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Dioxin-Dependent Activation of Murine *Cyp1a-1* Gene Transcription Requires Protein Kinase C-Dependent Phosphorylation†

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Transcriptional activation of the murine *Cyp1a-1* (cytochrome P_1450) gene by inducers such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (dioxin) requires the aromatic hydrocarbon (Ah) receptor and the interaction of an inducer-receptor complex with one or more of the Ah-responsive elements (AhREs) located about 1 kb upstream from the transcriptional initiation site. We find that treatment of mouse hepatoma Hepa-1 cells with 2-aminopurine, an inhibitor of protein kinase activity, inhibits CYP1A1 mRNA induction by TCDD as well as the concomitant increase in CYP1A1 enzyme activity. Formation of DNA-protein complexes between the Ah receptor and its AhRE target is also inhibited by 2-aminopurine, as determined by gel mobility shift assays. Phosphorylation is required for the formation of Ah receptor-specific complexes, since in vitro dephosphorylation of nuclear extracts from TCDD-treated Hepa-1 cells abolishes the capacity of the Ah receptor to form specific complexes with its cognate AhRE sequences. To determine whether any one of several known protein kinases was involved in the transcriptional regulation of the *Cyp1a-1* gene, we treated Hepa-1 cells with nine other protein kinase inhibitors prior to induction with TCDD; nuclear extracts from these cells were analyzed for their capacity to form specific DNA-protein complexes. Only extracts from cells treated with staurosporine, a protein kinase C inhibitor, were unable to form these complexes. In addition, staurosporine completely inhibited CYP1A1 mRNA induction by TCDD. Depletion of protein kinase C by prolonged treatment with phorbol ester led to the complete suppression of CYP1A1 mRNA induction by TCDD. We conclude that (i) phosphorylation is necessary for the formation of a transcriptional complex and for transcriptional activation of the *Cyp1a-1* gene; (ii) the phosphorylation site(s) exists on at least one of the proteins constituting the transcriptional complex, possibly the Ah receptor itself; and (iii) the enzyme responsible for the phosphorylation is likely to be protein kinase C.

The mammalian *CYP1A1* gene encodes an enzyme expressed endogenously during cell division, embryogenesis, and differentiation, suggesting that this gene as well as certain other P450 genes might be involved in distinct critical life processes (51, 53). The CYP1A1 enzyme also plays an important role in the detoxification of numerous polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene, a major component in the production of cigarette smoke and other combustion processes. Moreover, CYP1A1 is an essential enzyme in the detoxification of chemicals responsible for activation of the *CYP1A1* gene, and paradoxically, the enzyme also potentiates promutagens and procarcinogens by converting them into reactive intermediates (cf. references 53 and 60 and references therein). Aromatic hydrocarbons and TCDD are ligands for the cytosolic Ah receptor that is responsible for transcriptional activation of the *CYP1A1* gene (for reviews, see references 54 and 73). *CYP1A1* activation involves nuclear translocation of the receptor, possibly as a ligand-bound complex, and acquisition of chromatin-binding properties enabling the complex to bind to the AhREs located between positions −1090 and −880 from the transcriptional initiation site (14, 19, 26, 28, 40, 55, 56). As a consequence of gene activation, the rates of CYP1A1 mRNA and protein synthesis are increased 20- to 100-fold (for a review, see reference 54). Posttranslational modifications, such as ADP-ribosylation, have been shown to modulate the transcriptional induction process (65). A functional CYP1A1 enzyme appears to be required to maintain the basal level of constitutive transcription (66).

In addition to its role as a transcriptional activator, the Ah receptor shares many other biochemical and biophysical properties with the steroid receptors. Although the gene has not yet been cloned, the Ah receptor gene is expected to be a member of the steroid-thyroid hormone receptor gene superfamily (23, 51, 53). Unlike the steroid receptors, however, the Ah receptor appears to bind to the Ah-responsive domain recognition motifs as a monomer, not a dimer, and to require ligand-dependent activation to bind to its cognate DNA target site (15, 28, 34). A receptor-dependent increase in the accessibility of *CYP1A1* chromatin to restriction endonucleases does not require RNA or protein synthesis (20), and binding of the liganded Ah receptor to the Ah-responsive domain appears to cause bending of the DNA at or near the site of the protein-DNA interaction (22).

Many of the members of the steroid receptor superfamily have been shown to be phosphoproteins. Phosphorylation has been proposed as a regulatory mechanism that modulates receptor activation, hormone binding, nuclear translocation, and DNA binding (for a review, see reference 7). Formal proof that protein kinase-mediated phosphorylation is responsible for the activation of hormone binding has recently been obtained by using a human estrogen receptor

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† F.C. dedicates this article to the memory of Marc Cantin.

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synthesized in vitro (49). Presently, more than 70 protein kinases have been characterized, and many are essential elements of intracellular regulatory circuits (21, 33, 39). The balance between protein kinase and protein phosphatase activities can regulate gene expression (not just by controlling translational rates but also by affecting transcriptional initiation), mRNA accumulation (11, 36), and, conceivably, cytoplasmic transport and transcript stability. In addition, because not every protein kinase is expressed in every cell, phosphorylation of proteins is probably involved in developmental and cell-type-specific gene expression. The purpose of the present study was to explore the possibility that phosphorylation plays an important role in the regulation of transcriptional activation of the mammalian *CYP1A1* gene. We provide evidence here to suggest that phosphorylation by protein kinase C might be essential for sequence-specific binding of the Ah receptor and the concomitant transcriptional activation of the murine *Cyp1a-1* gene during induction by TCDD.

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MATERIALS AND METHODS

Definitions. The abbreviations and designations used in this report are defined as follows: *CYP1A1*, the mammalian generic cytochrome P_1450 (rat P450c) gene and cDNA; *Cyp1a-1*, the murine P_1450 gene and cDNA; CYP1A1, the corresponding mRNA and protein product; CYP1A1 activity, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.2); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin); TPA, 12-*O*-tetradecanoylphorbol-13-acetate (phorbol 12-myristate 13-acetate); 2-AP, 2-aminopurine; Ah receptor, aromatic hydrocarbon receptor; AhRE, aromatic hydrocarbon-responsive element. MDC, methyl-2,5-dihydroxycinnamate; HDB, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid; CIP, calf intestinal alkaline phosphatase; and PKI, the peptide inhibitor of PK-A.

Cell lines and treatments. The mouse Hepa-1c1c7 cell line is a subclone of the Hepa-1 hepatoma cell line (3, 4) and was a kind gift of O. Hankinson. It is referred to here as simply Hepa-1. Cells were grown in α-minimal essential medium supplemented with 2 mM L-glutamine and 10% fetal calf serum. Establishment of stable G418-resistant Hepa-1 cells by transfection of pSV2-*neo* has been described (66). The protein kinase inhibitors were dissolved in dimethylsulfoxide, except for H8 and PKI, which were dissolved in water and 2-AP (nitrate salt), which was dissolved in culture medium and neutralized before use. Following the manufacturers' specifications, we used all inhibitors at concentrations not greater than twice the 50% inhibitory concentration. Concentrations used were as follows: 2-AP, 10 mM; H8, 60 μM; staurosporine, 150 nM; quercetin, 2 μM; PKI, 150 nM; MDC, 0.77 μM; lavendustin A, 20 nM; genistein, 2.6 μM; HDB, 44 nM; and tyrphostin, 1.5 μM. Staurosporine and H8 were purchased from Calbiochem (San Diego, Calif.); quercetin, 2-AP, and PKI were from Sigma (St. Louis, Mo.); and all others were from GIBCO-BRL (Gaithersburg, Md.). None of the drugs used produced any apparent cytotoxicity during treatment. Cells grown in duplicate TC150 plates were preincubated with each kinase inhibitor for 1 h at 37°C. One plate was treated with 10 nM TCDD (a gift of the Dow Chemical Company, Midland, Mich.), and the other plate was kept as a control. Samples for preparation of nuclear extracts for gel mobility shift assays were removed after an additional 60 min at 37°C. Samples for RNA extractions were incubated for an additional 4.5 to 6 h. For studying down-regulation of protein kinase C, Hepa-1 cells were treated for 72 h with 150 nM TPA; in agreement with the results of others (18), we observed that treatment for 24 or 48 h was insufficient for complete down-regulation of the kinase in hepatoma cells.

Enzyme assays. CYP1A1 activity in crude extracts was measured spectrophotofluorometrically by determination of the rate of hydroxylated-benzo[*a*]pyrene formation (52). Benzo[*a*]pyrene was purchased from the National Cancer Institute Carcinogen Repository (Bethesda, Md.).

RNA quantitation. Cytoplasmic RNA was prepared by using a kit from 5 Prime-3 Prime, Inc. (Paoli, Pa.). Total cellular RNA was extracted by the acid guanidinium thiocyanate method (9). For slot blot hybridization, 1 or 5 μg of RNA was loaded on duplicate Zeta-Probe membranes with a Bio Dot SF slot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.). For Northern (RNA) blots, 20 μg of RNA was separated in formaldehyde-agarose gels and the gels were dried under vacuum at 60°C and processed for in-gel hybridization (66). DNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham Corporation, Arlington Heights, Ill.) by random priming (25) or by nick translation (47). The 3'-specific *CYP1A1* cDNA probe was derived from either the 334-bp *Bgl*II-*Xba*I fragment or the 1.2-kbp *Bgl*I-to-poly(A) tail fragment of the mouse cDNA (GenBank accession no. K02588) (41). The *neo* probe was derived from the 1.6-kbp *Hin*dIII-*Dra*I fragment of pSV2-*neo* (70). The source of the c-*myc* probe was the mouse c-*myc* cDNA clone pmMycP7, a derivative of pSV2*myc-dhfr* (64) that contains 420 bp of exon 2 and was a kind gift of Cindy Bachurski. The β-actin probe was from the chicken β-actin plasmid pBA1 (10). Conditions for prehybridization, hybridization, and washes were as previously described (66). Following autoradiography, each complete gel was counted in an AMBIS scanner (Automated Microbiology Systems, San Diego, Calif.), and the data were integrated and analyzed by using the manufacturer's software.

Gel mobility shift assays. Nuclear extracts were prepared by procedures described elsewhere (17, 56). For in vitro dephosphorylation, nuclear extracts from TCDD-treated cultures were preincubated for 15 min at room temperature with CIP in concentrations ranging between 0.2 and 4 U/20-μl reaction. DNA-binding reactions were carried out for 20 min at room temperature in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), 300 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 μg of poly(dI-dC) · poly(dI-dC) carrier, 10,000 dpm of DNA probe, and 10% glycerol. The probe used was the 76-bp *Bam*HI-*Hin*dIII insert in plasmid pJ2E69, containing AhRE3 within nucleotides −1019 to −943 of the *Cyp1a-1* gene (54). This region does not contain AhRE2 (nucleotides −1081 to −1055) or AhRE1 (nucleotides −913 to −888); all three AhREs are known to act cooperatively with the dioxin-Ah receptor complex (14, 27, 34, 56, 68). The probe was internally labeled by filling in the restriction enzyme sites with T4 DNA polymerase in the presence of [α-^{32}P]dCTP. Samples were loaded onto nondenaturing 4% polyacrylamide gels, and following electrophoresis, the gels were dehydrated and exposed to X-ray film.

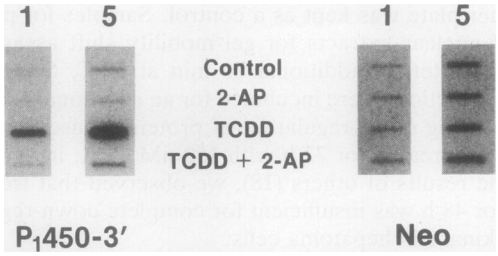

FIG. 1. Effect of 2-AP on TCDD-inducible CYP1A1 mRNA accumulation in mouse Hepa-1 cells. Cells stably transformed with pSV2-*neo* were treated with control medium alone, 2-AP, TCDD, or 2-AP plus TCDD for 6 h. Slots were loaded with 1 or 5 μg of cytoplasmic RNA, as indicated above each panel. Membranes were incubated with ^{32}P-labeled probes specific for CYP1A1 ($P_1$450-3′) or NEO, washed, and exposed to X-ray film.

RESULTS

Induction of *Cyp1a-1* expression is inhibited by 2-AP. 2-AP is a protein kinase inhibitor with a well-documented effect on the phosphorylation states of many proteins, although the effect is not yet fully elucidated by any means (13, 24, 44). To investigate the possibility that a protein kinase might be involved in the regulation of *Cyp1a-1* gene expression, we examined the effect of 2-AP pretreatment of Hepa-1 cultures on TCDD inducibility of CYP1A1 enzyme activity. We found that 2-AP strongly inhibits TCDD induction. Results were as follows: no treatment, 13 ± 3; 2-AP, 16 ± 4; TCDD, 540 ± 50; 2-AP plus TCDD, 24 ± 6 (activity is expressed as picomoles of benzo[*a*]pyrene hydroxylated per minute per milligram of total cellular protein; values are means ± standard deviations of three separate experiments).

To determine whether 2-AP affects the transcriptional induction of the *Cyp1a-1* gene, we examined steady-state levels of CYP1A1 mRNA in TCDD-treated cells that were also treated with 2-AP (Fig. 1). Constitutive CYP1A1 mRNA levels are not measurably affected by 2-AP, and as expected, transcription is induced more than 20-fold by TCDD treatment alone. In contrast, mRNA levels in cells treated with 2-AP prior to TCDD treatment are almost indistinguishable from those found in control cells, indicating that 2-AP pretreatment inhibits the TCDD induction effect by as much as 90 to 95%. The NEO mRNA levels, used as a control, are unaffected by all four experimental conditions.

Accumulation of CYP1A1 mRNA following TCDD treatment has been shown to result largely from transcriptional activation (54, 73). Since treatment with 2-AP does not lead to any apparent decrease in the basal CYP1A1 mRNA levels (Fig. 1), we conclude that the inhibitory effect of 2-AP on *Cyp1a-1* expression takes place primarily at the transcriptional level, resulting in the suppression of TCDD-induced mRNA synthesis, and not at the posttranscriptional level, by destabilization of an otherwise unstable transcript.

In vitro dephosphorylation of nuclear extracts abolishes the formation of Ah receptor-dependent DNA-protein complexes. 2-AP treatment of Hepa-1 cells also eliminates the capacity of nuclear extracts to form TCDD-dependent Ah receptor-DNA complexes (data not shown), suggesting that in vivo inhibition of phosphorylation impairs the capacity of the Ah receptor to be assembled in a transcriptional complex. The same effect would be observed, however, if inhibition of phosphorylation were to affect the synthesis, rather than the assembly, of an essential component of the complex. To establish that the effects observed represent the direct inhibition of phosphorylation of one or more of the components of the transcription complex, we examined the capacity of nuclear extracts to form DNA-protein complexes after in vitro phosphatase treatment. Incubation of nuclear extracts from TCDD-treated Hepa-1 cells with various concentrations of CIP prevents the formation of Ah receptor-specific complexes, as determined by the gel mobility shift assay (Fig. 2, band A). Even at higher CIP concentrations, CIP treatment does not affect the formation of two of the other complexes that contain constitutively expressed proteins (Fig. 2, bands C and E); the complexes in bands B and D of Fig. 2 appear to be diminished at the highest concentrations.

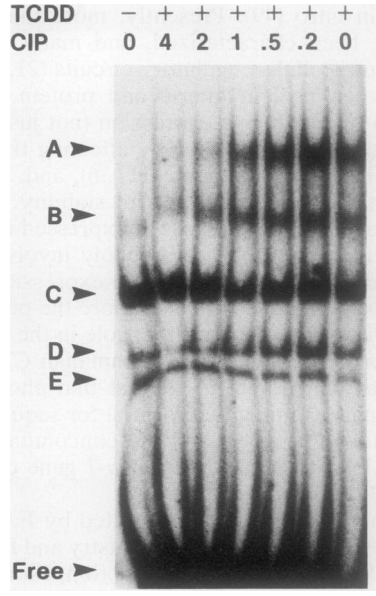

FIG. 2. In vitro CIP treatment of nuclear extracts from TCDD-induced cells prevents the formation of specific DNA-protein complexes. Nuclear extracts from TCDD-treated Hepa-1 cells were incubated with various concentrations of CIP, as indicated above each lane (in units/20-μl reaction), before the DNA-binding reaction. Controls include CIP-untreated extracts from cells not treated with TCDD (leftmost lane) and from TCDD-treated cells (rightmost lane). Each reaction contained 20 μg of protein and 10,000 dpm of probe. The lettered arrowheads denote the various DNA-protein complexes, of which A is the TCDD-dependent, Ah receptor-dependent complex. The identity of these DNA-protein complexes has been well established by work from this and other laboratories (14, 16, 19, 26–28, 34, 40, 55, 56, 68).

These data therefore indicate that phosphorylation is required for the assembly of the transcriptional complex. We conclude that formation of the complex depends on the phosphorylation state of a preexisting protein component of the transcriptional complex itself.

Staurosporine inhibits formation of specific protein-DNA complexes as well as induction of *Cyp1a-1* expression by TCDD. To determine which of several known protein kinases are responsible for the phosphorylation of one (or more) protein in the CYP1A1 transcriptional complex, we used gel mobility shift assays to screen a panel of nine commercially available protein kinase inhibitors (Fig. 3). Three of the compounds—H8, staurosporine, and PKI—are inhibitors of serine/threonine protein kinases, with H8 and PKI having a marked preference for protein kinase A and staurosporine having a preference for protein kinase C (8, 37, 69). The remaining six—lavendustin A, tyrphostin, quer-

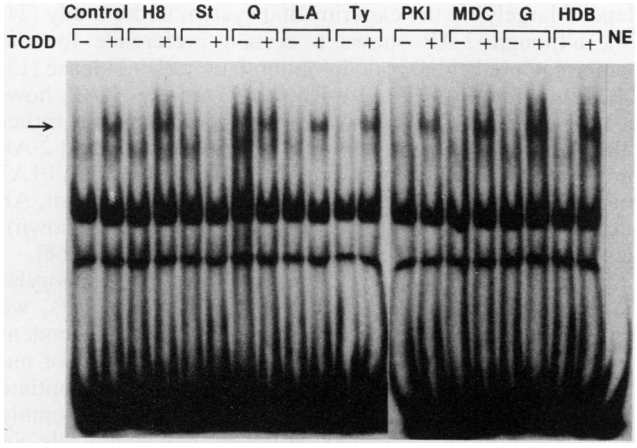

FIG. 3. Gel mobility shift assays with nuclear extracts from Hepa-1 cells treated with various protein kinase inhibitors. Cells were pretreated with the inhibitors indicated and then induced with TCDD (+). As a control, each inhibitor treatment was continued in the absence of TCDD (−). Control, samples from cells not treated with protein kinase inhibitors. Abbreviations: St, staurosporine; Q, quercetin; LA, lavendustin A; Ty, tyrphostin; G, genistein; and NE, no extract. Each binding reaction contained 20 to 25 μg of extract and 10,000 dpm of the pJE269 probe. The arrow indicates the position of the TCDD-dependent Ah receptor-dependent complex.

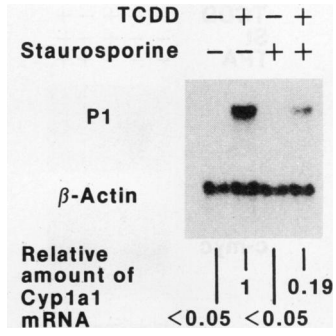

FIG. 4. Staurosporine inhibits the TCDD-induced accumulation of CYP1A1 mRNA. Cells were treated in the absence (−) or presence (+) of 150 nM staurosporine for 1 h and then incubated in the presence (+) or absence (−) of TCDD for a further 4.5 h. Total cellular RNA was separated in formaldehyde-agarose gels. One gel was probed with the 1.2-kb 3′-specific *Cyp1a-1* cDNA probe (P1), and another was probed with a chicken β-actin-1 cDNA probe (β-actin). Following autoradiography, the radioactivity hybridized to the RNA in the dried gel was measured with an AMBIS scanner, and the amount of CYP1A1 mRNA normalized to the counts hybridized to β-actin was determined relative to the amount present in TCDD-induced cells (bottom).

cetin, MDC, genistein, and HDB—are tyrosine kinase inhibitors.

Nuclear extracts from TCDD-induced Hepa-1 cells pretreated with the indicated inhibitors were analyzed for their capacity to form Ah receptor-dependent protein-DNA complexes (Fig. 3). The results show that extracts from untreated cells are capable of forming the expected TCDD-dependent Ah receptor-dependent complex, a result also observed with most of the inhibitors tested. In contrast, pretreatment with staurosporine prevents the formation of the TCDD-dependent complex (Fig. 3, lanes St).

We examined the effect of staurosporine on *Cyp1a-1* expression by Northern blot hybridization of total RNA extracted from cells treated with staurosporine prior to TCDD induction (Fig. 4). Control RNA samples included RNA from cells treated with TCDD alone, treated with staurosporine alone, or left untreated. The level of CYP1A1 mRNA is barely detectable in untreated or in staurosporine-treated cells but, as expected, is very high in TCDD-induced cells. When staurosporine was added before TCDD, mRNA induction was markedly inhibited. The extent of this inhibition was estimated to be 80%, as determined from the radioactive counts hybridized. The levels of β-actin mRNA, used as a control, remained unaffected (Fig. 4). The other eight inhibitors tested failed to block CYP1A1 mRNA induction by TCDD (data not shown).

Inhibition of induction was incomplete at the dose (150 nM) of staurosporine used in the experiments described above. We carried out dose-response experiments in order to determine whether the residual mRNA level was insensitive to the drug. RNA samples from cells treated with staurosporine at different concentrations and then induced with TCDD were analyzed in Northern blots. The data were normalized to β-actin mRNA levels, and the extent of inhibition was calculated by comparison with CYP1A1 mRNA levels in cells induced by TCDD in the absence of staurosporine. The results shown in Fig. 5 establish that complete inhibition of CYP1A1 mRNA induction occurs at a concentration of 5 μM staurosporine, with a 50% inhibitory concentration of approximately 60 nM.

Since staurosporine has a marked preference for the inhibition of protein kinase C, these results strongly suggest that the phosphorylation event(s) required for TCDD-induced activation of the *Cyp1a-1* gene is carried out by this kinase.

Down-regulation of protein kinase C abolishes the induction of *Cyp1a-1* expression by TCDD. Tumor-promoting phorbol diesters, such as TPA, are analogs of diacylglycerol, the

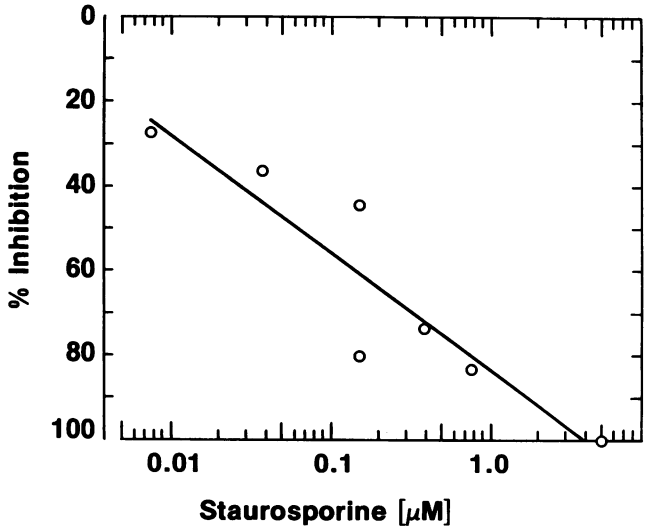

FIG. 5. Effect of staurosporine dose on the inhibition of CYP1A1 mRNA induction by TCDD. Cells were treated with different doses of staurosporine, and the RNA was analyzed as indicated in the legend to Fig. 4. Results are expressed as the percentage of inhibition of CYP1A1 mRNA induction, with 0% corresponding to the value for cells treated with TCDD in the absence of staurosporine. Data were fitted to a straight line by the linear regression method.

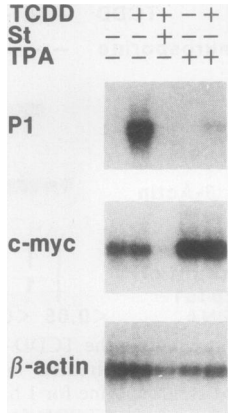

FIG. 6. Protein kinase C depletion blocks CYP1A1 mRNA induction by TCDD. Cells were treated with 150 nM TPA for 72 h or left untreated. Untreated cells were induced with TCDD after 1 h of staurosporine treatment (St) or in the absence of prior staurosporine treatment. TPA-treated cells were induced with TCDD or left untreated. Total RNA from these cells was analyzed by Northern blot hybridization with the 3'-specific *Cyp1a-1* cDNA probe (P1), the c-*myc* probe, or the β-actin probe.

biological activator of protein kinase C. Prolonged treatment of cell cultures with TPA is known to cause the down-regulation and eventual depletion of protein kinase C in TPA-treated cells (2, 18, 35, 48). To test the hypothesis that protein kinase C activity is required for *Cyp1a-1* activation, we analyzed CYP1A1 mRNA levels in Hepa-1 cells treated with 150 nM TPA for 72 h prior to TCDD induction. As a control for these experiments, we used the c-*myc* gene, whose transcriptional status in hepatoma cells is very different from that found in other cultured cell lines. Steady-state C-MYC mRNA levels are high in untreated hepatoma cells (18, 38, 58, 67, 74, 75) and, after an initial rise, do not change during prolonged exposure to TPA (18). We found that prolonged TPA treatment, however, has a dramatic effect on TCDD inducibility of the *Cyp1a-1* gene (Fig. 6). In the absence of TPA treatment, TCDD induces the accumulation of CYP1A1 mRNA, and, as described above, this induction is abolished by staurosporine. In contrast, after 72 h of TPA treatment, the capacity of TCDD to induce CYP1A1 mRNA is eliminated (Fig. 6, top). Increases in C-MYC mRNA are unaffected by TCDD, repressed by staurosporine, and, as shown by others (18), unchanged or slightly increased by prolonged TPA treatment (Fig. 6, middle). Transcription of β-actin is not significantly affected by any of the treatments (Fig. 6, bottom).

These results provide strong support for our hypothesis that transcriptional activation of the *Cyp1a-1* gene requires protein kinase C-mediated phosphorylation.

DISCUSSION

The experiments described in this report show that TCDD-induced activation of the murine *Cyp1a-1* gene is suppressed by inhibitors of protein kinase activity. Of ten compounds tested, two were highly effective suppressors, namely, 2-AP and staurosporine. The mechanism of 2-AP inhibition of cellular phosphorylation is not well understood, but it is believed to block protein kinases by modifying serine and threonine residues (71, 74). Whether 2-AP acts as a general or a specific protein kinase inhibitor seems to depend largely on the experimental system under study (24, 76), although 2-AP shows a certain preference for the heme-regulated eukaryotic initiation factor eIF-2 kinase (13, 24). Inhibition of CYP1A1 mRNA induction by 2-AP, however, does not result from a translational block but rather takes place at the pretranslational level. We found that 2-AP prevents both the accumulation of TCDD-induced CYP1A1 mRNA (Fig. 1) and the formation of a TCDD-dependent, Ah receptor-dependent DNA-protein complex (data not shown), neither of which requires de novo protein synthesis (68).

To verify that a causal relationship between phosphorylation and specific Ah receptor binding to DNA exists, we demonstrated that formation of the Ah receptor-dependent complex is abolished by in vitro dephosphorylation of nuclear extracts with CIP (Fig. 2). These results substantiate the conclusion that phosphorylation is required for assembly of the transcriptional complex, although they do not rule out other possible effects of phosphorylation on *Cyp1a-1* transcription.

Staurosporine, an inhibitor of protein kinase C, is the only compound other than 2-AP that blocked both the formation of specific DNA-protein complexes (Fig. 3) and the increases in CYP1A1 mRNA after TCDD treatment (Fig. 4 and 5). Seven other protein kinase inhibitors tested were ineffective. The specificity of staurosporine has been disputed, since in addition to inhibiting protein kinase C activity (69), staurosporine can also inhibit the epidermal growth factor receptor tyrosine kinase (46). In the experiments reported here, six different tyrosine kinase inhibitors had no effect on the TCDD-dependent formation of the Ah receptor-AhRE DNA complex (Fig. 3). Two of these compounds—tyrphostin and MDC—are competitive inhibitors of substrate binding to epidermal growth factor receptor tyrosine kinase (29, 72), whereas three others—lavendustin A, HDB, and genistein—are competitive inhibitors at the ATP-binding site (12, 59). Genistein also inhibits tyrosine kinases other than the epidermal growth factor receptor-associated enzyme (1), and quercetin inhibits an unspecified tyrosine kinase (30, 37, 45). Since none of these tyrosine kinase inhibitors affects the formation of specific Ah receptor-DNA complexes, we conclude that the activity inhibited by staurosporine is that of protein kinase C. Two serine/threonine kinase inhibitors, H8 and PKI, both known to block cyclic nucleotide-dependent kinase activities (8, 37), have no effect on the formation of specific Ah receptor-AhRE complexes.

We cannot rule out completely the possible participation of other protein kinases in *Cyp1a-1* transcriptional activation. Our results indicate that protein kinase C plays an essential role in induction of this gene, but it is always possible that other kinases participate in *Cyp1a-1* activation. We have not conducted permeability studies for each inhibitor, and it is possible that the failure of one or more of the inhibitors tested to cause an effect may result, for example, from a failure of the chemical to cross the cell membrane. This may apply in particular to PKI, which, unlike the rest of the inhibitors used, has not yet been shown to penetrate cells.

Depletion of protein kinase C by prolonged TPA treatment completely eliminates TCDD inducibility of CYP1A1 mRNA (Fig. 6). These results strengthen our conclusion that protein kinase C-dependent phosphorylation of one or more of the components of the transcriptional complex is an essential step in the activation of the *Cyp1a-1* gene. A similar effect of phorbol ester treatment in mice has recently been observed (57), although in mice the effect took place within 1 h after administration of the drug (57).

Our observations do not rule out the possibility that other events in the regulatory cascade of *Cyp1a-1* expression may also be dependent on phosphorylation-dephosphorylation cycles. By analogy with the evidence available for members of the steroid receptor superfamily (7), it is appealing to speculate that the Ah receptor itself is one of the phosphorylation targets. As is the case with steroid hormone induction, Ah induction does not require de novo protein synthesis (68) but involves the transformation of an inactive to an active form of the Ah receptor, followed by nuclear translocation of the activated receptor (for reviews, see references 32 and 43). Protein kinase C activity, coupled with the presence of the ligand, may be necessary not only for DNA binding but also for nuclear translocation. It has been established that protein kinase C activity is not necessary for receptor activation (16).

TCDD is an extremely potent tumor promoter and teratogen (42, 50, 51, 61–63). Polycyclic hydrocarbons such as benzo[*a*]pyrene, which are proven ligands for the Ah receptor, can cause mutation and tumor initiation following metabolic potentiation (60). It has been shown that TCDD stimulates kinase activities in vivo, particularly those of protein kinase C and various tyrosine kinases (5, 6). An understanding of the events leading to the simultaneous activation of the protein kinase C second messenger pathway (and perhaps others) and of mammalian *CYP1A1* transcription may provide valuable insight into the molecular basis of Ah receptor-mediated toxicity, tumor initiation and promotion, and perturbation of growth and differentiation.

ADDENDUM

After this work was submitted for publication, a report on the inhibition of the specific DNA-binding activity of the dioxin receptor by phosphatase treatment was published (63a). Our conclusions are in complete agreement with the conclusions presented in that publication.

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