Inhibition of the Specific DNA Binding Activity of the Dioxin Receptor by Phosphatase Treatment*

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The dioxin receptor stimulates transcription of the cytochrome P-450IA1 gene in response to dioxin. Exposure of the intracellular dioxin receptor to dioxin leads to a rapid conversion of the receptor from a latent form to a DNA binding species which specifically recognizes dioxin-responsive positive control elements in vitro. In this report, we show that treatment of in vivo or in vitro ligand-activated receptor with potato acid phosphatase significantly reduced or abolished its specific DNA binding activity. This effect was inhibited in the presence of sodium phosphate. In control experiments, the ligand-activated glucocorticoid receptor was not inactivated by phosphatase treatment. Moreover, phosphatase treatment did not induce any detectable degradation of covariantly labeled dioxin receptor, arguing against protease contamination as a cause for receptor inactivation. Finally, phosphatase-inactivated dioxin receptor exhibited bona fide levels of ligand binding activity. Taken together, these data suggest that phosphorylation may regulate the DNA binding activity of the ligand-occupied dioxin receptor.

Dioxin1 and structurally related environmental contaminants induce transcription of a number of genes encoding drug-metabolizing enzymes such as cytochrome P-450IA1. This activation response is mediated by the intracellular dioxin receptor which, upon exposure to ligand, is translocated to the nucleus in vivo and converted from a latent species to a DNA binding form in vitro (for review, see Nebert and Gonzalez, 1987). Both nuclear translocation of the dioxin receptor (Denison et al., 1988) and receptor-dependent activation of the cytochrome P-450IA1 promoter (Israel et al., 1985) are induced rapidly and can occur in the presence of protein synthesis inhibitors. Following ligand-dependent activation, the dioxin receptor recognizes in vitro dioxin-inducible positive control elements (XREs) which modulate transcription of target promoters (Fujisawa-Sehara et al., 1987; 1988; Denison et al., 1988; Hapgood et al., 1988; Neubold et al., 1990; Paulson et al., 1990).

It is possible to reconstitute ligand-dependent activation of the dioxin receptor to a DNA binding form in vitro (Fujisawa-Sehara et al., 1988; Nemoto et al., 1990; Cuthill et al., 1991). Under these conditions, the XRE binding activity of the dioxin receptor is regulated by ligand in a manner that directly reflects the affinity of the ligand for the receptor (Cuthill et al., 1991). We have recently shown that conversion of the latent form of dioxin receptor to an XRE binding species can be achieved by release from an inhibitor protein, possibly the 90-kDa heat shock protein, hsp90 (Wilhelmsson et al., 1990). To further explore the mechanism of regulation of dioxin receptor activity, we have investigated the effect of phosphatase treatment on ligand and DNA binding activities of the receptor. We show that a dephosphorylated form of dioxin receptor exhibited control levels of ligand binding activity. However, the XRE binding activity could be significantly reduced or abrogated by phosphatase treatment of either crude in vivo or in vitro ligand-activated forms of receptor. Ligand cross-linking experiments demonstrated that the phosphatase-treated, non-DNA binding receptor form was a physically intact 95-kDa protein. Thus, we propose that specific DNA binding activity of the dioxin receptor may require receptor phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cells and Preparation of Cellular Extracts—**Wild-type Hepa 1c1c7 hepatoma cells (Hankinson, 1979), obtained from Dr. Oliver Hankinson (UCLA), were used throughout. Cells were grown and treated with 1 μM dioxin for 1 h as described (Cuthill and Poellinger, 1988; Wilhelmsson et al., 1990). Nuclear extracts from untreated or dioxin-treated cells were prepared as described by Dignam et al. (1983) with minor modifications (Wilhelmsson et al., 1990). Cytosolic extracts from untreated cells were prepared as described previously (Wilhelmsson et al., 1990).

**Phosphatase Treatment of Nonactivated and Ligand-activated Dioxin Receptor—**The cytosolic form of dioxin receptor in Hepa 1c1c7 cells was activated to a DNA binding form by ligand treatment in vitro (Nemoto et al., 1990). In vivo-activated dioxin receptor was recovered in a nuclear extract from Hepa 1c1c7 cells treated with dioxin as described above. Enzymatic dephosphorylation of dioxin receptor was performed by incubation of the receptor with the indicated concentrations of potato acid phosphatase (Boehringer Mannheim) at 25 °C for 20 min in 20 mM Tris-HCl, pH 7.2, 1 mM EDTA 10% (v/v) glycerol, and 2 mM 2-hydroxyethylmercapto-ethanol.

**DNA Binding Assay—**The specific DNA binding activity of the dioxin receptor was monitored by a gel mobility shift assay employing as specific probe a 3'-P-labeled, double-stranded oligonucleotide XRE (5'-AATTCGAGG TCTTTTAC CAATTCGG GC-3') spanning the dioxin-responsive XRE1 element (Fujisawa Sehara et al., 1987) from positions -1,026 to -999 relative to the transcription start site of the rat cytochrome P-450IA1 gene. The DNA binding reactions were performed at 30 °C for 30 min, and bound and free DNA was electrophoretically separated under conditions described previously (Hapgood et al., 1988; Nemoto et al., 1990). A similar gel

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1The abbreviations and trivial names used are: dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic response element; GRE, glucocorticoid response element; [3H]dioxin, [1,6-3H]2,3,7,8-tetrachlorodibenzo-p-dioxin; [22]methyl-dioxin, 2-azido-3-[22]methyl-7,8-dibromodibenzo-p-dioxin; SDS, sodium dodecyl sulfate.

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mobility shift assay was used to characterize the DNA binding activity of purified glucocorticoid receptor. In these experiments, a radio-labeled, double-stranded oligonucleotide \(5'\text{-AGCTTCCTAGAGATCTGTACAGATCGAATT-3'}\) spanning the palindromic GRE of the rat tyrosine aminotransferase gene (Jantzen et al., 1988) was used as specific probe. In indicated DNA binding competition experiments, a synthetic octamer element from the BCL1 immunoglobulin promoter (Poellinger et al., 1989) was used as an unrelated sequence motif.

Ligand Binding Experiments—The cytosolic dioxin receptor was labeled by incubation of crude cytosol for 3 h at 25 °C with 10 nM \([3H]dioxin\) (Chemsyn, Lenexa, KS) in the absence or presence of a 200-fold molar excess of the unlabeled high affinity receptor ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin. A hydroxylapatite adsorption assay (Poellinger et al., 1985) was used to quantitate the levels of protein-bound \([3H]dioxin\). Specific binding activity was determined by subtracting the binding in the presence of an excess of the unlabeled ligand (nonspecific binding) from the levels of totally bound.

Photoaffinity Labeling of the Dioxin Receptor—\([125I]\)Dioxin was synthesized and purified essentially as described (Poland et al., 1986), and the dioxin receptor was covalently labeled by incubation of crude cytosol from untreated Hepa 1c1c7 cells with \([125I]\)-dioxin and subsequent UV irradiation as described by Pendew (1988).

Purification and Immunoblot Analysis of Glucocorticoid Receptor—Glucocorticoid receptor was purified from rat liver (Wrangle et al., 1986) and treated with potato acid phosphatase as described above for the dioxin receptor. Following electrophoretic separation on SDS-polyacrylamide gels, proteins were electrophoretically transferred to nitrocellulose filters. Immunocomplexes were visualized by incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulins.

Safety Precautions—In cell culture and biochemical experiments involving the use of dioxin, special handling procedures were employed (Wilhelmsson et al., 1990, and references therein). Contaminated materials were disposed of by high temperature incineration.

RESULTS

Inactivation of the Dioxin Receptor by Phosphatase Treatment—The dioxin receptor was activated to a nuclear, DNA binding form by treatment of Hepa 1c1c7 cells with dioxin (Fujisawa-Sehara et al., 1988; Hapgood et al., 1989). The ability of the receptor to bind to DNA was monitored by a gel mobility shift assay using as probe a synthetic XRE sequence. This XRE probe matches the XRE1 sequence of the rat cytochrome P-450IA1 gene (Fujisawa-Sehara et al., 1987). Fig. 1A shows the dioxin-induced, receptor-dependent XRE binding activity (lane 1, receptor-XRE complex indicated by an arrow) in nuclear extract from treated cells. Binding could be abolished by the addition of excess unlabeled probe to the binding reaction, but not by addition of an excess of an unrelated sequence motif (compare lanes 1–3). We next added potato acid phosphatase to the extract containing in vivo activated receptor to remove O-linked phosphates. This treatment resulted in a concentration-dependent decrease in formation of the specific receptor-XRE complex (Fig. 1B, compare lanes 1–4).

Similar results were obtained when we examined the effect of phosphatase treatment on the XRE binding activity of the cytosol in vitro-activated dioxin receptor. In vitro activation of the receptor was achieved by incubation of crude Hepa 1c1c7 cytosol with 10 nM dioxin for 3 h at 25 °C prior to assembly of the DNA binding reaction (Nemoto et al., 1990; Cuthill et al., 1991). We confirmed that the in vitro-induced XRE binding activity was sequence-specific by competition with unlabeled oligonucleotides. In the presence of an excess of the unlabeled XRE sequence, dioxin-induced XRE complex formation was abrogated, whereas no effect on XRE binding was observed in the presence of an excess of an unrelated sequence motif (Fig. 2A, compare lanes 1–3). The relative mobility of the XRE-specific complex generated by in vitro-
activated receptor (Fig. 2A, lane 1) is identical with that generated by the in vivo-activated receptor (Wilhelmsson et al., 1990). Moreover, the DNA binding properties of the in vitro ligand-activated receptor form are indistinguishable from those of the in vivo-activated nuclear receptor (Cuthill et al., 1991), arguing that all the components required for receptor-XRE interaction are present in the cytosolic extract. Phosphatase treatment nearly abolished the XRE binding activity of the in vitro-activated receptor. This effect was, however, inhibited by addition of 10 mM phosphate (Fig. 2B, compare lanes 1-3).

These results could be explained if our conditions for dephosphorylation of the receptor simply resulted in accidental degradation of the receptor protein. To address this issue, the dioxin receptor was covalently labeled with [125I]dioxin prior to incubation with phosphatase. The [125I]dioxin-labeled receptor was treated with or without phosphatase and analyzed in parallel for ligand binding and DNA binding activities by nondenaturing gel electrophoresis. Although the phosphatase-treated, [125I]dioxin-labeled receptor did not generate a complex with the 32P-labeled XRE probe (Fig. 3A, lane 2), the high specific activity of the iodinated ligand permitted us to visualize the ligand-receptor complex by autoradiography and to qualitatively compare the relative mobilities of untreated and phosphatase-treated [125I]dioxin-receptor complexes, respectively, following nondenaturing gel electrophoresis in the absence of the radiolabeled XRE probe. Under these conditions, a significant amount of the [125I]dioxin-receptor complex appeared to be aggregated and was recovered in the application slots of the gel (Fig. 3A, lanes 3 and 4). However, the untreated and phosphatase-treated [125I]dioxin-receptor complexes which penetrated the gel exhibited identical relative mobilities and very similar levels of ligand binding activity (compare lanes 3 and 4). Importantly, these ligand-receptor complexes were detected in the very same region of the gel as the 32P-labeled XRE complex generated by the phosphatase-unsubtracted [125I]dioxin-receptor complex (compare lanes 1, 3, and 4).

We next examined the M.sub, of untreated and phosphatase-treated forms of [125I]dioxin-labeled receptor by SDS-polyacrylamide gel electrophoresis. Although the XRE binding activity of the treated form of receptor was virtually undetectable (Fig. 3A, lane 2), both the untreated and treated forms migrated as 95-kDa proteins on SDS-polyacrylamide gels (Fig. 3B). Moreover, no change in apparent amounts of the [125I]dioxin-receptor complex was detected by this assay after phosphatase treatment. Thus, these experiments strongly argue against simple protein degradation (possibly due to contaminating proteases) as the cause of phosphatase-induced loss in XRE binding activity of the receptor.

A number of DNA binding transcription factors appear to be differentially modified by proteases, kinases, or phosphatases in a DNA-bound state as opposed to an unbound configuration (Prywes et al., 1988; Schreiber et al., 1988; Jackson et al., 1990). In the case of the ligand-activated dioxin receptor, preformation of the receptor-XRE complex prior to addition of phosphatase did not significantly protect the receptor from enzyme-dependent inactivation (data not shown). Thus, the configuration of either unbound or DNA-bound receptors appears to permit access of the enzyme to key phosphorylated residues.

The Specific DNA Binding Activity of the Glucocorticoid Receptor Is Not Altered by Phosphatase Treatment—In control experiments, we examined the effect of phosphatase treatment on the DNA binding activity of the glucocorticoid receptor which represents a prototypical ligand-activated intracellular receptor (reviewed in Gustafsson et al., 1987; Evans, 1988; Beato, 1989). Importantly, the glucocorticoid receptor has been shown to be phosphorylated in a hormone-dependent manner at amino acids within its DNA binding domain (Hoeck and Groner, 1990). Moreover, the rat glucocorticoid receptor is very sensitive to proteolysis, generating several distinct protease-induced degradation products (Carlstedt-Duke et al., 1987 and references therein), and thus serves as a control of the possible level of protease contamination in the phosphatase preparation used.

In agreement with the results obtained with affinity-labeled dioxin receptor (Fig. 3B), treatment of purified, ligand-activated glucocorticoid receptor with phosphatase resulted in no change in the apparent M.sub, (94 kDa) or amount of the receptor, as assessed by immunoblot analysis of untreated and treated receptor forms using monoclonal antibodies directed against either the amino terminus (Fig. 4A) or the centrally located DNA binding domain (data not shown) of the receptor. Furthermore, the specific DNA binding activity of the glucocorticoid receptor toward a synthetic GRE probe was not altered by phosphatase treatment (Fig. 4B). Thus, although the glucocorticoid and dioxin receptors both represent ligand-activated DNA binding factors, the effect of phosphatase appears to be specific for the dioxin receptor.

Dephosphorylated Dioxin Receptor Exhibits Differential Ligand and XRE Binding Activities—To address the mechanism underlying the phosphatase-induced reduction in XRE binding activity of the dioxin receptor, we treated the ligand-unoccupied form of cytosolic receptor with phosphatase. This form or receptor shows very low levels of specific XRE binding activity in gel mobility shift experiments (Nemoto et al., 1990; Fig. 5B, lane 2). As schematically diagrammed in Fig. 5A, the
The glucocorticoid receptor is not inactivated by phosphatase treatment. Ligand-activated glucocorticoid receptor was purified to apparent homogeneity from rat liver cytosol. A, effect of phosphatase treatment on the specific DNA binding activity of the glucocorticoid receptor. Purified receptor was treated as described under B, indicated on top of the autoradiograph, and analyzed by a gel mobility shift assay for specific GRE binding activity (lanes 5–8). Lane 1 shows the mobility of unbound (Free) GRE probe without any added protein. In control reactions, untreated receptor was incubated with the labeled and phosphatase-treated receptor. Gel mobility shift assay for specific GRE binding activity (lanes 5–8). Lane 1 indicates the presence of an excess of phosphate. Moreover, phosphatase and phosphatase/pAP activity was selectively inhibited ligand-induced activation of the dioxin receptor. Cytosol (about 2 mg of protein/ml) was prepared from untreated Hepa 1c1c7 cells and incubated in the absence of any. A schematic diagram of the protocol used for phosphatase treatment prior to receptor-ligand interaction and ligand-dependent activation of the dioxin receptor. Cytosol (about 2 mg of protein/ml) was prepared from untreated Hepa 1c1c7 cells and incubated in the absence of any 0.02 unit of potato acid phosphatase/μl (PAP) as indicated. Treated material was analyzed for ligand and DNA binding activities. See "Experimental Procedures" for details. B, pretreatment with phosphatase inhibits ligand-induced activation of the dioxin receptor. Untreated (lanes 2 and 3) and phosphatase-treated (lanes 4 and 5) cytosol was incubated in the absence (lanes 2 and 4) or in the presence (lanes 3 and 5) of 10 nM [3H]dioxin. Untreated (lanes 2 and 3) and phosphatase-treated (lanes 4 and 5) cytosol was incubated in the absence (lanes 2 and 4) or in the presence (lanes 3 and 5) of 10 nM [3H]dioxin prior to the addition of radiolabeled XRE probe. The specific XRE binding activity was monitored by gel mobility shift analysis. The receptor-induced XRE complex is indicated by an arrow. Lane 1 shows the mobility of the XRE probe in the absence of any added protein. C, pretreatment with phosphatase does not alter the ligand binding activity of the dioxin receptor. Untreated (columns 1 and 2) and phosphatase-treated (columns 3 and 4) cytosol was incubated with 10 nM [3H]dioxin in the absence (columns 1 and 3) or presence (columns 2 and 4) of a 200-fold excess of the unlabelled dioxin analogue 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Ligand binding activity was determined by an hydroxylapatite adsorption assay. Binding in the presence of an excess of unlabelled competitor represents nonspecific ligand binding activity. The columns represent the average value from duplicate determinations.

**DISCUSSION**

**Regulation of the DNA Binding Activity of the Dioxin Receptor**—Both XRE binding activity and nuclear localization of the dioxin receptor are modulated by ligand (Denison et al., 1988; Fujisawa-Sehara et al., 1988; Happood et al., 1989). In this report, we show that the XRE binding activity of both cytosolic and nuclear ligand-occupied dioxin receptor is significantly reduced or abolished by treatment with potato acid phosphatase. Inactivation of the receptor was inhibited in the presence of an excess of phosphate. Moreover, phosphatase treatment of the cytosolic, latent form of receptor selectively inhibited ligand-induced conversion to an XRE binding species but did not alter its ligand binding activity. In agreement with these results, the ligand binding activity of the dioxin receptor has been reported to be insensitive to treatment with alkaline phosphatase (Denison et al., 1989). Finally, no proteolysis of either dioxin receptor or purified glucocorticoid receptor was detected upon incubation with phosphatase, suggesting that the observed effects are not due to receptor dephosphorylation and not protein degradation due to contaminating proteases. Taken together, these results imply that one or more O-linked phosphorylations may be necessary for generating the dioxin receptor species with high affinity for the XRE target sequence. In this context, it is interesting to note that a conserved pattern of charge heterogeneity has recently been observed for both the mouse and rat dioxin receptors (Perdew and Hollenback, 1990).

Several steroid hormone receptors, including the glucocorticoid receptor, have been demonstrated to be phosphorylated at multiple sites (reviewed in Auricchio, 1989; Denner et al., 1990). In the case of the glucocorticoid and estrogen receptors, it has been suggested that phosphorylation may be important for hormone binding activity (Mendel et al., 1986; Auricchio et al., 1987; Migliaccio et al., 1989). Moreover, phosphorylation of the amino-terminal trans-activating domain of the glucocorticoid receptor is strongly increased by hormone treatment (Hoeck and Groner, 1990), indicating that this modification may be important for the function of the receptor as a transcriptional regulator. In contrast, the nonspecific DNA binding activity of the glucocorticoid receptor has been reported to be independent of phosphorylation (Tienrungroj et al., 1987), in agreement with our observation that binding of the receptor to a GRE sequence was not altered by phosphatase treatment. Thus, phosphorylation appears to affect different functional properties of ligand-activated intracellular receptor proteins.
Phosphorylation of Transcription Factors—Phosphorylation has been implicated in transcriptional activation by a number of transcription factors. The effect of protein phosphorylation on functional properties of these factors appears to fall into two classes. For instance, protein phosphorylation which leads to increased DNA binding activity has been shown for the serum response factor (Prywes et al., 1988; Manak et al., 1990), and the adenovirus-induced E4F (Raychadhuri et al., 1989) and E2F factors (Bagchi et al., 1989). On the other hand, phosphorylation of the heat shock (Sorger and Pelham, 1988), CREB (Yamamoto et al., 1988), GAL4 (Mylin et al., 1990), and Oct-2 (Tanaka and Herr, 1990) transcription factors correlates with transcriptional activation, rather than DNA binding activity. In summary, phosphorylation has been implicated in transcriptional activation by a mechanism. Thus, it will be interesting to establish which leads to increased DNA binding activity has been shown for the serum response factor (Prywes et al., 1988), CREB (Yamamoto et al., 1988), and Oct-2 (Tanaka and Herr, 1990) transcription factors. Our present data raise the possibility that the dioxin receptor is also regulated by a mechanism. Thus, it will be interesting to establish which sites on the receptor are modified and to see which protein kinases are involved. Finally, the question whether protein phosphorylation itself is regulated by dioxin must be addressed.

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