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Cross-Coupling of Signal Transduction Pathways: the Dioxin Receptor Mediates Induction of Cytochrome P-450IA1 Expression via a Protein Kinase C-Dependent Mechanism

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Signal transduction by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is mediated by the intracellular dioxin receptor which, in its dioxin-activated state, regulates transcription of target genes encoding drug-metabolizing enzymes, such as cytochrome P-450IA1 and glutathione *S*-transferase Ya. Exposure of the dioxin receptor to dioxin leads to an apparent translocation of the receptor to the nucleus in vivo and to a rapid conversion of the receptor from a latent, non-DNA-binding form to a species that binds to dioxin-responsive positive control elements in vitro. This DNA-binding form of receptor appears to be a heterodimeric complex with the helix-loop-helix factor Arnt. In this study, we show that activation of the cytochrome P-450IA1 gene and minimal dioxin-responsive reporter constructs by the dioxin receptor was inhibited following prolonged treatment of human keratinocytes with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. Inhibition of the receptor-mediated activation response was also achieved by treatment of the cells with a number of protein kinase inhibitors, one of which, calphostin C, shows selectivity for protein kinase C. Taken together, these data suggest that protein kinase C-dependent phosphorylation may play an essential role in the dioxin signaling pathway. This hypothesis is supported by the observation that pretreatment of the cells with 12-*O*-tetradecanoylphorbol-13-acetate inhibited the DNA-binding activity of the dioxin receptor in vivo. In vivo, the dioxin receptor was found to be a phosphoprotein. In vitro, dephosphorylation of the ligand-activated, heteromeric dioxin receptor form or dephosphorylation of the individual ligand-binding and Arnt receptor subunits inhibited the xenobiotic response element-binding activity. Moreover, dephosphorylation experiments with the individual receptor subunits prior to assembly of the xenobiotic response element-binding receptor form indicated that phosphorylation seemed to be important for the DNA-binding activity per se of the receptor, whereas Arnt appeared to require phosphorylation to interact with the receptor. Finally, a protein kinase C inhibitor-sensitive cytosolic catalytic activity that could restore the DNA-binding activity of the dephosphorylated dioxin receptor form was identified.

Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and several related halogenated aromatic hydrocarbons are members of a class of environmental pollutants that give rise to a plethora of teratogenic, tumorigenic, and toxic responses in rodents. In humans, a well-documented toxic effect of this class of compounds is a hyperkeratotic and metaplastic response of the hair follicles and interfollicular epidermis, leading to persistent acne-like lesions called chloracne (reviewed in reference 58). On a molecular level, dioxin induces transcription of genes encoding drug-metabolizing enzymes, such as glutathione *S*-transferase Ya, quinone reductase, and cytochrome P-450IA1, in both rodent and human cells (for a review, see reference 30).

Signal transduction by dioxin is mediated by the intracellular dioxin receptor protein (reviewed in references 47 and 57). The receptor represents a ubiquitous regulatory factor that harbors a ligand-binding subunit of about 100 kDa (8) and that is distinct from members of the steroid receptor family of nuclear receptors but bears similarity (22) to the broad class of helix-loop-helix regulatory factors (reviewed in reference 68). In the absence of a ligand, the receptor is present in the cytoplasmic compartment of target cells in a latent, inactive (non-DNA-binding) configuration. The process of ligand binding, however, induces translocation of the receptor to the cell nucleus and activation of the receptor to a DNA-binding form (reviewed in reference 57). In vitro, the activated receptor specifically recognizes dioxin-inducible transcriptional control elements (xenobiotic response elements [XREs]), which confer dioxin responsiveness to target promoters (18, 25, 26, 34, 51, 55). Activation of the receptor is stimulated by ligands in a manner that reflects their relative binding affinities for the receptor protein in vitro and their relative potencies to induce cytochrome P-450IA1 transcription in vivo (16).

At present, the mechanism of activation of the dioxin receptor to a DNA-binding form is poorly understood. The DNA-binding activity of the receptor is regulated by the formation of a heteromeric complex with the 90-kDa heat shock protein (hsp90), which functions as an inhibitory protein preventing the receptor from binding to DNA recognition elements (69). Thus, dioxin-induced activation of the receptor requires release of hsp90. In addition, an ~85-kDa non-dioxin-binding helix-loop-helix factor, Arnt, has been reported to be required for nuclear translocation of the ligand-activated form of the receptor and may possibly interact with the receptor via the putative helix-loop-helix dimerization interface (40). Consistent with this model, Arnt has been shown to be a component (63) of the ~200-kDa ligand-activated nuclear receptor form (34), and we recently

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observed that the receptor strictly requires physical interaction with Arnt for DNA-binding activity to occur (68a). Moreover, it is possible that the phosphorylation state of the receptor and/or Arnt is important for receptor function, since dephosphorylation of the ligand-occupied dioxin receptor by phosphatase treatment in vitro generates a non-DNA-binding receptor form without inducing any detectable degradation of the receptor protein (59). Interestingly, expression of the cytochrome P-450IA1 gene in human keratinocytes is modulated by extracellular Ca^{2+} and serum (3), and treatment of mice with 12-*O*-tetradecanoylphorbol-13 acetate (TPA) has been reported to down-regulate induction of cytochrome P-450IA1 activity by dioxin receptor ligands in the liver (62) and epidermis (60). The major cellular target for the action of phorbol esters is the protein kinase C (PKC) family of serine-threonine protein kinases, which has been implicated as a modulator of many cellular control mechanisms, including proliferation, differentiation, and tumorigenesis (reviewed in references 46 and 52).

In this paper, we show that prolonged treatment of normal human keratinocytes with TPA or inhibitors of PKC activity negatively interfered with activation by dioxin analogs of the endogenous cytochrome P-450IA1 gene and transfected minimal dioxin-responsive reporter constructs. These data suggest that induction of target gene expression by the dioxin receptor may require a PKC-dependent pathway. Consistent with this model, pretreatment of keratinocytes with TPA resulted in inhibition of the DNA-binding activity of the dioxin receptor. In vitro, the DNA-binding activity of the ligand-activated dioxin receptor was inhibited by dephosphorylation. Moreover, Arnt appeared to require phosphorylation to interact with the receptor, indicating that dioxin receptor function is regulated by a complex pattern of phosphorylation. Finally, the XRE-binding activity of the dephosphorylated receptor could be restored by incubation with a cytosolic activity.

MATERIALS AND METHODS

Reporter plasmids. Dioxin-responsive reporters were constructed as follows. The XRE1 element, extending from nucleotides −1026 to −999 relative to the transcription start site of the rat cytochrome P-450IA1 gene (25), was inserted into the *Bam*HI site of pUC19, subsequently excised with *Sac*I and *Sma*I, and ligated into *Sma*I- and *Xho*I-digested pT81 (53), containing the herpes simplex virus thymidine kinase (TK) promoter and the luciferase reporter gene. The resulting construct (pTX.ONE) was then digested with *Sac*I, and an additional XRE1 element was inserted to generate pTX.DIR and pTX.INV. The orientation of the XREs in all constructs was confirmed by dideoxy sequencing. pRNH11C (38) contains a fragment of the human cytochrome P-450IA1 gene (extending from nucleotides −1140 to +2435 relative to the transcription start site) fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Plasmid pON249, containing the cytomegalovirus promoter-driven *lacZ* gene (13), was used as an internal control for efficiency of transfection.

Cell culture and treatment of cells. Human keratinocytes were isolated from adult donors as described previously (36). To allow for maximal growth, we cultured human keratinocytes with a final concentration of 70 μM Ca^{2+} in MCDB 153 medium (see below) supplemented with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.4 μg of hydrocortisone per ml, 10 ng of epidermal growth factor per ml, 100 μg of bovine pituitary extract per ml, 100 μg of streptomycin per ml, 100 IU of penicillin per ml, and 0.25 μg of amphotericin B per ml (Fungizone; Nordcell) until near confluence; they were then subcultured and plated onto fibronectin- and collagen I-coated cell culture plastic. The medium and all chemicals and growth factors were purchased from Sigma, unless stated otherwise. The pituitary extract was prepared as described previously (6) from bovine pituitaries purchased from Pel-Freeze. Human keratinocytes were routinely used for induction and dioxin receptor experiments at the third to fifth passage of cells. To maximize the dioxin-dependent cytochrome P-450IA1 induction response in the keratinocytes, we altered the medium as described previously (3) to contain 2 mM Ca^{2+} and substituted 5% bovine serum (Hyclone) for the pituitary extract 2 days prior to treatment. The cells were treated with the high-affinity dioxin receptor ligand 2,3,7,8-tetrachlorodibenzofuran (TCDF; Cambridge Isotope Laboratories), TPA (Pharmacia), and calphostin C (Kamiya Biomedical Co.) dissolved in dimethyl sulfoxide (DMSO). Inhibition of PKC activity by calphostin C has been reported to be light dependent (9). Calphostin C-treated cells were therefore incubated at 37°C for 2 h with a 15-W light source 30 cm above the culture dish prior to the addition of TCDF and withdrawal of the light source. Control cells were treated only with solvent, the final concentration of which never exceeded 0.2%. Staurosporine (Calbiochem) was diluted in ethanol, and H-7 (GIBCO/BRL) was diluted in water. The dioxin-responsive Hepa-1 hepatoma cell line and the non-dioxin-responsive (nuclear translocation-deficient [nt^-]) mutant cell line derived from it (C4) (32 and references therein) were grown in minimal essential medium (GIBCO) as previously described (69). For metabolic labeling experiments, Hepa-1 cells were grown to near confluency in 15-cm dishes. Cellular proteins were labeled with 1 mCi of $^{32}P_i$ (Amersham) per ml by incubation for 2.5 h at 37°C in phosphate-free Dulbecco's modified Eagle's medium (GIBCO) supplemented with 1% bovine serum.

DNA transfection and transient expression assays. Keratinocytes were transiently transfected by use of 1.2 μg of cationic liposomes (lipofectin; BRL) (23) per cm^2 with various concentrations of luciferase, CAT, or LacZ reporter constructs. The cells were incubated with the DNA-liposome mixture overnight and subsequently incubated for 24 h in fresh medium containing 5% serum and 2 mM Ca^{2+} prior to treatment with solvent alone or 50 nM TCDF in the presence or absence of 50 nM TPA. CAT, luciferase, and β-galactosidase activities were assayed as described previously (64) with the modification that the cells were lysed on ice with a sonicator (Branson Sonic Power Corp.). ^{14}C-Chloramphenicol and luciferin were purchased from Amersham and Bio Thema, respectively.

Isolation of RNA and RNA blot analysis. Total RNA was isolated by acid-phenol extraction as described previously (15). The RNA was fractionated on formaldehyde-agarose gels, blotted onto nylon membranes by capillary flow, and UV cross-linked, and the filters were prehybridized, hybridized with labeled DNA probes, and washed prior to autoradiography by standard protocols (64). For hybridization analysis, the insert of phP1-4503′ (encoding human cytochrome P-450IA1 [42]) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA (24) were isolated and ^{32}P labeled by a random priming procedure (64).

Determination of PKC activity. For determination of total cellular PKC activity, whole-cell extracts were prepared as described previously (37, 44). In brief, cells were treated in the absence or presence of 50 nM TPA for 4 and 16 h, harvested in 10 mM Tris-HCl (pH 7.5)–10 mM 2-mercaptoeth-

anol–2 mM EDTA–10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–240 mM sucrose–10% (vol/vol) glycerol–10 μg of leupeptin per ml–100 kIU of aprotinin per ml–1 μM phenylmethylsulfonyl fluoride (PMSF)–0.2% (vol/vol) Triton X-100, homogenized, and subsequently shaken at 4°C for 30 min. The supernatant obtained after centrifugation at 14,000 × *g* for 30 min at 4°C was passed through a DEAE-Sephacel (Pharmacia) column preequilibrated with buffer B (10 mM Tris-HCl [pH 7.5], 10 mM 2-mercaptoethanol, 5 mM EDTA, 10% glycerol). The column was washed in buffer B containing 50 mM NaCl, and PKC was eluted with buffer B containing 120 mM NaCl. PKC activity was determined with a PKC assay system (Amersham), in which the PKC-catalyzed transfer of the γ-phosphate group of [γ-^{32}P]ATP to the threonine residue of a peptide substrate is measured. This assay represents a modification of a mixed-micelle assay (33). PKC activity was calculated by substraction of the activity obtained in the absence of Ca^{2+} and phospholipids. The activity was normalized to the protein content of the DEAE-Sephacel eluates.

Preparation of cellular extracts. Nuclear and whole-cell extracts were prepared from untreated or treated keratinocytes essentially as described by Dignam et al. (20). For the whole-cell extract, the cells were lysed, after a washing step, by Dounce homogenization in a low-ionic-strength KCl extraction buffer (20), KCl was added to a final concentration (including the cell volume) of 300 mM, and proteins were extracted for 30 min on ice prior to centrifugation and dialysis in accordance with the protocol for nuclear extract preparation. Cytosolic extracts were prepared from Hepa-1 cells (both wild-type and nt$^-$ cells) by homogenization of untreated cells in 1 volume of 20 mM Tris-HCl (pH 7.4)–10% (wt/vol) glycerol–1 mM EDTA–2 mM 2-mercaptoethanol. The homogenates were centrifuged for 1 h at 105,000 × *g*, and the resulting supernatant was taken as the cytosolic fraction. The cytosolic dioxin receptor was activated in vitro by dioxin (Chemsyn) to a DNA-binding form as described previously (16).

DNA-binding assays. The specific DNA-binding activity of the dioxin receptor was monitored by an electrophoretic gel mobility shift assay. DNA-binding reactions were carried out with either cytosolic or nuclear extracts (~10 μg of protein) in a total volume of 20 μl, with 2 fmol of a radiolabeled double-stranded synthetic XRE (5′-CTCCG GTCCT TCTCACGCAA CGCCTGGGCA-3′) spanning the dioxin response element at about bp −1000 of the human cytochrome P-450IA1 gene (45) as a specific probe. The reactions were performed in the presence of 0.1 μg of poly(dI-dC) per μl and 12.5 ng of poly(dA-dT) per μl (nonspecific competitor DNAs) with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9)–75 mM KCl–5% glycerol–4% Ficoll–0.2 mM EDTA–2 mM $MgCl_2$–1 mM dithiothreitol–0.1 mM PMSF. In oligonucleotide competition experiments, a double-stranded oligonucleotide (5′-TCTAGTGTTG GAGAACGAAT CAGCAT CTGA GTAC-3′) was used as a non-XRE-related sequence motif. The DNA-binding activity of adenovirus major late transcription factor USF was assayed by electrophoretic gel mobility shift analysis as described above by use of, as a probe, a double-stranded oligonucleotide containing the USF recognition sequence of the adenovirus major late promoter (31). DNA-protein complexes were separated under nondenaturing conditions in 5% polyacrylamide (29:1) gels with 0.35× TBE (1× TBE is 90 mM Tris-borate plus 1 mM EDTA).

Dephosphorylation of the dioxin receptor and subsequent reactivation. The DNA-binding activity of the dioxin receptor was inhibited by treatment of in vitro- or in vivo-activated dioxin receptor with potato acid phosphatase (Boehringer Mannheim) as described previously (59). To permit reactivation of the DNA-binding activity of the receptor, we coupled phosphatase to cyanogen bromide-activated Sepharose (Pharmacia) and then removed the phosphatase by centrifugation prior to incubation of the dephosphorylated receptor with cellular extracts for 20 min at room temperature. Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS; Boehringer) was found to optimally inhibit the reactivation of the receptor DNA-binding activity at a concentration of 400 μM. In the experiment shown in Fig. 6E, the cellular extract from the nt$^-$ cells had been preincubated for 20 min at room temperature in normal light with increasing concentrations of calphostin C.

In vitro expression of Arnt. Arnt mRNA was generated from pBM5-NEO-M1-1 (40) by use of T7 polymerase and used for in vitro synthesis of labeled or unlabeled Arnt proteins in rabbit reticulocyte lysates (Promega) in the presence of either [^{35}S]methionine (New England Nuclear) or 20 to 30 μM unlabeled methionine under the conditions suggested by the manufacturers. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, prestained and ^{14}C-labeled M_r marker proteins were purchased from Bio-Rad and Amersham, respectively.

Dioxin receptor antiserum and immunoblotting and immunoprecipitation experiments. A peptide corresponding to amino acids 12 to 31 of the murine dioxin receptor (22) was synthesized and coupled to ovalbumin (68b), and antisera were produced in rabbits by standard techniques (35). For immunoblot analysis, proteins were separated by SDS-PAGE (7.5% polyacrylamide), transferred to nitrocellulose membranes, incubated with antisera, and stained with peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako). In immunoprecipitation experiments, cytosolic extracts (~6 mg of protein per ml) from nt$^-$ mutant hepatoma cells were treated with 10 nM dioxin at 25°C for 3 h. In vitro-translated, [^{35}S]methionine-labeled Arnt (5 μl) was added to 10 μl of ligand-treated C4 cytosol, and the mixture was incubated at 25°C for 20 min. Immune or preimmune serum (20 μl) was added, and the mixture was shaken gently for 1 h. Immunoprecipitation was carried out by the addition of 100 μl of a 50% slurry of protein A-Sepharose (Pharmacia) in 20 mM sodium phosphate (pH 7.2)–1 mM EDTA–10% (wt/vol) glycerol–150 mM NaCl–0.1% Triton X-100–1 mM dithiothreitol. After incubation for 30 min, the resin was washed four times with the same buffer and the immunoprecipitated proteins were separated by SDS-PAGE. The gel was subsequently fixed in 20% methanol–10% acetic acid, immersed in Amplify (Amersham) for 30 min, dried, and fluorographed. For immunoprecipitation of the metabolically labeled dioxin receptor, the cells were scraped into 500 μl of lysis buffer (20 mM sodium phosphate [pH 7.2], 50 mM β-glycerophosphate, 10% [wt/vol] glycerol, 1 mM EDTA, 150 mM NaCl, 0.1 mM Na_3VO_4, 0.2 mM PMSF, 5 μM pepstatin, 10 μg of leupeptin per ml, 100 kIU of aprotinin per ml, and 0.1% Nonidet P-40 [NP-40]) and aspirated through an 0.8-mm-inner-diameter needle, and the lysates were cleared by centrifugation for 45 min at 15,000 × *g* and 4°C. Aliquots of the lysates (70 μl) were incubated on ice for 30 min with 5 μl of immune serum that had been preincubated with either the dioxin receptor peptide or an unrelated peptide (amino acids 779 to 795 of the rat glucocorticoid receptor [49]). Protein A-Sepharose was used to immunopre-

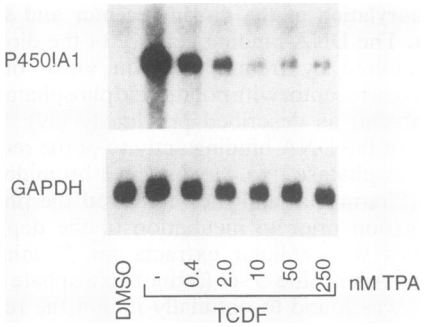

FIG. 1. TPA inhibits in a dose-dependent manner the induction of cytochrome P-450IA1 mRNA expression by TCDF. Human keratinocytes were treated for 24 h before being harvested with solvent (DMSO) alone or with 50 nM TCDF in the absence or presence of the indicated concentrations of TPA. Cytochrome P-450IA1 and control GAPDH mRNA levels were assayed by RNA blot analysis of 10 μg of total cellular RNA per lane.

cipitate the receptor; however, a more harsh washing procedure was necessary to reduce nonspecific background activity. The resin was washed once with lysis buffer supplemented with 1% NP-40–0.5% sodium deoxycholate–0.1% SDS, once with 1 M NaCl (pH 7.4), and intervening washes with 20 mM sodium phosphate (pH 7.2)–10% glycerol–150 mM NaCl–1 mM EDTA–1% NP-40. The proteins were analyzed by SDS-PAGE as described above.

Safety precautions. In cell culture and biochemical experiments involving the use of TCDF and dioxin, special handling procedures were used (69 and references therein). Contaminated materials were disposed of by high-temperature incineration.

RESULTS

Inhibition of PKC interferes with induction of cytochrome P-450IA1 mRNA expression. Given the observation that TPA appears to down-regulate induction of cytochrome P-450IA1-dependent enzyme activities in mouse liver (62) and epidermis (60), we investigated the effect of TPA on dioxin receptor-mediated induction of target gene expression in human keratinocytes. To this end, we studied the induction of cytochrome P-450IA1 mRNA expression by TCDF, a compound that binds to the dioxin receptor with a high affinity and is more water soluble than dioxin (29 and references therein). A dramatic (more than 50-fold) induction of cytochrome P-450IA1 mRNA levels was detected by RNA blot analysis following exposure of the cells to 50 nM TCDF (Fig. 1, compare the two left lanes). In virtually all experiments, the basal-level expression of the cytochrome P-450IA1 gene was below the limit of detection. It is thus difficult to reach a quantitative estimate of the level of induction following exposure to TCDF. In the presence of TPA, however, the induction elicited by TCDF treatment was significantly reduced. This inhibition of cytochrome P-450IA1 mRNA induction by TPA was dose responsive and, at a TPA concentration of 10 nM, only about 10% induction was produced by TCDF compared with the level generated by TCDF alone (Fig. 1). It was not possible to further reduce this level of induction by increasing the dose of TPA.

Although phorbol esters are known to rapidly activate PKC, the effect is transient, since the long-term response is characterized by down-regulated levels of PKC activity (46 and references therein). This effect has been observed in keratinocytes (14, 44). We therefore examined the time course of inhibition produced by administration of TPA prior to or concomitantly with the addition of TCDF. In the absence of any TPA treatment, maximal levels of cytochrome P-450IA1 mRNA were induced after 4 h of incubation of human keratinocytes with TCDF (Fig. 2A). When TPA was administered together with TCDF to the keratinocytes, maximal inhibition of TCDF induction response

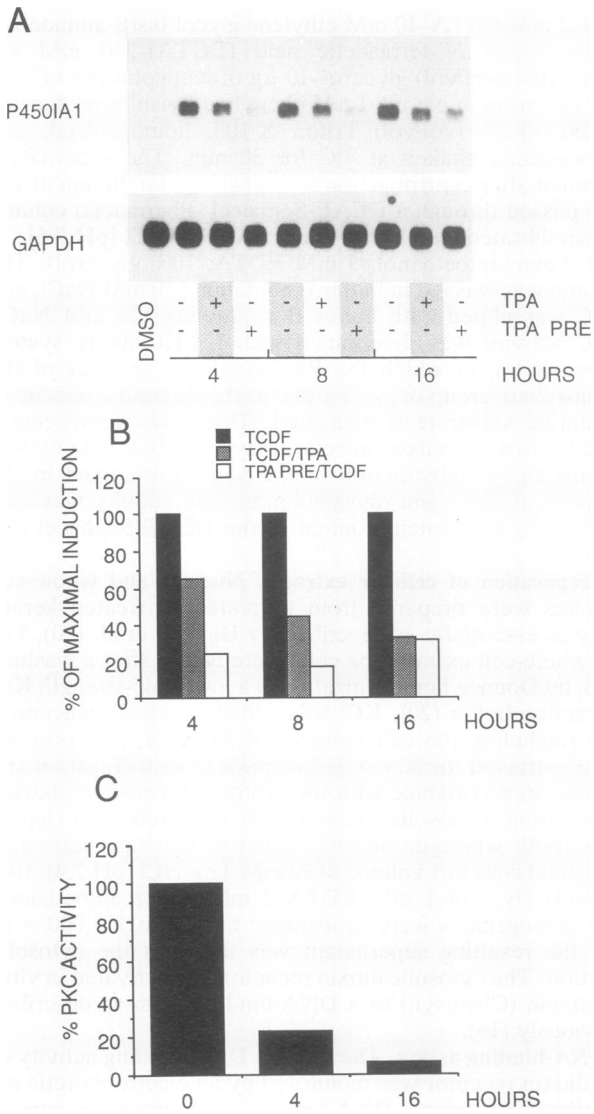

FIG. 2. Time course of inhibition of the cytochrome P-450IA1 mRNA induction response by pretreatment or simultaneous treatment with TPA. (A) RNA blot analysis of total RNA prepared from cells treated either with the solvent DMSO alone or with 50 nM TCDF in the absence or presence of simultaneously added 50 nM TPA for the indicated times. In lanes marked TPA PRE, the cells were incubated with 50 nM TPA for 24 h prior to the addition of TCDF. (B) Densitometric determination of the P450IA1 mRNA signals obtained in panel A, normalized to the GAPDH signal. The TCDF-induced P450IA1 signal at each time point was set to 100%. (C) Total cellular PKC activity measured in extracts prepared from control cells and from cells treated with 50 nM TPA for 4 and 16 h. The values represent averages for two independent experiments. The activity at 0 h of TPA treatment was set to 100%.

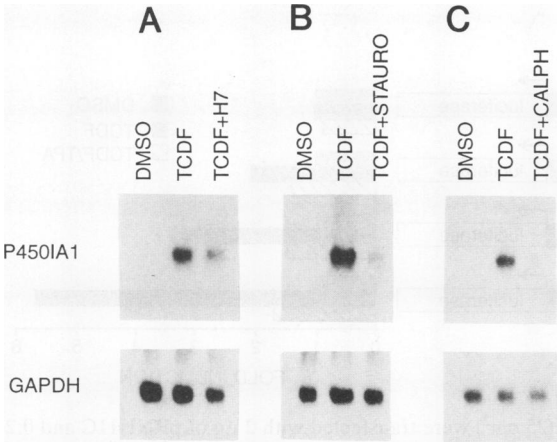

FIG. 3. Inhibition of the cytochrome P-450IA1 mRNA induction response by protein kinase inhibitors. Keratinocytes were treated for the indicated times with 50 nM TCDF in the absence or presence of the following protein kinase inhibitors: A, 100 μM H-7 (24 h); B 200 nM staurosporine (STAURO) (8 h). (C) Cells were pretreated (see Materials and Methods for details) with 1 μM calphostin C (CALPH) prior to the addition of TCDF and incubation for an additional 6 h. Subsequently, the cells were analyzed for expression of cytochrome P-450IA1 or GAPDH mRNA by RNA blot analysis.

was observed after a prolonged (16-h) treatment of the cells (Fig. 2A and B). Significant inhibition of the TCDF induction response was also detected following 24 h (Fig. 1) or 48 h (data not shown) of coincubation of the cells with TPA and TCDF. Interestingly, pretreatment of the cells with TPA for 24 h prior to the administration of TCDF resulted in maximal inhibition of the TCDF induction response by 4 or 8 h of treatment with TCDF (Fig. 2A and B). In control experiments, the cellular levels of the GAPDH control mRNA were not significantly altered by any of the TPA treatment protocols used (Fig. 1 and 2A). We confirmed that TPA treatment in fact led to a down-regulation of PKC activity in human keratinocytes by determining the total cellular activity at different time points of treatment. By 4 h, the PKC activity was decreased to 22% of the control, and by 16 h, only 6% remained (Fig. 2C). Thus, in conclusion, pretreatment with TPA was more effective in inhibiting the cytochrome P-450IA1 induction response than simultaneous treatment of the cells with TPA and TCDF, lending support to the idea that TPA-induced down-regulation of PKC renders the cells insensitive to induction by dioxin receptor ligands.

To investigate further the role of PKC in induction of cytochrome P-450IA1 expression by TCDF, we next examined the effect of a number of protein kinase inhibitors on the induction response. Staurosporine and H-7 represent common examples of potent, albeit not very selective, inhibitors of PKC activity (reviewed in reference 4). Both of these compounds effectively inhibited cytochrome P-450IA1 mRNA induction by TCDF (Fig. 3A and B). Similarly, calphostin C, a novel PKC inhibitor with a higher specificity (67), produced strong inhibition of the TCDF induction response (Fig. 3C). None of these substances significantly altered the cellular levels of the GAPDH control mRNA (Fig. 3). Densitometric quantitation in two independent experiments of the autoradiographic P450IA1 mRNA signals, normalized to those of GAPDH, revealed that H-7 reduced the TCDF induction response to 26%, staurosporine reduced it to 16%, and calphostin C reduced it to 17%. Taken together, these data indicate that induction of cytochrome P-450IA1 expression by dioxin receptor ligands involves a PKC-dependent pathway.

The XRE sequence is sufficient to mediate sensitivity to TPA. To investigate the mechanism underlying the inhibitory effect of TPA, we transfected into human keratinocytes a reporter construct, pRNH11C (38), containing a fragment of the human cytochrome P-450IA1 gene (extending from nucleotides −1140 to +2435 relative to the transcription start site) fused to the CAT reporter gene. In contrast to the virtually undetectable levels of endogenous cytochrome P-450IA1 promoter activity in untreated cells (Fig. 1, left lane), transfection with the pRNH11C reporter construct resulted in significant levels of constitutive expression of CAT activity (Fig. 4A, left lane). Exposure of the transfected cells to TCDF resulted in up to a sixfold induction of CAT activity (Fig. 4A). When the transfected cells were coincubated with both TCDF and TPA for 24 h, it was not possible to detect any induction of CAT activity. Importantly, treatment of the transfected cells with TPA alone did not significantly alter the basal levels of CAT activity observed in the control transfected cells incubated with the solvent DMSO (Fig. 4A). We therefore wished to examine the possibility that TPA directly regulated the TCDF induction response.

To this end, we constructed minimal dioxin-responsive reporter vectors containing single or dimerized XRE sequences (corresponding to the XRE1 element of the rat cytochrome P-450IA1 promoter [25]) in front of a minimal (extending from nucleotide −81 relative to the transcription start site) herpes simplex virus TK promoter and a luciferase reporter gene (shown schematically in Fig. 4B). The luciferase activity was analyzed after transient transfection of these reporter constructs into keratinocytes. As shown in Fig. 4B, the promoter containing the single XRE motif (pTX.ONE) responded to TCDF treatment with about a twofold induction of luciferase activity, whereas the construct with either a direct (pTX.DIR) or an inverted (pTX.INV) repeat of the XRE motif showed a stronger TCDF induction response. Interestingly, the most potent (about sixfold) induction response was obtained with the pTX.INV reporter construct (Fig. 4B).

For all the tested XRE-TK promoter constructs, the TCDF induction response was completely abolished when the cells were simultaneously treated with TPA over an extended period of time (40 h; Fig. 4B). The levels of expression for reporter construct pT81, containing only the minimal TK promoter and the luciferase reporter gene, (53) were not affected by any of the treatments (Fig. 4B). Finally, the pTX promoter constructs were also activated by TCDF treatment following transient transfection into wild-type Hepa-1 hepatoma cells. However, no induction of transiently transfected pTX promoter constructs by TCDF was observed in the C4 mutant Hepa-1 subclone (40 and references therein), which expresses a dioxin receptor phenotype deficient in nuclear translocation in vivo and DNA-binding activity in vitro (data not shown). Thus, TCDF induction of these reporter constructs was mediated by the dioxin receptor.

The DNA-binding activity of the dioxin receptor is repressed by TPA treatment. To assess the effect of TPA on functional properties of the dioxin receptor in human keratinocytes, we analyzed the DNA-binding activity of the nuclear dioxin receptor following treatment of the keratinocytes with TPA. The DNA-binding activity of the dioxin receptor was monitored by an electrophoretic gel mobility shift assay with a synthetic XRE sequence as a specific probe. The dioxin

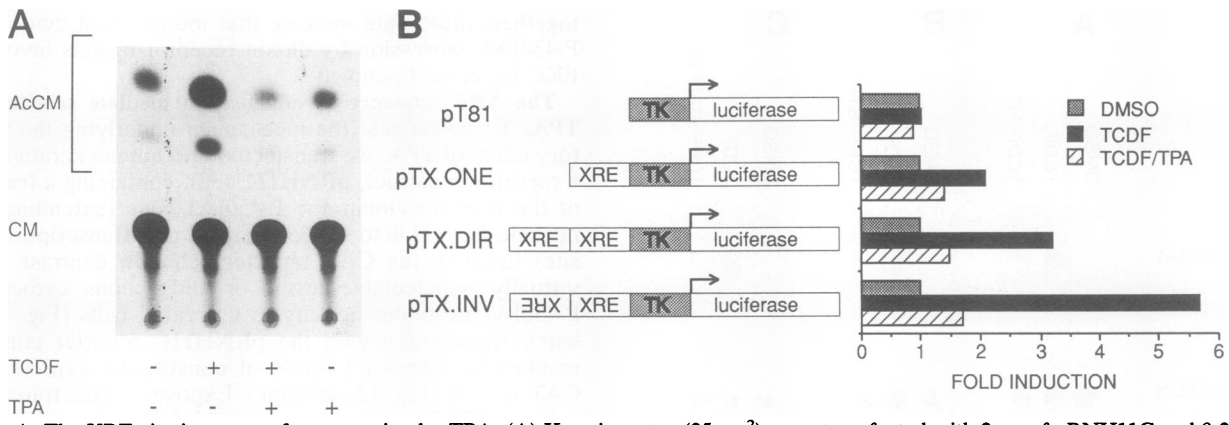

FIG. 4. The XRE site is a target for repression by TPA. (A) Keratinocytes (25 cm^2) were transfected with 2 μg of pRNH11C and 0.2 μg of the control *lacZ* reporter plasmid pON249. The transfected cells were treated for 24 h with 50 nM TCDF and/or 50 nM TPA as indicated. The amount of cell extract used for CAT assays was normalized on the basis of the level of β-galactosidase expression obtained from pON249, which was used to monitor transfection efficiency. A representative analysis by thin-layer chromatography of CAT activity is shown. The mobilities of ^{14}C-chloramphenicol (CM) and acetylated products (AcCM) are indicated. (B) Keratinocytes (10 cm^2) were transfected with 1 μg of TK promoter-containing dioxin-responsive reporter plasmids. Reporter construct pT81 lacks the XRE site, and the activity obtained from this construct was >100 times higher than the background activity, in agreement with previously published data (53). Luciferase activity was assayed after 40 h of treatment of the cells with DMSO or 50 nM TCDF in the absence or presence of 50 nM TPA as indicated. The cell extracts were normalized on the basis of protein content. Reporter activity is shown as fold induction over the DMSO control for each construct. The bars represent average values for six to eight independent transfection experiments.

receptor was activated to a nuclear DNA-binding form by exposure of the cells to TCDF for 1 h (26, 34). The in vivo-activated receptor generated a complex with the XRE probe that was not detected in nuclear extracts from control cells (Fig. 5A, compare lanes 1 and 2; the receptor-dependent complex is indicated by an R). In contrast, the protein-DNA complex generated by adenovirus major late transcription factor USF (31 and references therein) was unaffected by TCDF treatment and served as a control for the quality of the extracts (Fig. 5B, compare lanes 1 and 2). The specificity of the TCDF-induced XRE complex was characterized by DNA competition experiments. Formation of the dioxin receptor-dependent XRE complex could be abolished by the addition of a 100-fold molar excess of the unlabeled XRE sequence motif but not by the addition of an identical molar excess of an unrelated sequence (Fig. 5A, compare lanes 8 and 9). Moreover, the XRE complex generated by nuclear extracts from TCDF-treated human keratinocytes comigrated with that generated by the in vitro-activated cytosolic or in vivo-activated nuclear dioxin receptor from mouse hepatoma cells (data not shown).

When the keratinocytes were pretreated with TPA prior to the addition of TCDF, a marked decrease in the level of the dioxin receptor-dependent XRE complex was observed (Fig. 5A, compare lanes 6 and 7). However, the DNA-binding activity of the dioxin receptor was not significantly affected by a brief (1-h) simultaneous treatment of the cells with TCDF and TPA (Fig. 5A, compare lanes 2 and 3). The relative levels of the USF-dependent DNA-binding activity in nuclear extracts from the keratinocytes were not altered by any of the treatments used (Fig. 5B).

To exclude the possibility that pretreatment with TPA interfered with the nuclear translocation process of the TCDF-activated dioxin receptor, we prepared whole-cell extracts from untreated human keratinocytes or cells exposed to either TCDF alone or TPA prior to the addition of TCDF. As shown in Fig. 5C, the TPA-induced decrease in the formation of the dioxin receptor-dependent XRE complex was also observed in whole-cell extracts of treated cells (compare lanes 2 and 3), strongly arguing that pretreatment of the cells with TPA negatively affected the DNA-binding activity rather than the subcellular localization of the ligand-activated receptor. In control experiments, the USF-dependent DNA-binding activity in whole-cell extracts was not regulated by the TPA pretreatment protocol (Fig. 5D, compare lanes 1 to 3).

To control for effects of prolonged TPA treatment on the relative levels of the dioxin receptor, we next analyzed the receptor content in whole-cell extracts by immunoblot analysis with an anti-receptor antiserum. These antibodies specifically recognized the ~120-kDa receptor, which was present at equal levels in the extracts from untreated and TPA-treated cells (Fig. 5E, compare lanes 2 and 3).

A cytosolic catalytic activity restores DNA binding of the dephosphorylated dioxin receptor. We previously showed that treatment with potato acid phosphatase inhibited the DNA-binding activity of the ligand-activated dioxin receptor from hepatoma cells without affecting the ligand-binding activity or inducing any detectable degradation of the receptor (59). Therefore, we wished to investigate whether the dioxin receptor is phosphorylated in vivo. Mouse hepatoma cells were metabolically labeled with $^{32}P_i$, and the dioxin receptor was immunoprecipitated with an anti-dioxin receptor antiserum. As can be seen in Fig. 5F, a labeled protein of the expected size (~95 kDa) was detected by autoradiography. The precipitation of the receptor was specifically inhibited when the antiserum was preincubated with the dioxin receptor peptide used for immunization, while an unrelated peptide (i.e., a portion of the C-terminal domain of the glucocorticoid receptor) was ineffective. Next, we examined the effect of phosphatase treatment on the DNA-binding activity of the in vivo-activated dioxin receptor in nuclear extracts from human keratinocytes. The DNA-binding activity of the receptor was induced by exposing the keratinocytes to TCDF and visualized by electrophoretic gel mobility shift analysis (Fig. 6A, compare lanes 1 and 2). Incubation of the receptor with potato acid phosphatase, however, completely abolished this activity (Fig. 6A, com-

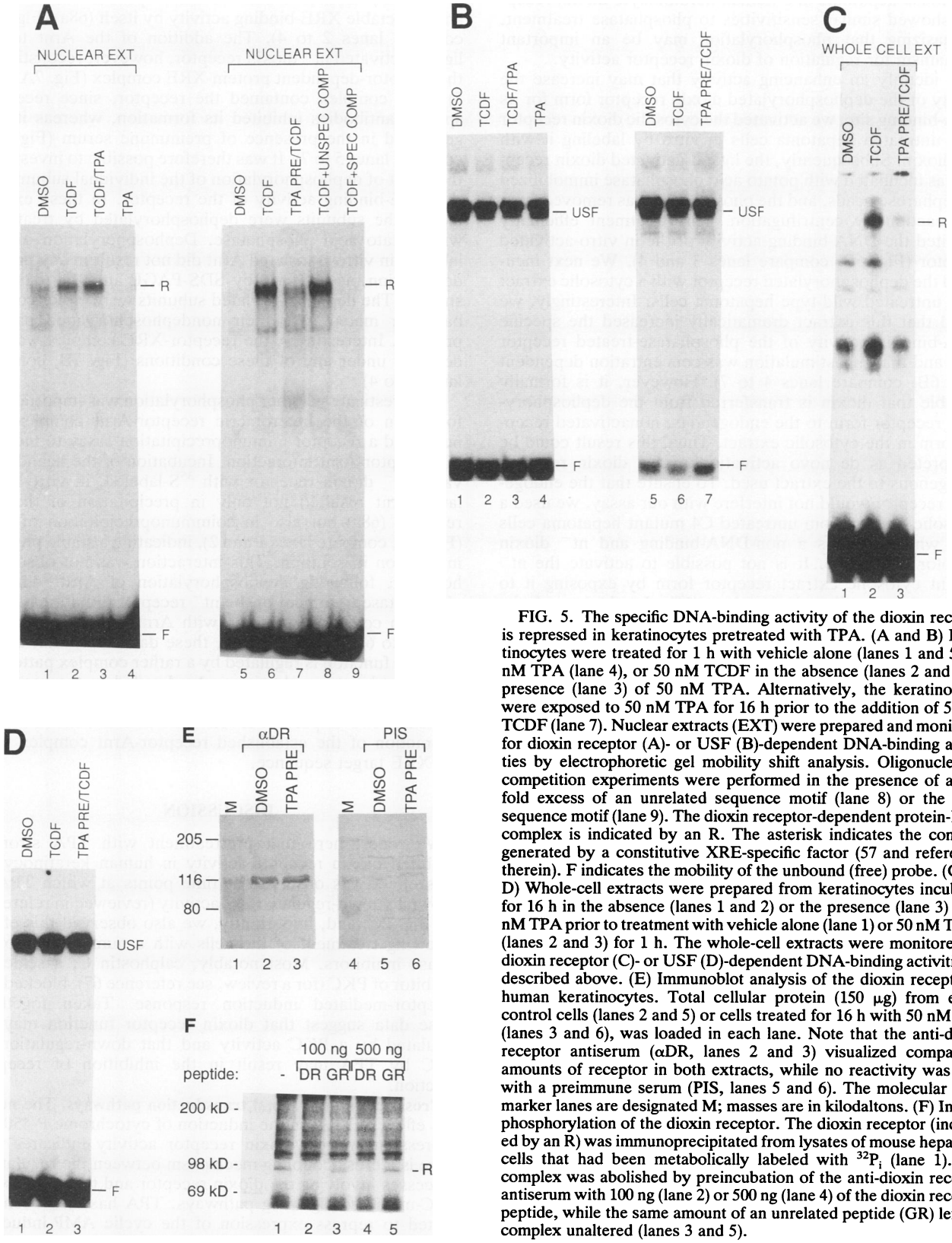

FIG. 5. The specific DNA-binding activity of the dioxin receptor is repressed in keratinocytes pretreated with TPA. (A and B) Keratinocytes were treated for 1 h with vehicle alone (lanes 1 and 5), 50 nM TPA (lane 4), or 50 nM TCDF in the absence (lanes 2 and 6) or presence (lane 3) of 50 nM TPA. Alternatively, the keratinocytes were exposed to 50 nM TPA for 16 h prior to the addition of 50 nM TCDF (lane 7). Nuclear extracts (EXT) were prepared and monitored for dioxin receptor (A)- or USF (B)-dependent DNA-binding activities by electrophoretic gel mobility shift analysis. Oligonucleotide competition experiments were performed in the presence of a 100-fold excess of an unrelated sequence motif (lane 8) or the XRE sequence motif (lane 9). The dioxin receptor-dependent protein-DNA complex is indicated by an R. The asterisk indicates the complex generated by a constitutive XRE-specific factor (57 and references therein). F indicates the mobility of the unbound (free) probe. (C and D) Whole-cell extracts were prepared from keratinocytes incubated for 16 h in the absence (lanes 1 and 2) or the presence (lane 3) of 50 nM TPA prior to treatment with vehicle alone (lane 1) or 50 nM TCDF (lanes 2 and 3) for 1 h. The whole-cell extracts were monitored for dioxin receptor (C)- or USF (D)-dependent DNA-binding activities as described above. (E) Immunoblot analysis of the dioxin receptor in human keratinocytes. Total cellular protein (150 μg) from either control cells (lanes 2 and 5) or cells treated for 16 h with 50 nM TPA (lanes 3 and 6), was loaded in each lane. Note that the anti-dioxin receptor antiserum (αDR, lanes 2 and 3) visualized comparable amounts of receptor in both extracts, while no reactivity was seen with a preimmune serum (PIS, lanes 5 and 6). The molecular mass marker lanes are designated M; masses are in kilodaltons. (F) In vivo phosphorylation of the dioxin receptor. The dioxin receptor (indicated by an R) was immunoprecipitated from lysates of mouse hepatoma cells that had been metabolically labeled with $^{32}P_i$ (lane 1). The complex was abolished by preincubation of the anti-dioxin receptor antiserum with 100 ng (lane 2) or 500 ng (lane 4) of the dioxin receptor peptide, while the same amount of an unrelated peptide (GR) left the complex unaltered (lanes 3 and 5).

pare lanes 2 and 3). Thus, the XRE-binding activities of both the mouse hepatoma and human keratinocyte dioxin receptors showed similar sensitivities to phosphatase treatment, emphasizing that phosphorylation may be an important mechanism for regulation of dioxin receptor activity.

To identify an enhancing activity that may increase the affinity of the dephosphorylated dioxin receptor form for its DNA-binding site, we activated the cytosolic dioxin receptor from untreated hepatoma cells in vitro by labeling it with [^{3}H]dioxin. Subsequently, the ligand-activated dioxin receptor was incubated with potato acid phosphatase immobilized on Sepharose beads, and the phosphatase was removed from the reaction by centrifugation. This treatment efficiently inhibited the DNA-binding activity of the in vitro-activated receptor (Fig. 6B, compare lanes 3 and 4). We next incubated the dephosphorylated receptor with a cytosolic extract from untreated wild-type hepatoma cells. Interestingly, we found that this extract dramatically increased the specific DNA-binding activity of the phosphatase-treated receptor form and that this stimulation was concentration dependent (Fig. 6B, compare lanes 4 to 7). However, it is formally possible that dioxin is transferred from the dephosphorylated receptor form to the endogenous, nonactivated receptor form in the cytosolic extract. Thus, this result could be interpreted as de novo activation of the dioxin receptor endogenous to the extract used. To ensure that the endogenous receptor would not interfere with our assay, we used a cytosolic extract from untreated C4 mutant hepatoma cells (32), which express a non-DNA-binding and nt$^-$ dioxin receptor phenotype. It is not possible to activate the nt$^-$ mutant cytosolic extract receptor form by exposing it to dioxin receptor ligands in vitro (16). Incubation of the dephosphorylated receptor with a cytosolic extract from untreated mutant nt$^-$ cells yielded results very similar to those obtained with a cytosolic extract from untreated wild-type cells (Fig. 6B, compare lanes 5 to 7 with lanes 8 to 10). This effect was temperature dependent, arguing that it reflects a catalytic activity (data not shown). Antibody inhibition experiments confirmed that the nt$^-$ mutant cytosolic extract stimulated the DNA-binding activity of the dioxin receptor (Fig. 6C, compare lanes 4 to 8). The addition of excess exogenous ATP did not have any effect on the reconstituting activity in the nt$^-$ cytosol (data not shown). However, this activity was inhibited by the addition of the slowly hydrolyzable ATPγS (Fig. 6D, compare lanes 5 and 6) (21 and references therein). In addition, the reconstituting activity was sensitive to the action of the PKC inhibitor calphostin C (Fig. 6E). Preincubation of the nt$^-$ cytosol with increasing concentrations of calphostin C for 20 min prior to addition of the dephosphorylated receptor resulted in a dose-dependent inhibition of the reconstituting activity (Fig. 6E, compare lanes 2 and 6). Similar results were obtained with H7 (data not shown). Interestingly, the reconstituting activity did not require the addition of PKC cofactors, suggesting that the PKC activity in the cells under the culture conditions used was sufficient for receptor function. In agreement with this suggestion, untreated in vitro-cultured cells have been reported to express substantial basal PKC activity (1, 10). It will therefore be important of further characterize this activity and to identify which functional step in the reconstitution process requires a PKC-like activity.

Distinct effects of dephosphorylation of the dioxin receptor and the Arnt subunits. The DNA-binding activity of the ligand-activated dioxin receptor is regulated by a physical interaction with the structurally related Arnt coregulator. Neither in vitro-translated Arnt nor the dioxin receptor present in Arnt-deficient nt$^-$ mutant hepatoma cells showed any detectable XRE-binding activity by itself (68a) (Fig. 7A, compare lanes 2 to 4). The addition of the Arnt to the ligand-activated nt$^-$ dioxin receptor, however, reconstituted the receptor-dependent protein-XRE complex (Fig. 7A, lane 5). This complex contained the receptor, since receptor-specific antibodies inhibited its formation, whereas it was generated in the presence of preimmune serum (Fig. 7A, compare lanes 5 to 7). It was therefore possible to investigate the effect of dephosphorylation of the individual subunits on the XRE-binding activity of the receptor. In these experiments, the subunits were dephosphorylated by treatment with potato acid phosphatase. Dephosphorylation of ^{35}S-labeled, in vitro-translated Arnt did not result in any protein degradation, as assessed by SDS-PAGE analysis (data not shown). The dephosphorylated subunits were either coincubated or mixed with their nondephosphorylated partner proteins. Interestingly, the receptor-XRE complex was not detected under any of these conditions (Fig. 7B, compare lanes 1 to 4).

To investigate whether phosphorylation was important for formation of the heteromeric receptor-Arnt complex, we next used a receptor immunoprecipitation assay to monitor the receptor-Arnt interaction. Incubation of the ligand-activated nt$^-$ dioxin receptor with ^{35}S-labeled, in vitro-translated Arnt resulted not only in precipitation of the nt$^-$ receptor (68a) but also in coimmunoprecipitation of Arnt (Fig. 7C, compare lanes 1 and 2), indicating a stable physical interaction in solution. This interaction was not observed, however, following dephosphorylation of Arnt, whereas phosphatase treatment of the nt$^-$ receptor did not have any effect on complex formation with Arnt (Fig. 7C, compare lanes 3 to 6). Taken together, these data imply that dioxin receptor function is regulated by a rather complex pattern of phosphorylation mechanisms. As detailed in the model in Fig. 7D, protein phosphorylation appears to be important at (at least) two important levels: interaction with Arnt and interaction of the established receptor-Arnt complex with the XRE target sequence.

DISCUSSION

We report here that pretreatment with TPA strongly inhibited dioxin receptor activity in human keratinocytes. This effect was observed at time points at which TPA is known to down-regulate PKC activity (reviewed in reference 46; Fig. 2C) and, importantly, we also observed this effect following treatment of the cells with a number of protein kinase inhibitors. Most notably, calphostin C, a selective inhibitor of PKC (for a review, see reference 67), blocked the receptor-mediated induction response. Taken together, these data suggest that dioxin receptor function may be regulated by a PKC activity and that down-regulation of PKC by TPA may result in the inhibition of receptor function.

Cross-coupling of signal transduction pathways. The negative effect of TPA on the induction of cytochrome P-450IA1 expression and on dioxin receptor activity indicates that there is a cross-coupling mechanism between the regulatory processes involving the dioxin receptor and those involving PKC-mediated signaling pathways. TPA has also been reported to repress expression of the cyclic AMP-inducible cytochrome P-450scc (P45011A1) gene (50). However, basal cytochrome P-450scc promoter activity is repressed by TPA, and the TPA and cyclic AMP regulatory pathways are

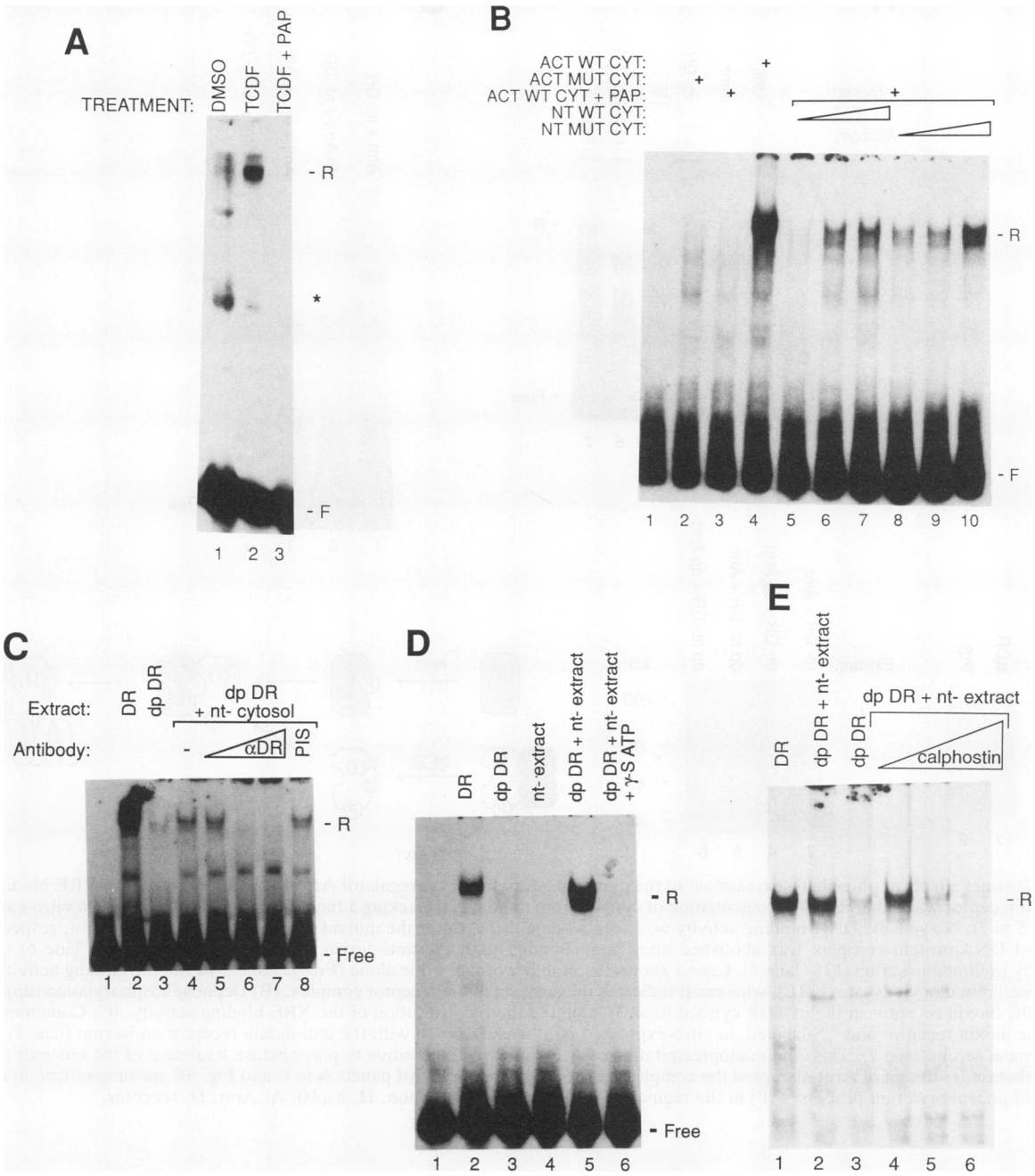

FIG. 6. A cytosolic activity enhances the DNA-binding activity of the dephosphorylated dioxin receptor. (A) Human keratinocytes were treated for 1 h with DMSO alone or 50 nM TCDF prior to the preparation of nuclear extracts. Subsequently, the in vivo-activated dioxin receptor (designated R) was incubated with potato acid phosphatase (PAP) immobilized on Sepharose (lane 3) prior to electrophoretic gel mobility shift analysis with a ^{32}P-labeled XRE probe as described in the legend to Fig. 5. The asterisk and F are as defined in the legend to Fig. 5. (B) Inactivation and reactivation of the dioxin receptor. Cytosolic extracts (CYT) from noninduced wild-type (WT) or mutant (MUT) hepatoma cells were activated in vitro (ACT) by exposure to [^{3}H]dioxin prior to electrophoretic gel mobility shift analysis. The in vitro-activated wild-type receptor was treated with potato acid phosphatase prior to incubation with increasing concentrations (2.5 to 10 μl; ~2 μg of protein per μl) of cytosolic extracts from either untreated (NT) wild-type (lanes 5 to 7) or untreated mutant (lanes 8 to 10) hepatoma cells. The mobility of the free probe (designated F) in the absence of any added protein is shown in lane 1. (C) The reactivated dioxin receptor is recognized by the anti-dioxin receptor antiserum. An experiment similar to that described for panel B was performed; however, after reactivation, the receptor (lane 4) was incubated with increasing amounts of the anti-dioxin receptor antiserum (αDR; 1, 2.5, and 5 μl of a 1:10-fold dilution [lanes 5 to 7, respectively]), which disrupted the receptor-XRE complex. Preimmune serum (PIS) had no effect on the DNA-binding activity (lane 8). DR, input wild-type receptor; dp DR, phosphatase-treated DR. (D) Reactivation of the dioxin receptor is inhibited by ATPγS. The dephosphorylated receptor was incubated with nt$^-$ cytosol to reactivate the receptor (lane 5). This process was effectively inhibited by the addition of 400 μM ATPγS (lane 6). (E) The PKC inhibitor calphostin C inhibits the reactivation of the dioxin receptor. The dephosphorylated receptor (lane 3) was incubated with nt$^-$ cytosol that had been preincubated in the absence (lane 2) or presence of 0.1 (lane 4), 0.4 (lane 5), or 1 μM (lane 6) calphostin.

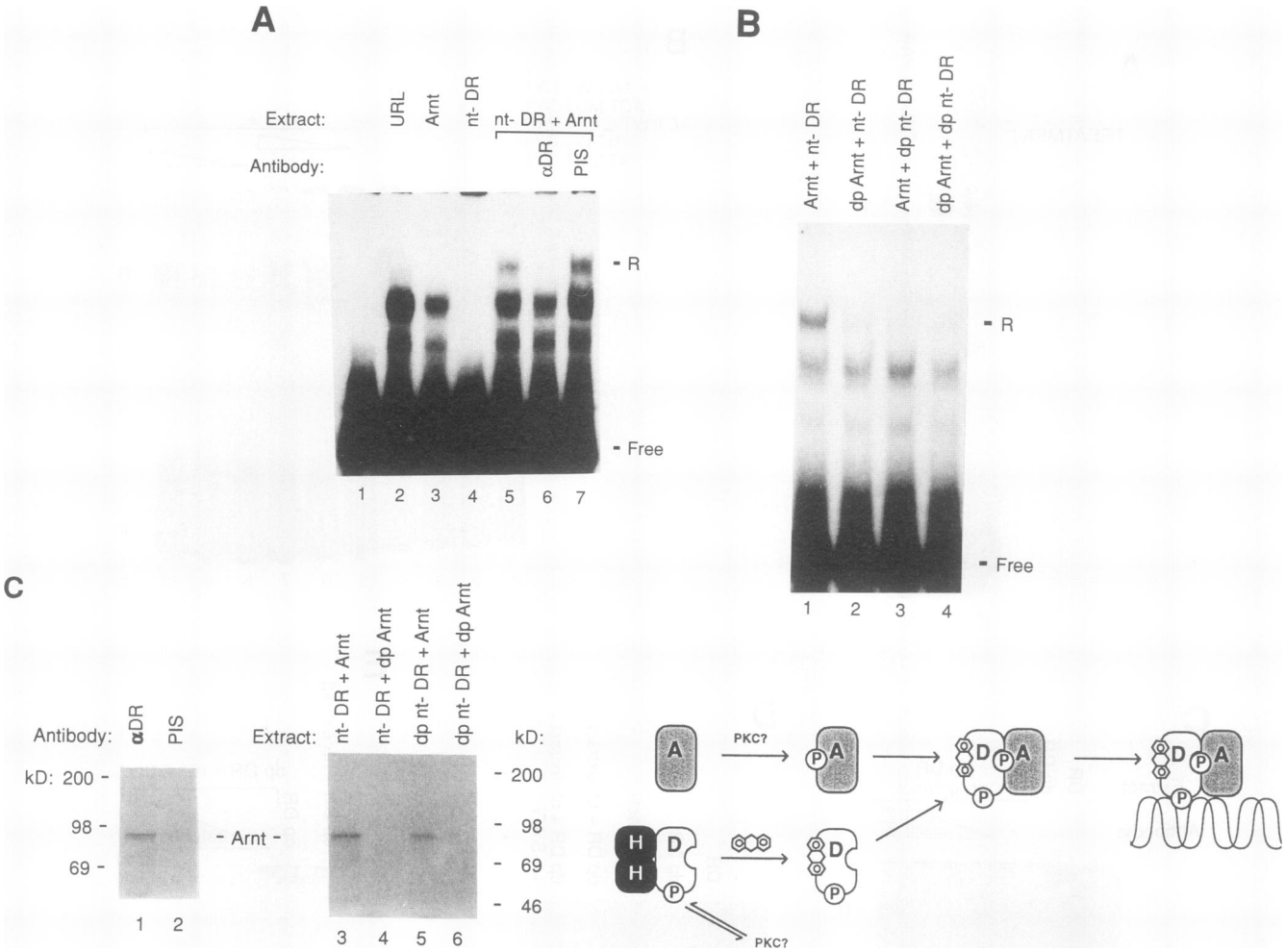

FIG. 7. Distinct effects of phosphatase treatment of the dioxin receptor and its coregulator Arnt. (A) Restoration of the XRE-binding form of the dioxin receptor was achieved after coincubation of cytosol from mutant cells lacking a functional Arnt (nt^- DR) and in vitro-expressed Arnt (lanes 5 to 7). No specific DNA-binding activity was seen when either Arnt or the mutant cytosol was used (lane 3 or 4, respectively). The restored DNA-protein complex was abolished after preincubation with the anti-dioxin receptor antiserum (αDR; lane 6) but was unaffected by preimmune serum (PIS: lane 7). Lane 1 shows the mobility of the probe alone (Free). Lane 2 shows the binding activity of the unprogrammed reticulocyte lysate (URL), whereas R indicates the position of the receptor complex. (B) Dephosphosphorylation (dp) of Arnt (lane 2) or the dioxin receptor in the nt^- DR cytosol (lane 3) inhibited the reconstitution of the XRE-binding activity. (C) Coimmunopecipitation of the dioxin receptor and ^{35}S-labeled, in vitro-expressed Arnt was observed with the anti-dioxin receptor antiserum (lane 1) but not with preimmune serum (lane 2). This coimmunoprecipitation of Arnt appeared insensitive to phosphatase treatment of the receptor (lane 5), while phosphatase treatment of Arnt disrupted the complex (lane 6). (D) The results of panels A to C and Fig. 5F are summarized in a model of the role of phosphorylation processes (P) in the regulation of dioxin receptor action. H, hsp90; A, Arnt; D, receptor.

mediated through independent sequence elements of this gene (50). Thus, the mechanism appears to be distinct from that negatively regulating the inducibility of the cytochrome P-450IA1 gene.

In this context, it is important to point out that the TPA inhibition in human keratinocytes of induction by the dioxin receptor of both the endogenous cytochrome P-450IA1 gene and the minimal XRE-TK promoter constructs correlates well with the reported negative effect of TPA on cytochrome P-450IA1 activity in the mouse epidermis (60). In the mouse epidermis, TPA treatment induces a hyperproliferative response (61). It is therefore tempting to speculate that the signal transduction cross-coupling mechanism described here may serve to protect the proliferating cells from high levels of cytochrome P-450IA1 activities which, in turn, have been correlated with an increased risk of metabolic activation of xenobiotics to mutagenic forms (reviewed in reference 30). In support of this hypothesis, expression of the cytochrome P-450IA1 gene is not inducible by dioxin receptor ligands in undifferentiated, proliferative keratinocytes (3).

In addition to repression of the cytochrome P-450IA1 induction response, TPA has been shown to negatively regulate the function of the nuclear glucocorticoid, thyroid hormone, and retinoic acid receptors on certain target promoters (reviewed in reference 65). This cross-coupling phenomenon, however, appears to involve interference via a direct protein-protein interaction between the nuclear receptor and the two components of the AP-1 transcription factor, c-Jun and c-Fos (43, 66, 71). Moreover, although the glucocorticoid receptor becomes hyperphosphorylated upon hormone treatment, this phosphorylation event involves

mainly amino acids in the amino-terminal transactivation domain of the receptor, and the DNA-binding activity of the glucocorticoid receptor appears to be independent of phosphorylation (5, 39 and references therein). Similar observations have been made with the progesterone receptor (12, 19). From the present work, we conclude that the mode of regulation of steroid hormone receptors by phosphorylation appears to be different from that of the dioxin receptor and that the regulation of dioxin receptor function by TPA and the apparent involvement of PKC in this process may represent a distinct and novel mechanism of cross-coupling of the signal transduction pathways of nuclear receptor ligands and phorbol esters.

Inhibition of dioxin receptor activity by TPA: a link to protein phosphorylation. Interestingly, treatment of human keratinocytes with TPA resulted in negative regulation of the DNA-binding activity of the dioxin receptor. Although it is formally possible that this effect may be attributed to an altered cellular partitioning of the dioxin receptor (resulting in a predominantly cytoplasmic localization), we believe this possibility to be very unlikely, since the DNA-binding activity of the receptor was also down-regulated in whole-cell extracts of cells treated with TCDF in the presence of TPA.

It is noteworthy that dephosphorylation of the ligand-activated dioxin receptor by phosphatase treatment in vitro also results in efficient inhibition of the DNA-binding activity of the receptor (59) (Fig. 6A). In this context, our results imply that PKC activity may modulate the DNA-binding activity, rather than the transactivating activity, of the receptor. In support of this model, it is possible to restore the DNA-binding activity of the in vitro-dephosphorylated receptor by incubation with a cytosolic activity extracted from untreated hepatoma cells. During the review process of the manuscript, it was reported that the DNA-binding activity of the dioxin receptor is repressed in mouse liver and hepatoma cells by prolonged treatment with TPA and staurosporine, respectively (11, 54). Although these authors did not identify the XRE as the target of negative transcriptional regulation by TPA, their data, together with the present results, strongly suggest a critical role of PKC in dioxin receptor function.

Distinct phosphorylation events have been described to selectively regulate either the DNA-binding or the transactivating activity of a number of transcription factors (reviewed in reference 41). For instance, the DNA-binding activity of the 67-kDa serum response factor appears to be modulated by phosphorylation by casein kinase II (27, 48). Conversely, phosphorylation of the CREB factor enhances its transcriptional activity rather than its DNA-binding activity (70). It should be pointed out, however, that transcription factor activity can be controlled by protein kinase pathways that do not involve direct phosphorylation of the transcription factor itself. Thus, both the NF-κB and the AP-1 (c-Fos and c-Jun) transcription factors appear to be derepressed by phosphorylation of their corresponding inhibitory proteins, IκB (28) and IP-1 (2). In addition, the DNA-binding activity of c-Jun is positively regulated by PKC-dependent dephosphorylation (7).

We showed here that the DNA-binding activity of the dioxin receptor from nt$^-$ cells, lacking functional Arnt, could be reconstituted by the addition of in vitro-translated Arnt. Phosphorylation of both the dioxin receptor subunit and Arnt appeared to be required for efficient DNA-binding activity. However, our data indicate that phosphorylation processes may modulate dioxin receptor function by controlling two distinct and important steps during the receptor activation process (outlined in Fig. 7D). Thus, although no DNA-binding activity was detected after phosphatase treatment of the nt$^-$ dioxin receptor, the receptor could still physically interact with Arnt. Dephosphorylation of Arnt, on the other hand, resulted in a loss of heterodimer formation.

Mechanism of activation of the dioxin receptor. The DNA-binding activity of the dioxin receptor appears to be regulated by several distinct mechanisms. First, in the absence of a ligand, the DNA-binding activity of the dioxin receptor is inhibited by the formation of a nonproductive, heteromeric complex with hsp90 (69). A similar negative regulatory strategy appears to control the activity of the glucocorticoid receptor (17, 56). Second, the ligand-induced activation process of the dioxin receptor involves heterodimerization of the receptor with Arnt to yield an ~200-kDa form of active receptor with a high affinity for the DNA target sequence. It is conceivable that formation of the 200-kDa form of active receptor is initiated by the release of hsp90 and that this event is a critical target of regulation. We have demonstrated that the dioxin receptor subunit is phosphorylated in control cells, and it will therefore be interesting to investigate whether this subunit itself, Arnt or, in fact, both proteins are subject to changes in their phosphorylation status prior or subsequent to the ligand-induced release of hsp90 and the factor dimerization event. Since PKC action represents a starting point of a cascade of phosphorylation events, it will now be important to characterize further the cytosolic activity that restores the DNA-binding activity of the dephosphorylated dioxin receptor form and to determine at which regulatory level it modulates dioxin receptor function.

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