specific antibody reacting to only c-Src, as it is directed against the N-terminal 8-amino-acid sequence of c-Src, which is not shared with any other Src-type protein tyrosine kinases (as described by the supplier, Santa Cruz Biotechnology Co.).

Since the action of TCDD on c-Src antibody immunoprecipitate could also be abolished with co-administration of 1 μM α-naphthoflavone, which at this concentration is known to block the AhR specifically (Table 6), the above results appear to favor the idea that c-Src immunoprecipitate also contains AhR. Consistent with these results, we found that the action of TCDD and three other dioxin congeners in this regard followed the expected rank order for their potency in terms of the stimulation of c-Src kinase activity. This rank order matches with their AhR binding affinities: 2,3,7,8-TCDD > 1,2,3,7,8-PCDD > 1,2,4,7,8-PCDD > 2,7-DCDD (Table 7). These data showed that the immunoprecipitate obtained by incubating the specific c-

TABLE 8. TCDD effect on AhR-associated kinase activity and c-Src kinase activity in immunoprecipitates from cytosol of liver from guinea pig and C57Bl/6J mouse, and NIH 3T3 fibroblast cells tested under cell-free conditions

	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein)			
	Supernatant		Pellets	
	Control	TCDD	Control	TCDD
Experiment I				
With AhR antibody				
Guinea pig	2040 ± 90	4630 ± 130*	1440 ± 80	2720 ± 240*
Mouse C57Bl/6J	3270 ± 260	5220 ± 130*	2430 ± 520	4700 ± 160*
NIH 3T3 fibroblast cells	840 ± 110	2100 ± 200*	900 ± 130	800 ± 70
With non-specific IgG				
Guinea pig	410 ± 50	540 ± 90	580 ± 30	600 ± 40
Mouse C57Bl/6J	700 ± 60	680 ± 30	430 ± 40	540 ± 60
NIH 3T3 fibroblast cells	240 ± 20	310 ± 20	300 ± 30	340 ± 30
Experiment II				
With c-Src antibody				
Guinea pig	2350 ± 200	4150 ± 310*	ND†	ND
Mouse C57Bl/6J	3640 ± 420	5080 ± 530*	ND	ND
NIH 3T3 fibroblast cells	910 ± 50	2520 ± 170*	ND	ND
With non-specific IgG				
Guinea pig	450 ± 70	490 ± 90	ND	ND
Mouse C57Bl/6J	580 ± 60	640 ± 80	ND	ND
NIH 3T3 fibroblast cells	200 ± 40	200 ± 30	ND	ND

Data are the means ± SD of nine assays. Aliquots (150 μl) of AhR-immunoprecipitate (Expt. I) or c-Src-immunoprecipitate (Expt. II) were incubated with 10 nM TCDD or the same volume of vehicle only (dioxane) at 30°. After 60 min, the supernatant and the pellets from each immunoprecipitate were isolated at 14,000 rpm using an Eppendorf microfuge at 4° for 10 min. The supernatant was carefully collected and saved on ice, while the pellets were washed one more time with MENG buffer and resuspended in 150 μL 50 mM HEPES buffer, pH 7.4. Aliquots (40 μL) from supernatant and pellets were used to measure kinase activity with RR-SRC and tyrosine kinase assay buffer using the phosphocellulose paper method.

* Significantly different from the corresponding control values at $P \leq 0.01$ (Cochran t-test).

† ND = not determined.

TABLE 9. Effect of MC on TCDD-induced AhR/c-Src kinase activity in NIH 3T3 fibroblast cells*

	Kinase activity (dpm ^{32}P incorporated into 1 nmol RR-SRC/60 sec/mg equivalent of cytosolic protein			
	Without MC		With MC	
	Control	TCDD	Control	TCDD
With AhR antibody				
Supernatant	700 ± 50	1490 ± 110†	500 ± 30	500 ± 40
Pellets	810 ± 50	760 ± 40	540 ± 50	570 ± 40
With non-specific antibody				
Supernatant	410 ± 20	400 ± 30	440 ± 40	430 ± 50

* NIH 3T3 fibroblast cells were treated with 0.1 mM MC or vehicle (dioxane) for 3 hr followed by preparation of 100,000 *g* cytosol. Aliquots of 15 mg cytosolic protein were eluted through a CM-cellulose column as described in Materials and Methods. The eluates were immunoreacted with AhR antibody or non-specific antibody exactly as described in Table 1. The AhR-immunoprecipitates or the non-specific antibody immunoprecipitate were washed and incubated with 10 nM TCDD or vehicle only for 60 min at 30° followed by isolation of supernatant and pellets as described in Table 3. Tyrosine kinase activity was measured in each fraction separately using RR-SRC, [γ-^{32}P]ATP and the phosphocellulose paper method. Data are the mean ± SD of nine assays (N = 9).

† Significantly different from the corresponding control value at $P \leq 0.01$ (Cochran *t*-test).

Src antibody with cytosolic proteins contained the AhR protein (sometimes called the ligand-binding subunit of the AhR complex).

To ascertain that this AhR-associated tyrosine kinase was really c-Src, we analyzed endogenous ^{32}P-phosphorylated protein products in the AhR immunoprecipitate-supernatant (preparation equivalent of Table 3, Expt. II, but ^{32}P-phosphorylated without RR-SRC) through 2D, PAGE. The resulting autoradiographed electrophoretograms showed that there was a predominant ^{32}P-phosphoprotein spot at 60 kDa and approximately pI 6.1 (Fig. 3A), which matches with the major ^{32}P-phosphoprotein spot in the identically prepared electrophoretogram of c-Src immunoprecipitate (Fig. 3B). Additional antibody treatment on the ^{32}P-phosphorylated supernatant with c-Src antibody removed this particular spot (data not shown). The identical ^{32}P-phosphorylation treatment on control supernatant produced no ^{32}P-phosphoprotein spots as judged by 2D-autoradiograph (data not shown), indicating that the action of TCDD on AhR indeed induced autophosphorylation on c-Src kinase, which is responsible for the appearance of ^{32}P-phosphoprotein at the spot matching c-Src in Fig. 3A.

To investigate whether the same phenomenon occurred in other organs and other species, we repeated the same experiment as described in Table 3, Expt. II, using liver from guinea pig and C57Bl/6J mouse, and NIH 3T3 fibroblast cells. The data (Table 8) clearly showed that the pattern of TCDD-induced AhR-dependent activation of c-Src kinase activity was essentially identical in all of these biological materials. In addition, pretreatment of NIH 3T3 fibroblast cells with 0.1 mM MC abolished the inducible effect of TCDD on AhR-associated kinase activity (Table 9). When the same experiment was repeated using c-Src antibody instead of AhR antibody during the immunoprecipitation steps, TCDD did not affect c-Src immunoprecipitate kinase activity (data not shown). These data suggest that pretreatment of intact 3T3 cells with MC either dissociated c-Src from AhR or depleted AhR from intact cell cytosol.

DISCUSSION

In the present work, we found that c-Src was specifically associated with AhR as indicated by several lines of evidence: (a) by using 2D electrophoresis, ^{32}P-phosphorylated AhR immunocomplex protein was found to be associated with M_r = 60 kDa and pI 6.1 phosphoprotein that matches c-Src protein, (b) this phosphoprotein disappeared when the AhR immunocomplex supernatant was immunodepleted of c-Src protein by anti-c-Src antibody as judged by 2D autoradiograph, (c) an identical phosphoprotein with M_r = 60 kDa was found in the ^{32}P-phosphorylated immunoprecipitate's supernatant when the cytosol was first immunoreacted with anti-c-Src antibody followed by treatment with TCDD for 1 hr as judged by 2D-autoradiogram, (d) TCDD-induced cytosolic kinase(s) activity was abolished when TCDD was administered directly to immunodepleted cytosol of c-Src protein by anti-c-Src antibody, (e) the structure–activity relationship studies revealed that the increase of c-Src kinase activity is consistent with the known AhR affinity of several dioxin congeners, and (f) the TCDD-induced kinase activity was abolished when c-Src immunoprecipitate was incubated with α-naphthoflavone (AhR blocker) for 10 min prior to the addition of TCDD. The balance of evidence available, therefore, clearly supports the view that c-Src is the main protein kinase that is specifically associated with the AhR and is activated upon ligand binding. The association of c-Src with AhR-complex is not likely to be a mere artifact created during homogenization. This conclusion is based on the observation by Hutchison *et al.* [32] that Src association with hsp90 and p50 protein in cell-free systems could be created only by the use of an elaborate rabbit reticulocyte reconstitution

system and not by mere coincidence. In addition, treatment of intact cells with 0.1 mM MC prior to homogenization and preparation of cytosol totally eliminated the ability of TCDD to activate c-Src kinase activity (Table 9), indicating that c-Src/AhR association is no longer found in the AhR-immunoprecipitate. Since most of the constituents of the AhR complex such as hsp90, p50 protein, and c-Src are expected to be associated in cytosol, this lack of TCDD's action on AhR-associated kinase activity argues against artificial assembly of such a complex during homogenization.

In support of such a conclusion, we have shown previously that the mediated activation of protein tyrosine kinase occurs in immunoprecipitates obtained using antibodies to AhR, hsp90, or c-Src from the cytosolic preparation, demonstrating thereby that such cell-free activation of c-Src kinase activity by TCDD is likely to take place in a protein complex consisting at least of c-Src, hsp90, and the AhR [7]. Such a conclusion also helps to explain the past observations by other scientists. In addition, an earlier report from this laboratory showed that TCDD has the capacity to increase Src activity in guinea pig *in vivo* as well as in NIH 3T3 cells *in vitro* [33]. In cytosol, both c-Src and v-Src are known to be associated with hsp90 and 50 [10]. A key question we must now raise is whether the main function of AhR ligand-induced kinase activation is to facilitate growth factor signal transduction activities or not. Our current opinion is definitely in favor of such a possibility. The main reasons are that (a) it is very common to find a kinase specifically associated to a receptor to act as the key transducer; (b) c-Src is a very well established kinase capable of transducing growth factor signals, including tyrosine phosphorylation of growth factor receptors and recruitment of SH2-containing helper proteins for further transduction; (c) other intracellular/cytosolic receptors such as glucocorticoid and the estrogen receptor are now also shown to be associated with specific kinases; and (d) there are many sets of data both *in vivo* and *in vitro* indicating that TCDD causes activation of protein tyrosine kinases, particularly those associated with the growth factor signal transduction pathway [7, 34].

The cellular events that result from extracellular signaling by growth factors are initiated primarily by members of the transmembrane protein-tyrosine kinase family [35]. Stover *et al.* [36] found that epidermal growth factor receptor is phosphorylated *in vivo* by c-Src at nonautophosphorylation sites and that these novel sites can act as additional docking sites for Src, P85α, and potentially other SH2-containing proteins. Indeed, probably the most significant outcome of the current finding of c-Src association with the AhR is that it may now become possible to logically follow the cascade of events occurring upon activation of this well studied protooncogene through mitogenic signaling into the nucleus or any other routes of action of c-Src known to occur in a variety of cell types [37]. For instance, c-Src is known to be absolutely required for the signal transduction pathways of the platelet-derived growth factor. Also, c-Src acts as a part of the tyrosine phosphorylation cascade for many other growth factors as well as hormones such as insulin, thyroid hormone, and estrogen [35, 37]. In fact, one of the known major routes of action for c-Src is activation of c-ras, p42-p44mapk2 and AP-1 proteins, all of which have been shown previously to be activated by TCDD in adipose tissue of male guinea pigs [7, 14, 15, 38, 39].

As for the meaning of TCDD-induced dissociation of c-Src from AhR and concomitant activation of its kinase, we favor the view that these two events are functionally connected to each other, i.e. releasing c-Src from AhR appears to coincide with the activation of its kinase activity. However, it is not certain whether mere dissociation of c-Src without the action of a ligand or simple free floating of c-Src would provide active c-Src or not. In this regard, the observation that treatment with high NaCl concentrations shifts the action site of TCDD to the supernatant (Table 4) merits some comments. The fact that TCDD-inducible kinase shifted to the supernatant upon treatment with NaCl indicates that AhR is still likely to be associated with the c-Src released to the supernatant, despite their release from the immunocomplex. The site of NaCl-induced dissociation must be, therefore, somewhere between AhR and protein-A Sepharose since the latter protein is covalently attached to Sepharose. One pertinent observation in this regard is that at these concentrations of NaCl, the bond between hsp90 and AhR is likely to be broken off [20]. If such is the case, a possible scenario is that the AhR complex without hsp90 is still capable of responding to TCDD. However, much more data would be needed to prove such a possibility. In addition, the data in Table 9 illustrate that pretreatment of 3T3 cells with MC abolished the TCDD-induced kinase activation in subsequently isolated cytosol. Such data support our conclusion that c-Src association with AhR is functionally important in transducing the signal of AhR ligands in intact cells. Therefore, at least this model offers an approach to future study of some intricate aspects of the ligand-induced receptor rearrangement. One question we have not addressed thus far is the possibility of c-Src acting as the initial or main kinase to directly phosphorylate AhR. The subject of AhR phosphorylation is still somewhat controversial, though most scientists seem to agree that phosphorylation is important to AhR action. For instance, Pongratz *et al.* [22] concluded that dephosphorylation of the AhR by phosphatase treatment inhibited the specific DRE-DNA-binding activity of the AhR. Berghard *et al.* [17] have investigated the importance of phosphorylation of various subunits of the AhR complexes by examining the effect of acid phosphatase-dependent dephosphorylation on the ability of these subunits to form heterodimers and to bind to DNA. The results of their mixing experiments suggest that phosphorylation of the ligand-binding subunit is necessary for DNA binding, while phosphorylation of Arnt appears to be necessary for dimerization. Thus, dephosphorylation of either AhR or Arnt appears to result in inhibition/loss of DNA-binding

activity by the TCDD:AhR complex. On the other hand, Mahon and Gasiewicz [19] reported that TCDD binding to the AhR does not alter the total level of phosphorylation on the AhR. However, the same group [19] has localized phosphorylation sites of the AhR in Hepa 1 cells to two regions located in the C-terminal half of the protein. One region is centrally located between amino acids 368 and 605 and the other is located at the Gln-rich carboxyl terminus between amino acids 636 and 759 [19]. Thus, while c-Src phosphorylation/activation at the time of AhR/ligand binding appears to be logical, our current thinking is that one must be very cautious about assuming the *in vivo* event to be identical to the one occurring in this cell-free model system.

In conclusion, we have established that c-Src is specifically associated with AhR complex and that TCDD at toxicologically relevant concentrations causes a significant rise in c-Src kinase activity in an AhR-dependent manner. Such an arrangement of AhR appears to be rather common among other types of cells and other species, judging by the results of the current study (Table 8) as well as those from additional studies ongoing in our laboratory. Therefore, knowing the importance of the AhR in transducing the messages of many toxic chemicals, we judge that it is very worthwhile to study the meaning of AhR ligand-induced activation of c-Src in the development of various toxic expressions in the future.

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