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EVIDENCE FOR A SECOND PATHWAY IN THE ACTION MECHANISM OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD)

SIGNIFICANCE OF Ah-RECEPTOR MEDIATED ACTIVATION OF PROTEIN KINASE UNDER CELL-FREE CONDITIONS

ESSAM ENAN and FUMIO MATSUMURA*

Department of Environmental Toxicology and Center for Environmental Health Sciences, University of California, Davis, CA 95616-8588, U.S.A.

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Abstract—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) when administered directly to a nuclear-free subcellular homogenate of guinea pig adipose tissue, caused a significant rise in protein kinase activities within 1–10 min. Such a rapid response was not expected, based on the classic transcriptional mechanism of action for TCDD, i.e. TCDD first binds with its cytosolic Ah-receptor, translocates into the nucleus, dimerizes with "arnt" (a nuclear transcription factor), and activates genes containing "xenobiotic-responsive element" (XRE). The above actions of TCDD on protein kinases were clearly blocked by two specific Ah-receptor blockers, even under cell- and nucleus-free conditions. TCDD-induced increases in protein phosphorylation occurred mainly in cytosolic preparations (i.e. 100,000 *g* supernatant) devoid of nucleus, microsomes and plasma membranes and were still observed in the presence of inhibitors of protein phosphatases. Furthermore, TCDD caused a rise in protein tyrosine kinase activity in a purified Ah-receptor preparation, as well as in an isolated heat shock protein 90 complex preparation containing the Ah-receptor. This activation took place in the presence of actinomycin D and cycloheximide, indicating a portion of TCDD's action that is unrelated to *de novo* protein synthesis in this process. We have also obtained evidence indicating that this action of TCDD triggers the protein kinase mediated growth factor signal transduction pathway, such as stimulation of mitogen activated protein kinase 2 and tyrosine kinase activity. These results clearly support the view that the basic action pathway for such a TCDD-induced activation of protein kinases is distinctly different from its conventional action pathway involving changes in gene transcription in the nucleus.

Key words: TCDD; cell-free; Ah-receptor; kinases; gel-retardation; adipose tissue

Dioxin-type chemicals include serious environmental pollutants, such as polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, biphenyls (PCBs), naphthalenes, and polyaromatic hydrocarbons, all of which possess similar toxic properties [1–3]. There have been intensive studies on the mode of action of this class of chemicals, because of their potency as toxicants, their widespread occurrence in the environment, and their broad spectrum of toxic effects in many different vertebrate species. Particularly popular among scientists who are interested in the molecular aspect of their action is the use of TCDD† as a probe. Their interest in TCDD is understandable, since this is regarded as the most toxic congener of the dioxin-type chemicals and, because of its metabolic stability, is itself the acknowledged toxicant [1–3].

Despite the fact that TCDD is capable of causing a wide variety of toxic symptoms in many animal species, there is a near unanimous consensus that the initial event in the toxic effects produced by TCDD is its binding to the cytosolic Ah-receptor [3–5]. As far as it is known, upon TCDD-binding, this cytosolic Ah-receptor detaches itself from a complex with HSP 90, translocates to the nucleus, where it forms a dimer with another helix-loop-helix protein "arnt" (a nuclear transcription factor), and eventually interacts with the promoters of specific genes [6–8]. It has been demonstrated clearly, as the result of efforts of many scientists, that all of the active congeners belonging to the family of "dioxin-type" chemicals show significant affinities for the Ah-receptor, and that their interactions with this cytosolic receptor are the essential first step in the resulting toxic actions [9, 10].

Another common action of all these chemicals is the induction of xenobiotic metabolizing enzymes in various tissues, particularly in the liver [11]. Among the most studied is the induction of cytochrome P450 1A1 (CYP1A1) [12–14]. Some *CYP1A* genes have been shown to possess a specific sequence of DNA, 5′-GCGTG-3′, often termed "XRE" or "DRE"

* Corresponding author. Tel. (916) 752-4251; FAX (916) 752-3394.

† Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element; CYP1A1, cytochrome P_{450} 1A1; MAP-2 kinase, mitogen activated protein kinase 2; PKC, protein kinase C; Ah-receptor, cytosolic dioxin receptor; HSP, heat shock protein.

(xenobiotic- or dioxin-responsive element, respectively), to which the ligand bound Ah-receptor "arnt" complex binds [7–9], thereby activating transcription. This action of TCDD has been well documented and accepted.

Despite the great progress in describing the pathway for activation of gene transcription by TCDD, some scientists question that this is the sole toxic action route of dioxin-type chemicals. For instance, in the most susceptible species, the guinea pig, the induction of cytochrome P450s in the liver is not a prominent result of TCDD poisoning. In addition, Puga *et al.* [15] recently reported that TCDD causes a rise in mRNAs of immediate early genes such as c-*fos*, c-*jun*, *jun*-D and *jun*-B in Hepa-1 cells. Furthermore, they showed that TCDD could cause a rise in Ca^{2+} uptake within 5 min in those cells. Such a quick response could not be explained on the basis of gene expression changes.

Indeed, there exists a large gap in our knowledge between the initial event of TCDD binding to the Ah-receptor and the final expression of many overt toxicities and lethality. In this work, we have made an attempt to search for the existence of other pathways for TCDD action using *cell-free* preparations from adipose tissue of male guinea pigs. The reasons for selection of this biological material are: (a) adult male guinea pigs are the animals most susceptible to the lethal action of TCDD (LD_{50} = 0.6 μg/kg), (b) the reduction of its adipose tissue (i.e. "wasting syndrome") is one of the major manifestations of toxicity, and (c) TCDD at very low doses is known to cause rapid changes in protein phosphorylation [16], as well as glucose-transporting activities in this tissue [17, 18]. Unexpectedly, we have found that TCDD is capable of activating some protein kinases under strictly *cell-free* conditions even without the nucleus. Such an initial effect of TCDD takes place very rapidly (within 1–10 min) and is dependent on the Ah-receptor. Based on these data, we now propose an additional mechanism of action for this class of chemicals.

MATERIALS AND METHODS

Chemicals. TCDD was a gift from the Dow Chemical Co. (Midland, MI) with purity higher than 99.9%. [γ-^{32}P]ATP (~3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Monoclonal (mouse) anti-HSP 90 antibody (IgM, clone 3G3) was purchased from Affinity Bioreagents (Neshanic Station, NJ). MAP-kinase substrate peptide was purchased from UBI (Lake Placid, NY). Ac-MBP (4–14) protein kinase C substrate peptide, protein kinase A substrate peptide (Kemptide), and protein tyrosine kinase substrate peptide (RR-SRC) were purchased from GIBCO BRL (Grand Island, NY). Double-stranded AP-1 response element DNA (CTAGTGATGAGTCAGCCGGATC) was purchased from Stratagene (La Jolla, CA). 4,7-Phenanthroline and α-naphthoflavone were purchased from the Aldrich Chemical Co. (Milwaukee, WI). All other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Antibody for c-Src (SRC-2), Cat. No. SC-18, was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). Antibody for phosphotyrosine (PY20), Cat. No. 69-137, was purchased from ICN (Costa Mesa, CA). We synthesized a peptide corresponding to the published sequence of the Ah-receptor N-terminal amino acids and raised antibodies against the corresponding peptide according to the general protocol developed by Poland's group [19–21].

Animals. Four- to six-week-old (200–225 g) male English short hair guinea pigs (*Cavia porcellus*) were used throughout the study. Animals were housed in suspended stainless steel cages and provided with food and water *ad lib*. All animals were maintained on a 12-hr light/12-hr dark cycle at constant temperature (72 ± 1°F) and humidity (70%). *In vivo* treatments were carried out as previously described by us [17, 18]. *In situ* culture conditions for explant tissue of adipose tissue were exactly as described by Enan and Matsumura [18].

Preparation of cell fractions. Epididymal and abdominal adipose tissues of untreated guinea pigs were homogenized in 3 vol. (w/v) of buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.05 mM dithiothreitol (DTT)]. The homogenates were centrifuged at 7000 *g* for 10 min at 4° in a Sorvall SS34 rotor to pellet nuclei. The low speed (7000 *g*) supernatant from this step was designated as the postnuclear fraction, which was decanted carefully and either used in the assay as total extranuclear fraction or centrifuged for 60 min at 100,000 *g* (Beckman Type 50 rotor). The cytosol and pellet were obtained, and the latter was resuspended in enough volume of buffer A to give a protein concentration of 5 mg/mL and used as membrane (microsomal) fraction. The nuclear extract was prepared as described by Dignam *et al.* [22], with some changes. Briefly, the pellet obtained from the low speed centrifugation (7000 *g*) of the homogenate was resuspended in 3 vol. of buffer A and subjected to a second centrifugation for 20 min at 25,000 *g*. The pellet (crude nuclei) was resuspended in 500 μL of buffer B [20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M $NaCl_2$, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT plus 10 μg/mL of the other protease inhibitors, leupeptin, aprotinin and chymostatin, all of which were added to the buffer just before the extraction] with a glass/glass homogenizer. The suspension was stirred gently with a magnetic stirring bar at 4° for 30 min and then centrifuged for 30 min at 25,000 *g*. The resulting clear supernatant was used as a nuclear extract.

Cell-free phosphorylation studies. For SDS–PAGE studies, an aliquot usually containing 100 μg protein from each fraction (cytosol, microsomal and nuclear fractions) was incubated with 10 nM TCDD (or the same volume of acetone alone for control) *in vitro* at 30° in 80 μL of 50 mM HEPES, pH 7.9, 10 mM $MnCl_2$, and 10 μM Na_3VO_4. After 10 min, 1 μCi (final concentration 3 μM) of [γ-^{32}P]ATP was added, and the reaction was terminated after 60 sec by the addition of 40 μL of 4 × SDS-treatment buffer [16]. The samples were heated at 90° for 3 min. The entire volume of the reaction was analyzed using 10% SDS–PAGE at a constant current of 50 mA for 4–5 hr. The gel was stained in 1% Coomassie Blue with 50% methanol and 10% acetic acid and

destained in 50% methanol and 10% acetic acid. The dried gel was scanned using a computerized image scanner (Ambis, San Diego, CA) for the total protein phosphorylation. The bottom part of the gel, which contained unincorporated [γ-^{32}P]ATP and other labeled small molecular weight substances, was excluded from the scanning. The quantitative data presented are the means ± SD of three experiments, as shown in Fig. 1.

Protein phosphorylation assay using the phosphocellulose paper method. A portion (20 μg protein) of the 7000 *g* supernatant was incubated in 80 μL of 50 mM HEPES, pH 7.9, 10 mM $MnCl_2$ and 40 μg histone with and without various test agents, as shown in Table 1, Expt. I. The reaction was performed in the presence and absence of 10 nM TCDD. The control samples received the same volume of the vehicle (acetone). After 10 min at 30°, the reaction was initiated by the addition of 0.5 μCi [γ-^{32}P]ATP (final concentration 3 μM) for 60 sec and stopped by spotting 20 μL onto phosphocellulose paper (1 × 2 cm). The dried papers were washed three times in 3 mL of 85 mM H_3PO_4, and radioactivity was counted in 4 mL liquid scintillation fluid. Samples were tested in triplicate in the presence and absence of the exogenously added substrate peptide; the data presented in Tables 1, 3 and 4 are the total phosphorylation values (i.e. no background values being subtracted), and those shown in Figs. 2 and 4 are the differences in the level of phosphorylation in the presence and absence of the substrate peptide. In all cases, results were confirmed by at least two independent tests.

For the protein tyrosine kinase assay (Table 4), a specific substrate peptide (RR-SRC, Cat. No. 3124A, from Gibco BRL) was used at a 50 μM final concentration with 20 μg of 7000 *g* supernatant protein. All test chemicals were added simultaneously with TCDD (10 nM) and preincubated for 10 min at 30° prior to the addition of 0.5 μCi [γ-^{32}P]ATP and the substrate peptide. The reaction was terminated after 60 sec by spotting 20-μL aliquots onto phosphocellulose paper, as described above.

The time course of TCDD-induced activation of individual protein kinases was determined by incubating 500 μM of each specific kinase substrate peptide with an aliquot of 7000 *g* supernatant fraction (20 μg protein) in a buffer containing the most favorable ion composition of each kinase. The conditions for each kinase assay were exactly as recommended by each supplier of substrate. TCDD (10 nM) was preincubated with samples for different times at 30° (Fig. 4), and the reaction was initiated by simultaneous addition of [γ-^{32}P]ATP and the substrate peptide as above, followed by spotting onto phosphocellulose paper. The data are calculated as the means ± SD of nine independent assays.

Immunoprecipitation study. The method described by Enan and Matsumura [18] was used in the study of c-Src protein. In brief, 300 μg of 100,000 *g* supernatant (cytosolic) protein from untreated adipose tissue was reacted with 10 μg protein of c-Src antibody at 25°. After 1 hr, the antibody–protein complex was precipitated by the addition of 10 μL of protein A/G Sepharose (purchased from Santa Cruz Biotech Inc.). The precipitate was washed and phosphorylated with [γ-^{32}P]ATP (1 μCi, 3 μM) in the presence of 10 nM TCDD. The phosphoprotein was analyzed using 10% SDS–PAGE, and the autoradiograph was developed as described before [18].

HSP complexes (from 100,000 *g* cytosolic fraction of guinea pig adipose tissue) were isolated by immunoprecipitation with specific IgM antibody (clone 3G3) and antimouse IgM antibodies according to the method of Whitelaw *et al.* [23]. After washing the precipitate two times, an aliquot containing 50 μg protein was transferred to a tube with 50 μL of HEPES buffer containing 10 mM $MnCl_2$. TCDD, in some cases combined with Na_3VO_4 and/or genistein, was added, and the samples were incubated for 10 min at 30°.

Gel-retardation (gel mobility shift) assay. To prepare the samples for gel-retardation assay, TCDD (10 nM) was first added to the reaction buffer containing 20 μg protein from cytosol (i.e. 100,000 *g* supernatant from guinea pig adipose tissue) in 50 mM HEPES buffer and 10 mM $MnCl_2$ in a 15-μL volume, and incubated for 10 min at 30°. To initiate the phosphorylation reaction, an aliquot of 5 μL containing 5 μg of isolated nuclear protein extract, prepared according to Dignam *et al.* [22], and ATP (final concentration 1 μM) were added along with 5 μL of incubation buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol and 0.4 mg/mL sonicated salmon sperm DNA) with or without 2 μg of competitor oligonucleotide, unlabeled AP-1 response element DNA. The samples were preincubated at 4° for 15 min before the addition of 50,000 cpm of the ^{32}P-labeled AP-1 response element oligonucleotide and incubated at room temperature for 20 min. Two microliters of 0.1% bromophenol blue in 70% glycerol was added to each sample, which was analyzed on 6% polyacrylamide gels (29:1 acrylamide:bis) in 0.25 TBE (tris/borate/EDTA) buffer.

Ah-receptor binding assay. The method used for quantitation of tissue cytosolic Ah-receptor was that developed by Gasiewicz and Neal [24]. In brief, an aliquot containing 1 mg protein (200 μL) of guinea pig adipose tissue cytosol was incubated with different concentrations of 4,7-phenanthroline (10 μM), α-naphthoflavone (1 μM), or vehicle (acetone) in 1.5-mL Eppendorf tubes. In some experiments, different concentrations of unlabeled TCDD (1, 10 nM) were added in combination with 1 μM α-naphthoflavone or 10 μM 4,7-phenanthroline to the protein samples. In all cases, after preincubation for 20 min at 25°, 1 μL of [^{3}H]TCDD (30,000 dpm, 0.4 nM final concentration) was added to the mixture, and incubation was continued for 30 min at 25°, followed by addition of 250 μL of hydroxylapatite [HAP, 1 g suspended in 1 mL HEDG buffer, i.e. 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]. The suspension was mixed end-over-end at 4°. After 30 min, 1 mL of 0.5% Triton X-100 in HEDG buffer (v/v) was added to each tube, mixed and centrifuged in an Eppendorf microfuge at 3000 rpm for 3 min at 4°. The precipitate was washed twice with 1 mL of the same buffer, resuspended in 0.7 mL ethanol, and transferred into 4 mL liquid scintillation solution.

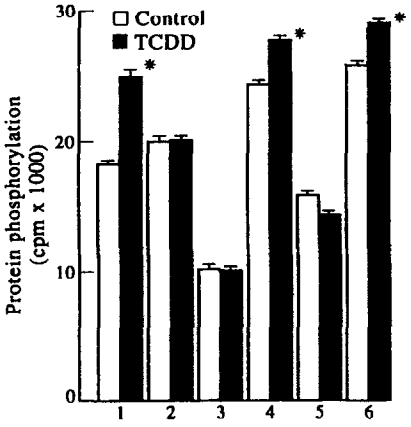

Fig. 1. Stimulation of protein phosphorylation by TCDD under *cell-free* conditions. An aliquot containing 100 μg protein from each fraction (cytosolic, microsomal and nuclear fractions) was incubated with vehicle alone (control) or the same volume of vehicle containing TCDD (to make 10 nM TCDD) for 10 min at 30° and ^{32}P-phosphorylated using [γ-^{32}P]ATP under the conditions described in Materials and Methods. The phosphorylation products were analyzed using SDS–PAGE and quantitative radioscanning [16]. The dried gel was scanned using a computerized radioscanner (Ambis). Bar designations are: a pair of control (open bars) and TCDD-treated (solid bars) samples of (1) cytosolic, (2) microsomal, (3) nuclear, (4) cytosolic plus microsomal, (5) cytosolic plus nuclear fractions, and (6) all three fractions added together. The results are the summary of three experiments and are shown as means ± SD. Key: (*) indicates a significant difference between control and TCDD-treated samples at $P \leq 0.05$.

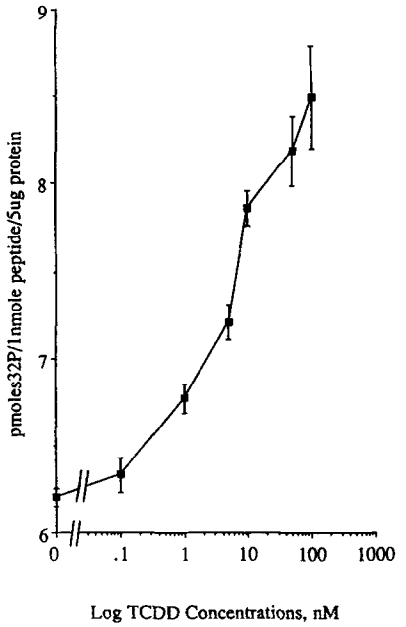

Fig. 2. Effects of changes in TCDD concentration on protein phosphorylation activities under *cell-* and *nucleus-free* conditions. The experimental conditions and the adipose tissue preparation method (7000 *g* supernatant) were exactly as described in Materials and Methods. Different concentrations of TCDD (0.1 to 100 nM) were added to cytosol and incubated for 10 min at 30° in 50 mM HEPES buffer, pH 7.9, containing 10 mM $MnCl_2$ and 20 μg protein of 7000 *g* supernatant fraction to make 80 μL final volume. The reaction was initiated by adding a 50 μM concentration of an artificial tyrosine kinase peptide substrate (RR-SRC) and 0.5 μCi [γ-^{32}P]ATP (final concentration 3 μM), continued by incubating at 30° for 60 sec, and terminated by spotting a 20-μL portion from each tube onto a piece of phosphocellulose paper (1 × 2 cm). The papers were washed with 85 mM H_3PO_4 and dried prior to the radioactivity counting, as described before. The background values (i.e. the same assay minus RR-SRC) were subtracted from these data. Values are means ± SD, N = 8.

Each tube was washed with 0.3 mL ethanol, which was combined with the previous 0.7 mL suspension. The radioactivity was counted using a Beckman Liquid Scintillation Spectrometer LS5801. Each determination was done in triplicate.

RESULTS

In the first series of experiments, we tested the effect of the addition of TCDD to isolated nuclei, cytosol and postnuclear membrane fractions (i.e. microsomal fraction) prepared from homogenate of adipose tissue. Aliquots containing 100 μg protein were incubated with 10 nM TCDD, and the changes in protein phosphorylation activities were assessed by addition of [γ-^{32}P]ATP [16]. Reactions were terminated with SDS and ^{32}P-phosphorylated proteins were analyzed by SDS–PAGE and quantitative radioscanning (Fig. 1). It was immediately noted that TCDD stimulated protein phosphorylation activity under these *cell-free* conditions even in preparations lacking nuclei. Of three fractions tested separately, TCDD caused an increase only in cytosolic protein phosphorylation, while it had no effect on the membrane or nuclear fractions. When combinations of fractions were tested, TCDD caused an increase only in the cytosol plus postnuclear membrane fractions. Overall protein phosphorylation activity in nucleus plus cytosol was reduced by TCDD under this experimental condition.

The concentration–response curve for TCDD induction of protein tyrosine kinase activity was studied using the 7000 *g* preparation (Fig. 2). The results showed that the stimulatory effect of TCDD was detectable at 1 nM and became significant ($P \leq 0.01$) at the 10 nM range.

We have characterized, using SDS–PAGE and autoradiography, the major endogenous substrate in the cytosol fraction, which was phosphorylated by [γ-^{32}P]ATP in the presence and absence of TCDD (Fig. 3). While phosphorylation reactions of endogenous proteins under cell-free conditions sometimes produce unnatural products (i.e. products that do not become phosphorylated in intact cells), some of these TCDD-stimulated ^{32}P-labeled phosphoproteins, such as the 120, 60–61 and 55 kDa proteins, matched with those also observed in intact

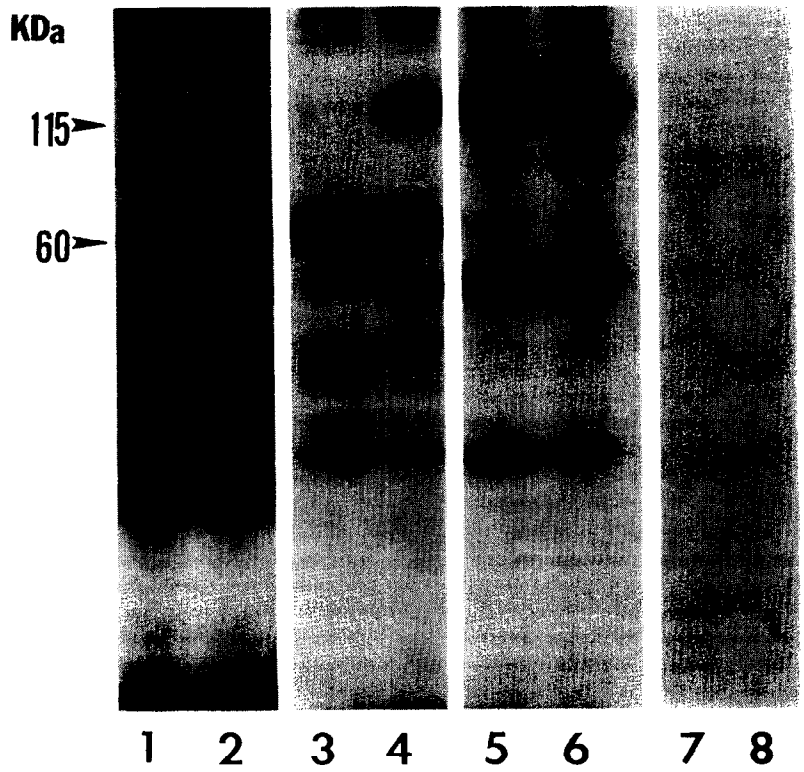

Fig. 3. Studies on the nature of endogenous cytosolic protein kinase substrate proteins whose states of phosphorylation were affected by TCDD treatment *in vitro*. The results shown are ^{32}P-autoradiograms of cytosol fractions treated or untreated with TCDD, phosphorylated in the presence of [γ-^{32}P]ATP, and analyzed by SDS–PAGE and autoradiography. Lanes 1 and 2: cytosol untreated (= control) and treated with TCDD, respectively; Lanes 3 (control) and 4 (TCDD treated): 7000 *g* supernatant treated with either the vehicle alone (control) or TCDD (10 nM) for 10 min at 4°, centrifuged at 100,000 *g* for 1 hr, and phosphorylated using [γ-^{32}P]ATP. Lanes 5 and 6: same as 3 and 4 except that 1 μg of protein of antiphosphotyrosine monoclonal antibody was added simultaneously with the vehicle or TCDD, respectively. Lanes 7 (control) and 8 (treated): isolated cytosol reacted with anti-Src antibody precipitated with protein A/G coupled to Sepharose, washed, phosphorylated using [γ-^{32}P]ATP, and analyzed on SDS–PAGE.

cells [16]. Of these, the 60–61 kDa protein band included a protein tyrosine kinase substrate, as judged by the ability of an antiphosphotyrosine antibody to block its phosphorylation (compare Lane 5 to Lane 3, Fig. 3). The pattern of phosphorylation on some proteins was not consistent. For instance, that on 90 kDa proteins varied from one run to the next. Previously, we observed [16] that TCDD caused a rise in tyrosine phosphorylation of a 60–61 kDa protein in intact adipose tissue, as determined by Western blotting using the same anti-phosphotyrosine antibody. In the current study, immunopurification with an anti-SRC antibody and protein A/G-Sepharose of ^{32}P-phosphorylated cytosol resulted in a single labeled band at 60–61 kDa (Lane 7, Fig. 3). Thus, TCDD-activated cytosolic phosphoproteins (Lane 8) include a 60–61 kDa tyrosine-phosphorylated SRC-type protein, which also undergoes an increase in phosphorylation after TCDD treatment in intact cells.

To gain an insight into the basic mechanism of TCDD-induced activation of protein phosphorylation, we tested phosphorylation activities in postnuclear and cytosol fractions using specific exogenous substrate proteins and peptides, each known to favor certain types of protein kinase (see Tables 1, 3, and 4). Note that the results of these studies are shown as raw values, including the background values. In the first series of tests, the effects of protein synthesis inhibitors and Ah-receptor blockers were tested using the same *cell-* and *nucleus-free* preparations with histone as an artificial substrate (Table 1, Expt. I). The results clearly showed that the stimulatory effect of TCDD could be abolished by these Ah-receptor blockers at appropriate concentrations, but not by actinomycin D or cycloheximide. These data are consistent with the idea that this aspect of the action of TCDD is mediated through the Ah-receptor but not through *de novo* protein synthesis.

To further characterize the effects of TCDD on protein phosphorylation and dephosphorylation under these conditions, we tested the effect of a phosphatase inhibitor, okadaic acid, and two protein tyrosine kinase inhibitors on TCDD-induced changes in cytosolic phosphorylation activity using histone as

Table 1. Effect of TCDD treatment under *cell-free* conditions on protein phosphorylation in postnuclear fraction (i.e. 7000 *g* supernatant) from the homogenate of guinea pig adipose tissue

Treatments	^{32}P (pmol/10 μg histone/5 μg protein/60 sec)	
	Control	TCDD (10 nM)
Experiment I		
No addition	10.3 ± 1.0	11.7 ± 0.2*
Actinomycin D (2 μg/mL)	10.3 ± 0.3	11.7 ± 0.4*
Cycloheximide (0.4 μM)	10.4 ± 0.3	11.7 ± 1.0*
4,7-Phenanthroline (10 μM)	10.3 ± 0.5	10.3 ± 0.7
α-Naphthoflavone (1 μM)	10.1 ± 0.7	10.1 ± 0.6
Experiment II		
No addition	13.4 ± 0.6	19.8 ± 1.3*
Heparin (100 nM)	14.5 ± 0.7†	15.0 ± 0.9
Genistein (15 μM) + heparin	11.7 ± 0.8†	15.2 ± 0.8*
Bombesin (10 nM) + heparin	15.5 ± 1.0‡	20.0 ± 1.9*
Okadaic acid (1 μM) + heparin	15.2 ± 0.9‡	18.3 ± 2.0*

Histone was used as the substrate under all conditions. Data are the means ± SD of nine assays. The Cochran *t*-test was used in all statistical analyses. Note that no background values (i.e. the level of ^{32}P-radioactivity found in phosphocellulose papers in tests where no histone was added, but otherwise identically prepared samples) have been subtracted from any of these values. The background values were 10.1 ± 0.6 for Expt. I and 11.7 ± 0.7 for Expt. II.

* Significantly different from the corresponding control value, $P \leq 0.01$.

†,‡ Significantly different from control, no addition value (13.4 ± 0.6): †$P \leq 0.05$, and ‡$P \leq 0.01$.

substrate (Table 1, Expt. II). Under this incubation condition with histone, which favors both serine and tyrosine protein kinases (i.e. 10 mM $MnCl_2$, 100 nM heparin, 1 mM EGTA), the stimulatory action of TCDD was apparent by 10 min. As expected, okadaic acid increased the level of histone phosphorylation as compared with control, but it did not eliminate stimulation by TCDD. Genistein, when added simultaneously with TCDD, reduced the stimulatory action of TCDD, whereas bombesin, a stimulator of protein tyrosine kinases, increased the control value. Heparin, at a concentration known to inhibit casein kinases [25], decreased the level of phosphorylation activity in TCDD-treated samples and increased the same in the control, but in the co-presence of either bombesin or okadaic acid, heparin did not antagonize the stimulatory effect of TCDD. These observations suggest the involvement of several protein kinases, such as tyrosine kinase(s) and casein kinase(s), in elevating protein phosphorylation activity in TCDD-treated preparations. However, it does not appear that okadaic acid-sensitive protein phosphatases (1A type) are involved.

To ascertain that Ah-receptor blockers, which have been demonstrated to be active in other species, can prevent TCDD binding in this species under this particular experimental condition, we conducted [^3H]TCDD binding tests (Table 2). At the concentrations employed, they were found to decrease significantly [^3H]TCDD binding in this preparation, and furthermore, in the presence of 1 μM α-naphthoflavone, the addition of 1 or 10 nM unlabeled TCDD did not cause a further displacement of [^3H]TCDD binding. On the other hand, in the presence of 10 μM 4,7-phenanthroline, a significant displacement (about 23% of the total specific binding) was observed when 10 nM unlabeled TCDD was added, indicating that at this concentration the blocking action of 4,7-phenanthroline is significant but not complete (Table 2).

To address the question of whether the ligand unbound Ah-receptor in cytosol is associated with protein kinase(s) and whether TCDD could affect their activity, a specific polyclonal anti-Ah-receptor antibody was used to immunoprecipitate the receptor and associated proteins. Their kinase activity was tested in the presence and absence of TCDD using histone as the exogenous substrate. The data in Table 3 show that TCDD could increase significantly the activity of kinase(s) associated with the Ah-receptor. Heparin (a casein kinase II inhibitor) greatly reduced the stimulatory action of TCDD on histone phosphorylation, indicating that casein kinase II is present in this fraction. When genistein and bombesin were added in combination with the same concentration of heparin, the effect of TCDD became apparent, showing that there are kinases other than casein kinase, genistein-sensitive and bombesin-inducible ones that are stimulated by TCDD in this preparation. The effect of TCDD was most significant in the presence of okadaic acid and heparin, indicating again that okadaic acid-sensitive phosphatase is not involved in the action of TCDD.

In a separate work, we reported other supporting evidence for the Ah-receptor-dependency of TCDD's stimulatory action on protein phosphorylation in cytosol from two different mouse strains under *cell-free* conditions [26]. In that work, we showed that

Table 2. Blocking effects of α-naphthoflavone and 4,7-phenanthroline on [^{3}H]TCDD binding

Treatments	[^{3}H]TCDD (fmol bound/mg cytosolic protein)		
	Unlabeled TCDD (nM)		
	0	1	10
No addition	3.45 ± 0.16	1.73 ± 0.13*	0.67 ± 0.03*
α-Naphthoflavone (1 μM)	0.40 ± 0.03*	0.42 ± 0.03	0.40 ± 0.03
4,7-Phenanthroline (10 μM)	0.94 ± 0.05*	0.96 ± 0.06	0.69 ± 0.04†

Values are means ± SD; N = 6 for each determination. The Cochran *t*-test was used in all statistical analyses.

* Significantly different from the "no addition" value (0 concentration of unlabeled TCDD, i.e. 3.45 ± 0.16 fmol/mg protein) at $P \leq 0.01$.

† Significantly different from 4,7-phenalthroline alone (0.94 ± 0.05 fmol) at $P \leq 0.01$.

Table 3. *Cell-free* effects of TCDD and other chemicals on isolated Ah-receptor associated protein kinase activity from the cytosol of guinea pig adipose tissue assayed using [γ-^{32}P]ATP and histone as an artificial substrate

	^{32}P (pmol/10 μg histone/19 pg equivalent of AH-R*/60 sec)	
	Control	TCDD (10 nM)
No addition	1.20 ± 0.13	1.81 ± 0.27†
With heparin		
Heparin (100 nM)	1.32 ± 0.03	1.37 ± 0.04
Genistein (15 μM) + heparin	1.07 ± 0.14	1.40 ± 0.05†
Bombesin (10 nM) + heparin	1.42 ± 0.34	1.83 ± 0.28
Okadaic acid (1 μM) + heparin	1.40 ± 0.10	1.67 ± 0.05‡

For this experiment, the Ah-receptor was isolated from supernatant (i.e. 100,000 *g* supernatant) of adipose tissue from untreated guinea pig, using a specific polyclonal anti-Ah-receptor antibody preparation. To initiate the reaction, all agents including TCDD, [γ-^{32}P]ATP, and histone were added to the isolated Ah-receptor preparation simultaneously and incubated for 5 min. The products were analyzed by the phosphocellulose assay method. No background values have been subtracted from any of these figures. Values are means ± SD of four different experiments. Each was run in triplicate, and the differences between control and TCDD-treated were analyzed by the Cochran *t*-test.

* The quantity of the Ah-receptor protein calculated from the results of a [^{3}H]-TCDD binding experiment using cytosolic and isolated Ah-receptor preparations based on the assumption that the K_D of the cytosolic Ah-receptor of the guinea pig adipose tissue is 0.5 nM, and the molecular weight of the receptor is 100 kDa.

†,‡ Significantly different from the corresponding control at $P \leq 0.05$ and 0.01, respectively.

10 nM TCDD significantly stimulated protein tyrosine kinase activity under *cell-free* conditions in cytosolic samples prepared from adipose tissue of the C57BL/6J mouse strain (a TCDD-responsive strain), but not in the DBA/2 strain (a less-responsive strain).

To study the nature of protein kinases involved, we employed another approach to specifically test protein tyrosine kinase activities in the same *cell-* and *nucleus-free* preparation using an artificial substrate specific for SRC-type protein tyrosine kinases (designated as RR-SRC) and a phosphocellulose paper method [16]. This approach has the advantage that it avoids the possibility of "cold phosphorylation" of endogenous proteins by residual ATP, which could occur prior to the addition of [γ-^{32}P]ATP. The results summarized in Table 4 (Expt. I) show that this system, unlike histone phosphorylation, was inhibited significantly by genistein, as expected. Under this experimental condition, herbimycin A, an inhibitor of adhesion-induced tyrosine phosphorylation [27], did not affect protein phosphorylation. Bombesin merely increased the basal value in both treatments, indicating that indeed this method is specific to genistein-sensitive SRC-type tyrosine kinases. Neither okadaic acid nor heparin affected the phosphorylation activity in TCDD-treated postnuclear fraction. The same

Table 4. *Cell-free* effect of 10 nM TCDD on protein tyrosine kinase activity in postnuclear fraction (Expt. I), or purified heat shock protein (HSP) 90 complex (Expt. II) prepared from the homogenate of guinea pig adipose tissue

Treatments	Phosphorylation on RR-SRC peptide	
	Control	TCDD
Experiment I: Postnuclear fraction	(pmol ^{32}P/nmol substrate/5 μg protein/60 sec)	
No addition	8.03 ± 0.12	9.56 ± 0.52*
Genistein (15 μM)	7.59 ± 0.32†	7.59 ± 0.45†
Bombesin (10 nM)	8.46 ± 0.61	9.93 ± 0.93
Herbimycin A (1 μM)	8.00 ± 0.56	8.95 ± 0.71
Heparin (100 nM)	8.88 ± 0.34	9.82 ± 0.52
Okadaic acid (1 μM)	8.88 ± 0.57	9.42 ± 0.81
Experiment II: HSP 90 complex	(fmol ^{32}P/nmol substrate/5 μg protein/60 sec)	
No addition	3.81 ± 0.02	4.34 ± 0.09*
Na_3VO_4 (10 μM)	4.23 ± 0.02	4.51 ± 0.04*
Na_3VO_4 + genistein	3.73 ± 0.10	3.43 ± 0.10

A specific protein tyrosine kinase substrate peptide (RR-SRC) was used in both experiments. Data are the means ± SD of six assays. The Cochran *t*-test was used in all statistical analyses. Data are the absolute values without subtracting background values.

* Significantly different from the corresponding control value, $P \leq 0.01$.

† Significantly different from the corresponding "no addition" value, $P \leq 0.01$.

phenomenon could be demonstrated using isolated HSP complexes, of which a small fraction is expected to be complexed with the ligand-free Ah-receptor. For this purpose, cytosol was immunoprecipitated with a mouse IgM antibody to HSP 90. After washing [23], the immunoprecipitate aliquots were incubated with TCDD (Table 4, Expt. II). The results showed that the stimulatory action of TCDD on protein phosphorylation was also observable in this preparation, and that such an action was abolished by genistein (Table 4). As expected, VO_4^{3-}, an inhibitor of protein phosphatases with relative substrate preference for phosphotyrosine residues, increased the overall level of phosphorylation on RR-SRC.

Thus far we have mainly concentrated on genistein-sensitive, SRC-type protein tyrosine kinases as the main candidates for the TCDD-activated protein kinase(s). In the experiments summarized in Fig. 4, the time courses of the response to TCDD of four different types of protein kinases were examined. It is interesting to note that while the overall extent of stimulation by TCDD was highest in the case of protein tyrosine kinase(s) (i.e. approximately to 300% in 20–30 min after the background values were subtracted), the quickest responding one was MAP-2 kinase (20–25% increase in 1–2 min). As for PKC, there was an initial decline in the activity immediately after addition of TCDD, followed by a stimulatory phase that started at 20–30 min. cAMP-dependent protein kinase responded to TCDD in a fashion similar to that of PKC.

The fact that MAP-2 kinase was elevated by addition of TCDD raises the possibility that the signal triggered by TCDD also activates the growth factor signal transduction pathway [28]. This was tested by first treating isolated cytosol with TCDD as before, then combining the cytosol with isolated nuclear proteins and analyzing binding to a ^{32}P-labeled AP-1 consensus oligonucleotide in a gel-retardation assay. Several protein kinases and phosphatases are known to alter the phosphorylation status of AP-1 proteins and thereby increase their overall DNA binding ability. Such phosphorylation changes are indispensable components of the growth factor signal transduction pathway [29]. As expected, the addition of protein kinase activators, Mg^{2+}, cAMP or PKC cofactors indeed increased the binding of the ^{32}P-labeled DNA probe to AP-1 proteins (Fig. 5A). Replacing Mg^{2+} with Mn^{2+}, to favor tyrosine kinase activity, reduced the overall extent of AP-1 binding in untreated samples, but under this condition TCDD clearly stimulated AP-1 binding, with the stimulatory action of TCDD most pronounced at the 5-min incubation time point (Fig. 5B). This stimulatory action of TCDD could be eliminated by simultaneous addition of genistein (data not shown). To confirm this observation on intact adipocytes, isolated adipose tissue slices were treated with TCDD in a tissue culture medium [16], and the same gel-retardation assay was conducted yielding very similar results (Fig. 5C).

DISCUSSION

The key observations of this study were: (a) TCDD activated protein kinase(s) in cytosol under *cell-free* conditions in the absence of nuclei, (b) this action of TCDD appeared to be mediated by the cytosolic Ah-receptor, (c) *de novo* protein synthesis was not required for this particular action of TCDD, and (d) TCDD induced a rapid increase in binding of nuclear transcription factors to an AP-1 consensus DNA sequence within a short time span, as judged by the gel-retardation assay. It is important to point out that such phenomena cannot be explained by a mere extension of the existing theory that TCDD-bound Ah-receptor must be translocated into the nucleus, forming a heterodimer with another helix-

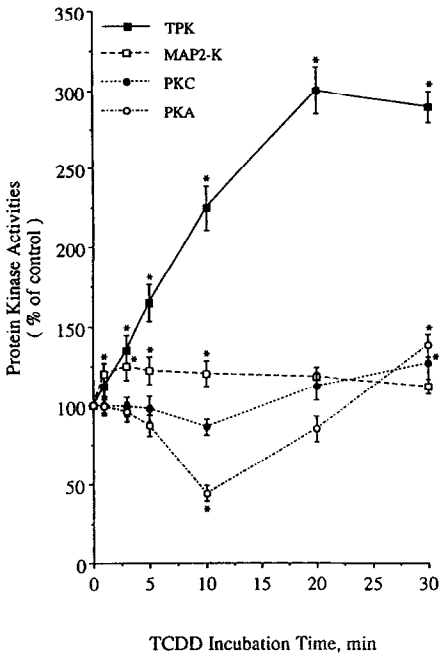

Fig. 4. Time course of TCDD-induced activation of individual protein kinases in the 7000 *g* supernatant under *cell-free* conditions. The phosphocellulose paper method was used to assay kinase activity in all cases. Data are the means ± SD of nine independent assays. Key: (*) significantly different from control at $P \leq 0.01$ (Cochran *t*-test). The substrate peptides used were: RR-SRC (RRLIEDAEYAARG) for protein tyrosine kinase; Ac-MBP (ACQKRPSQRSKTL) for protein kinase C; Kemptide (LRRASLG) for protein kinase A; and APRTPGGRR for MAP kinase. The net values of kinase activity were calculated by subtracting the mean of samples assayed in the presence or absence of each substrate peptide. The net value (pmol ^{32}P/nmol substrate/5 μg protein/60 sec) of the control: tyrosine kinase, 9.2 ± 0.4; protein kinase C, 6.5 ± 0.2; PKA, 3.7 ± 0.6; and MAP kinase 2.2 ± 0.04.

loop-helix protein, "arnt," which must finally bind to the XREs in promoters of TCDD responsive genes such as *CYP1A1*. Thus, it appears necessary to consider the presence of a second action pathway for TCDD in addition to the existing one (i.e. XRE pathway). One key observation that supports the existence of the second pathway is the rapidity of the protein kinase activation in response to the action of TCDD. The rise in MAP-2 kinase was observed within 1 min of the addition of TCDD, and by 3 min stimulation appeared to be maximal. These responses were not due to *de novo* protein synthesis, since actinomycin D and cycloheximide (Table 1, Expt. I) were unable to block these effects of TCDD.

The Ah-receptor dependency of such a TCDD-induced protein kinase change is a very important point, and therefore we have made several attempts in the current study to test this hypothesis. The results thus far are totally consistent with the Ah-receptor dependency theory. First, increased phosphorylation was observed whether TCDD was administered to the purified Ah-receptor complex or to the isolated cytosol fraction, both under a *cell-free* condition. Second, two Ah-receptor antagonists, 4,7-phenanthroline [30] and α-naphthoflavone [31, 32], blocked TCDD mediated activation of kinases at concentrations low enough to avoid their nonspecific actions. Third, TCDD-induced stimulation of protein kinase activities was also observed in purified Ah-receptor and HSP 90 complexes. The latter complexes are known to include Ah-receptors in a ligand-free state. Moreover, we have shown previously that in adipose tissue of male guinea pigs, all of the TCDD-mediated changes, such as protein kinase changes, glucose transporter activities [17, 18] and lipoprotein lipase activities [33], are Ah-receptor dependent, based on structure–activity relationships as well as receptor blocker studies. In addition, the stimulatory effect of TCDD on cytosolic protein phosphorylation activities was more pronounced in a TCDD-responsive mouse strain, C57, than that observed in a less TCDD-responsive strain, DBA [26]. Together these data strongly support the idea that most of the stimulatory actions of TCDD in the cytosol fractions of this material, if not all of them, are mediated through the Ah-receptor. The other possibility, that TCDD could directly interact with protein kinases, is unlikely, based on the observation that TCDD showed almost no effect on isolated microsomal and plasma membrane or nuclear protein kinases, which agrees with a similar observation made by Kramer *et al.* [34]. Altogether these data clearly support the view that these actions of TCDD, which occur in isolated cytosol, are mediated by the Ah-receptor.

Our current working hypothesis is that the Ah-receptor complex is organized in such a way that ligand binding to the resting form of the Ah-receptor itself serves as a trigger to activate the protein kinase(s). Under such a scheme, this form of Ah-receptor, still attached to HSPs [35], first binds TCDD, then the receptor is phosphorylated and leaves the HSP complex to travel to the nucleus. Somewhere in this sequence of events the ligand-dependent activation of protein kinase(s) could take place. Probably the closest example in such an arrangement would be the case of progesterone receptors, some of which have been shown to be associated with protein kinases and HSPs in their cytosolic, resting form [36]. The ligand-dependent activation of protein kinase(s) is also known to take place with these types of receptors [37]. In support of such a hypothesis, there are now several pieces of evidence to indicate that in the case of hormone receptors, some of the cytosolic forms of the ligand binding subunits are associated with specific protein kinase(s) [38–40]. To be sure, no one has shown the existence of the Ah-receptor, HSPs and protein kinase(s) in one complex, although the association of the Ah-receptor with HSP 90 [35] and the phenomenon of phosphorylation of the Ah-receptor subunit after TCDD binding and probably before entering the nucleus [41] have already been demonstrated. Therefore, it must be made clear that this working hypothesis is very speculative, and that much more work is needed along this line of approach.

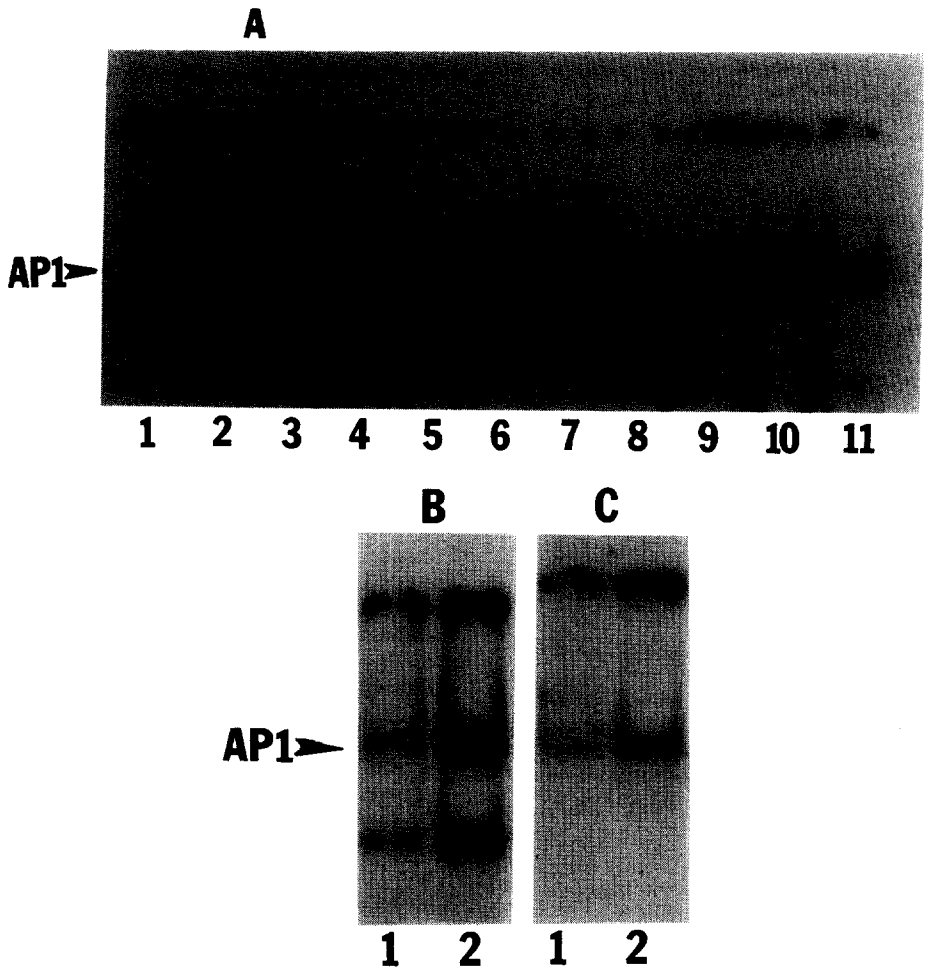

Fig. 5. Effect of TCDD under *cell-* and *nucleus-free* conditions on the abilities of isolated nuclear proteins to bind a specific ^{32}P-labeled AP-1 response element oligonucleotide in a gel-retardation assay. Panel A: Gel-retardation assay results showing *cell-free* activation of binding of AP-1 proteins to DNA by protein phosphorylation treatments. The cytosol fraction was first incubated with TCDD (in cell-free system) for 10 min, and then for 5 min with nuclear protein extract (prepared as described by Dignam *et al.* [22]) in the presence of 1 μM ATP under varying incubation conditions, basically following the method of Sadowski and Gilman [29]: (Lane 1) ^{32}P-probe incubated with unlabeled competitor oligonucleotide; (Lane 2) control in the presence of 10 mM $MnCl_2$; (Lane 3) TCDD-treated and incubated with 10 mM $MnCl_2$; (Lane 4) control with 10 mM $MnCl_2$ and 100 nM heparin; (Lane 5) the TCDD-treated counterpart of Lane 4; (Lane 6) control with 10 mM $MgCl_2$ in place of 10 mM $MnCl_2$; (Lane 7) the TCDD-treated counterpart of Lane 6; (Lane 8) control with 1.86 mM $CaCl_2$, 5 μg phosphatidylserine and 2 μg diocatanylglycerol; (Lane 9) TCDD-treated counterpart of Lane 8; (Lane 10) control with 100 μM cAMP; (Lane 11) the TCDD-treated counterpart of Lane 10. The quantitative data as scanned by Ambis in cpm were: 70 ± 5 (Lane 1); 530 ± 20 (Lane 2); 870 ± 10 (Lane 3), 420 ± 5 (Lane 4); 430 ± 5 (Lane 5); 2350 ± 10 (Lane 6); 1550 ± 10 (Lane 7); 920 ± 5 (Lane 8); 950 ± 10 (Lane 9); 1230 ± 10 (Lane 10); and 1050 ± 10 (Lane 11). Panel B: Gel-retardation assay results showing the cell-free effect of TCDD treatment on the DNA binding activity of AP-1 proteins in the presence of 10 mM $MnCl_2$ and 1 μM ATP. Cytosol was first incubated with TCDD (10 nM) under *cell-free* conditions for 5 min (Lane 2). The control sample (Lane 1) received the same volume of the solvent (acetone) only. Thereafter, nuclear extracts were added with 1 μM ATP and 10 mM $MnCl_2$ and incubated for 5 min before the gel-retardation assay. Panel C: gel-retardation assay of the effect of TCDD treatment of intact adipocytes on the binding properties of AP-1 proteins to an AP-1 response element. For this test 1-g pieces of intact adipose tissues were isolated and maintained in Dulbecco's modified Eagle's medium containing 13.3 mM glucose and gassed thoroughly with 95% O_2 and 5% CO_2 [17, 18]. Each piece was incubated in 10 mL of medium, treated with either TCDD (to make 10 nM) or acetone (for control), and incubated for 1 hr. The DNA binding reaction was performed in 20 μL total volume with 5 μg nuclear protein from control (Lane 1) or TCDD-treated (Lane 2) samples under the 10 mM $MnCl_2$ condition, as described above.

As to the nature of the initial protein kinase that acts as the TCDD-induced trigger for the subsequent cascade of activation of kinases in cytosol, the current data are insufficient to reach a definite conclusion. The genistein-sensitive group of SRC-type protein tyrosine kinases is one of the candidates for the initial protein kinase activated by TCDD, despite the fact that MAP-2 kinase showed an even quicker response than the overall protein tyrosine kinase activity. MAP-2 kinase is known to be activated by tyrosine kinases involved in the growth factor signal transduction pathway [32], and while it is a serine/threonine kinase itself, it is activated through phosphorylation on threonine and tyrosine residues by the action of MAP kinase kinase [42]. The fact that the SRC antibody reacted with the 60–61 kDa protein merely supports the view that an SRC protein may be involved as one of the components of the above cascade. Also based on the sensitivity of histone phosphorylation to 100 nM heparin, an involvement of casein kinase [25] could be surmised. Lastly, under our experimental conditions neither protein kinase C nor cAMP-dependent protein kinase(s) was activated at early time points, ruling out these kinases as the triggering events in TCDD-mediated activation of protein kinase activity.

While the precise identity of the trigger protein kinase could not be identified, the current study clearly shows that the major protein phosphorylation pathways activated by TCDD in the cell-free system could include the growth factor signal transduction pathway. Fortunately, two systems activated by TCDD, MAP-2 kinase and $p21^{ras}$, are the key members of this important pathway. In particular, MAP kinase represents the final cytosolic protein kinase to be activated, and is the sole messenger passing the growth factor signal into the nucleus [43]. Furthermore, its activation is strictly controlled by MAP kinase kinase and MAP kinase kinase kinase, and, therefore, no casual activation of MAP kinase is possible. Thus, it serves as a solid marker for the activation of this important transduction pathway by TCDD. Certainly, there are many unanswered questions remaining on the individual processes by which this protein phosphorylation cascade is turned on by TCDD. However, the involvement of the growth factor signal transduction pathway is apparent.

In addition to the analysis of the overall implication of the current results, a few technical comments may be necessary. First, as to the question of cold phosphorylation, we must mention here that in some instances we observed a totally opposite effect of TCDD (i.e. reduction rather than stimulation) on ^{32}P-phosphorylation on the endogenous substrate proteins after incubating with [γ-^{32}P]ATP. In a parallel experiment with the same preparation, however, both okadaic acid and Na_3VO_4 were also observed to reduce phosphorylation in such instances. Our interpretation is that during preincubation of the enzyme preparation with TCDD, those endogenous substrates were cold phosphorylated by the affected kinases using endogenous (non-radioactive) ATP present particularly when the preparations were fresh. This appears to be the only logical way to explain the effect of these phosphatase inhibitors to decrease ^{32}P-phosphorylation. To circumvent this problem in the experiments with exogenous, artificial substrate peptides (shown in Tables 1, 3 and 4), we added the substrates and [γ-^{32}P]ATP at the same time to make sure that no prior cold phosphorylation on the artificial substrate would take place. This treatment might have made the action of some slow-acting agent less potent, but always gave us directionally consistent results.

Second, the magnitude of the TCDD-induced changes in protein phosphorylation on these artificial substrates (Tables 1, 3 and 4) may appear to be small. Here, we have elected to present the total phosphorylation data, including the background values (i.e. phosphorylation on endogenous basic proteins that bind to phosphocellulose papers), despite the fact that the addition of these artificial substrates always increased the level of phosphorylation over the background, for two purposes: (a) to be able to analyze statistically the original data, and (b) to be inclusive, so that nothing would be missed, including potential effects of TCDD on endogenous proteins (which could occur even in the presence of these preferred exogenous substrates). In the end, we could show that when RR-SRC was used as a substrate, for instance, genistein, a specific tyrosine kinase inhibitor, could inhibit the TCDD-induced portion of phosphorylation, but heparin could not. This was not the case with histone phosphorylation, where heparin was a more effective inhibitor than genistein. These and other results showed that artificial substrates are useful in selectively expressing each type of protein kinase, and one can selectively favor each group of kinase by using these artificial substrates in combination with known specific inhibitors, activators and modulators.

The last technical comment pertains to the cell- and nuclear-free phosphorylation approach to AP-1 binding to DNA (Fig. 5, A and B). By eliminating the nuclear membrane barrier, we may be creating artificial situations where some nuclear proteins are phosphorylated by protein kinases that do not exist in the nucleus of intact cells. Thus, we must make it clear that the system is artificial, and that unnatural phosphorylation and dephosphorylation on AP-1 proteins could occur in such a system. Having acknowledged these shortcomings, we now must explain what the system is capable of demonstrating. First, it shows that phosphorylation does indeed change the binding of AP-1 to AP-1 response element DNA (i.e. "TRE") in this preparation from adipose tissue by virtue of the stimulatory potencies of ATP, cAMP, Mg^{2+} and Mn^{2+} and other cofactors. Second, TCDD indeed causes changes in AP-1 binding only when ATP and other cofactors for protein phosphorylation are present. Third, there are qualitative differences among the protein kinases in expressing the effect of TCDD. These valuable data help us to understand the importance of phosphorylation-mediated control of nuclear transcription factors. Furthermore, this is essentially the same approach taken recently by Sadowski and Gilman [29], who successfully demonstrated *cell-free*

activation of a DNA-binding nuclear protein by exogenously added epidermal growth factor.

In summary, we have shown clearly that the initiation of some part of TCDD's action pathway does not require the presence of the nucleus, although as far as the activation of protein kinase activities are concerned, such a pathway still requires the participation of the Ah-receptor. The possibility that this protein phosphorylation-mediated action pathway could play a significant role in at least some of the toxic actions of dioxin-type chemicals has been pointed out already [44]. While the relative importance of such a pathway would require much more work to establish, the key points of the current study may merit re-emphasis: i.e. TCDD interacts with the Ah-receptor in isolated cytosol in *cell-free* system, and activates certain protein kinases that are the key members of the growth factor signal transduction pathway. Thus, this TCDD-induced "protein phosphorylation pathway" must be considered as a separate route of action for dioxin-type chemicals from the well established nucleus translocation-dependent pathway, which activates various genes via "XRE."

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