Ah Receptor Phosphorylation: Localization, Regulation and Potential Role in the Activation to a DNA-binding State

Matthew J. Mahon and **Thomas A. Gasiewicz**
Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, New York 14642 U.S.A.

Introduction

The aryl hydrocarbon receptor (AhR) mediates the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related xenobiotics *via* a ligand-activated signal transduction pathway. The interaction of TCDD with the AhR results in the formation of a heterodimer complex with the AhR nuclear translocator protein (Arnt). This complex recognizes specific DNA sequences termed dioxin response elements (DREs) and elicits altered transcription of particular structural genes, such as that encoding cytochrome P4501A1. Several previous reports have implicated a role of phosphorylation during the TCDD-dependent transformation of the AhR to a DRE-binding form. Treatment of active AhR complex with phosphatase under conditions *in vitro* has been shown to result in the loss of DRE-binding activity [1,2]. Berghard *et al.* [3] also reported that the addition of cytosolic extracts from untreated wild type and Arnt mutant Hepa 1c1c7 (Hepa 1) cells were able to reactivate binding to the DRE following treatment with phosphatase. The authors suggested that this reconstitution may be dependent on a protein kinase C (PKC) type of activity since the activation was blocked by a PKC inhibitor and by a non-hydrolyzable ATP analogue. Although the mechanisms by which these deactivation and reactivation effects occur are not clear, these data do suggest that AhR phosphorylation may be instrumental in activating the receptor to a DNA-binding state. The purpose of the studies presented here was to clarify the role of phosphorylation during TCDD-elicited transformation of the AhR to a DRE-binding form. More specifically, we sought to determine the domains on the AhR that are phosphorylated, and if TCDD binding elicits major changes in the phosphorylation on these domains. In addition, we wished to further characterize the activity within extracts that is responsible for reestablishing binding to the DRE after dephosphorylation by phosphatase.

Methods

Cell ^{32}P and ^{32}S labeling and treatment. Prior to radiolabeling, Hepa 1 cells were rinsed and preincubated with either phosphate- or methionine-free MEM media

supplemented with 10% dialyzed fetal calf serum. Confluent plates were treated with fresh phosphate-free media containing ^{32}P-orthophosphate for 4 h. For ^{35}S-methionine labeling, 60-70% confluent plates were treated with 80% methionine-free media, 10% complete serum-free media containing ^{35}S-methionine, and 10% dialyzed fetal calf serum for 18-24 h. One h prior to the completion of labeling, the cells were treated with vehicle or 5 nM TCDD at 37°C.

Isolation and treatment of protein extracts. Transformed AhR was obtained from whole cell extracts of TCDD-treated (1 h, 2 nM, 37°C) Hepa 1 cells. Untransformed AhR was isolated from Hepa 1 cytosol and transformed *in vitro* with 2 nM TCDD for 2 h at room temperature. The protein extracts were incubated with aliquots of potato acid phosphatase (PAP) crosslinked to Sepharose for 30 min at room temperature followed by removal of the PAP-Sepharose by centrifugation. To block proteolytic activity present in the PAP preparation, the presence of various protease inhibitors was necessary. Phosphatase inhibitors were used in control samples to monitor the presence of proteolysis and nonspecific adsorption to the Sepharose beads.

Immunoprecipitation, gel electrophoresis, immunoblotting , and hydroxylamine and cyanogen bromide cleavage. These were carried out as described previously [4].

Gel retardation and specific binding assays. The binding of AhR contained in whole cell extract or cytosol to a DRE-containing, ^{32}P-labeled oligonuceotide was determined as previously described [5]. The presence of the AhR within the retarded complex was determined by a preincubation with an anti-AhR monoclonal antibody (gift from Dr. Gary Perdew). ^{3}H-TCDD specific binding to the AhR was determined as previously described [6].

Results

Effects of phosphatase treatment on binding of the AhR to the DRE. PAP blocked AhR binding to the DRE in a concentration-dependant manner, and at each PAP concentration the presence of phosphatase inhibitors reestablished DRE-binding to control levels. These results are consistent with those obtained by others [1,3]. However, phosphatase treatment failed to disrupt the AhR-Arnt complex since Arnt coimmunoprecipitated with the AhR. Together these data imply that phosphorylation positively regulates AhR binding to DNA.

TCDD-elicited changes in AhR phosphorylation. Additional studies determined whether ligand binding changes the degree of AhR total phosphorylation. ^{32}P-labeled AhR was isolated in the presence or absence of ligand and the relative intensity of labeling was quantitated by phosphorimaging. Using the same samples, immunoblot analysis was performed with AhR antibody and a ^{125}I-labeled secondary antibody. The ratios of ^{32}P to ^{125}I were similar for the unliganded and liganded AhR. These data indicated that the total level of AhR phosphorylation was not affected to a large degree by TCDD binding.

Cleavage of the ^{32}P-labeled AhR by hydroxylamine and cyanogen bromide. Hydroxylamine cleavage of the ^{32}P-labeled AhR revealed a consistent fragmentation pattern for both the unliganded and liganded AhR. Analysis of this fragmentation pattern suggested that the majority of the phosphorylated residues exist in a 31.5 kDa fragment between amino acids 368 and 650. Phosphorylation of a 17 kDa residue also suggested that amino acid(s) nearest ot the carboxyl terminus of the AhR is (are) phosphorylated. These data are consistent with the interpretation that the N-terminal half of the AhR, liganded and unliganded, is not phosphorylated. The cleavage pattern with cyanogen bromide was consistent with the interpretation that phosphorylated regions existed between amino acids 343 and 399, 479 and 535, and 549 and 605. A 13-kDa fragment likely represents a phosphorylated region between amino acids 636 and 759, or the glutamine-rich region.

DRE-binding reconstitution of PAP-treated AhR. The addition of untreated, wild-type Hepa 1 whole cell extracts reconstituted the ability of the PAP-treated transformed AhR to bind to the DRE. Whole cell extracts of BPRC1 (Arnt mutant) or TAO (AhR deficient) Hepa 1 mutant cell lines were also capable of reactivating the AhR binding to the DRE following PAP-Sepharose treatment. These data indicated that the reactivation was dependent on some factor other than AhR or Arnt. The addition of ATP did not enhance the ability of the extracts to reactivate the DRE binding. In addition, the removal of small molecular weight molecules by Sephadex G-50 had no effect on the ability of the extracts to reactivate this DRE binding following PAP treatment of the transformed AhR.

PAP treatment of the AhR prior to the addition of TCDD also inhibited ligand-elicited transformation of the DNA binding form, and this inhibition could be reversed by the addition of untreated whole cell extracts. However, PAP treatment did not affect the binding of ^{3}H-TCDD to the AhR. These data suggested that the mechanism by which PAP-Sepharose inhibits the ability of untransformed and transformed AhR to bind to the DRE is the same.

Partial characterization of the DNA binding reconstituting activity. When the activating extracts were treated with PAP-Sepharose, the ability to reconstitute AhR binding to the DRE was inhibited. This inhibition was prevented in the presence of phosphatase inhibitors. Heat inactivation and trypsin treatment also prevented the DRE-binding reconstitution. These experiments indicated that a proteinaceous factor interacts with the AhR complex in order to stabilize DRE binding and that dephosphorylation of this factor disrupts this interaction. Liver cytosol isolated from C57Bl/6-*Ah^{dd}* mice was also capable of reconstituting DRE binding of the PAP-treated AhR. The molecular weight of this factor was determined by gel filtration to be between 80 and 150 kDa.

Discussion

We have localized phosphorylation sites of the AhR contained in Hepa 1 cells to two locations located in the C-terminal half of the protein. These observations suggest

that one region is centrally located between amino acids 368 and 605, while the other is located at the glutamine-rich carboxyl terminus between amino acids 636 and 759. The exact functional significance of these post-translational modifications has yet to be determined. However, the proximity of the former site to the DNA-binding repressor domain previously described [7], suggest that phosphorylation may regulate DNA binding *via* this domain. The ligand-binding region is included in this domain, and thus agonists might act to suppress the repressor domain. Since the presence of ligand failed to produce a significant change in either total phosphorylation or phosphorylated cleavage patterns, it is unlikely that the agonist-induced suppression of the repressor domain is manifested *via* phosphorylation. Furthermore, since TCDD is able to transform the AhR is ATP-depleted cytosol, it is likely that a ligand-induced phosphorylation event is not required to achieve DNA binding.

However, we and others have observed that PAP treatment of the liganded or unliganded AhR clearly alters the ability of the AhR to bind to the DRE. This occurs without an effect on ligand binding or the abililty of the AhR to form a heterodimer complex with Arnt. Our results indicate that proteinaceous factor can restore the phosphatase-elicited inhibition of AhR binding to the DRE in an ATP-independent manner. The lack of ATP dependence implies that a kinase activity is not responsible fo the reconstitution, and suggests that another phosphorylated protein is required to stabilize the AhR-Arnt-DNA-binding complex.

References

1) Pongratz, I., Stromstedt, P.-E., Mason, G.F., and Poellinger, L. (1991) : Inhibition of the specific DNA binding activity of the dioxin receptor by phosphatase treatrnent. J. Biol. Chem. 266, 16813-16817.
2) Carrier, F., Owens, R.A., Nebert, D.W., and Puga, A. (1992) : Dioxin-dependent activation of murine *Cyp1a-1* gene transcription requires protein kinase C-dependent phosphorylation. Mol. Cell. Biol. 12, 1856-1863.
3) Berghard, A., Gradin, K., Pongratz, I., Whitelaw, M., and Poellinger, L. (1993) : Cross-coupling of signal transduction pathways: the dioxin receptor mediates induction of cytochrome P-4501A1 expression via a protein kinase C-dependent mechanism. Mol. Cell. Biol. 13, 677-689.
4) Mahon, M.J., and Gasiewicz, T.A. (1995) : Ah receptor phosphorylation: localization of phosphorylation sites to the C-terminal half of the protein. Arch. Biochem. Biophys. 318, 166-174.
5) Denison, M.S., Fisher, J.M., and Whitlock, J.P., Jr. (1989) : Protein-DNA interactions at recognition sites for the dioxin-receptor complex. J. Biol. Chem. 264, 16478-16482.
6) Gasiewicz, T.A., and Neal, R.A. (1982) : The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-p-dioxin using hydroxylapatite. Anal. Biochem. 124, 1-11.
7) Dolwick, K.M., Swanson, H.I., and Bradfield, C.A. (1993) : In vitro analysis of Ah receptor domains involved in ligand-activated DNA recognition. Proc. Natl. Acad. Sci. USA 90, 8566-8570.