Mechanism of action of the Ah receptor: characterization of the DNA binding form(s) of the Ah receptor complex.

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1. Abstract

The Ah receptor (AhR), a ligand-dependent transcription factor, binds with high affinity to a specific DNA recognition site (the dioxin responsive element (DRE)). The binding of the transformed AhR complex with the DRE is the first step in the activation of gene expression. To examine transcriptional activation by the AhR, the protein subunit composition of the complex must be established. It is believed that transformed AhR complexes exist as a heterodimer of the AhR and ARNT (Ah receptor nuclear translocator). Recent evidence suggests the involvement of other AhR subunits/factors. In previous crosslinking studies, we demonstrated that the transformed 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD):AhR complex exists as two distinct DNA binding forms, containing at least three protein subunits (97, 105 and 115 kDa) which inducibly binds to a DRE oligonucleotide. Here we have characterized these three protein-DNA cross-linked complexes using antibody recognition and precipitation experiments. Guinea pig hepatic cytosolic TCDD:AhR complexes, transformed *in vitro* were UV-crosslinked to a radiolabeled BrdU-containing DRE oligomer. Immunoprecipitation with an ARNT antibody identified the 97 kDa protein as ARNT while that using an AhR antibody identified the 105 kDa protein as the AhR. The lack of recognition of the 115 kDa protein-DNA complex by these and other antibodies suggests that it represents a unique protein which is associated with the DNA binding form of the AhR. The identity and functional activity of this protein remains to be elucidated.

2. Introduction

The aromatic hydrocarbon receptor (AhR) is a soluble, intracellular, ligand-dependent, DNA-regulatory protein that appears to mediate many of the biological and toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related halogenated aromatic hydrocarbons by differentially enhancing the expression of several genes.[1-4] The most extensively studied AhR-dependent response to TCDD, the induction of cytochrome P4501A1, has been used as a model system to dissect to characterize the mechanism of action of dioxin action.[1,2] Induction of P4501A1 by TCDD is regulated by the AhR, to which TCDD binds with high affinity. Following ligand-binding, the TCDD:AhR complex undergoes transformation, wherein the AhR acquires the ability to bind to DNA with high affinity and it is found tightly associated with the nucleus. The actual mechanistic events associated with transformation are not yet clear but it appears to involve dissociation of at least three proteins (including two molecules of hsp90 (a heat shock protein of 90 kDa) from the "untransformed" TCDD:AhR protein complex

(≈250 kDa), translocation of the AhR into the nuclear compartment and its high affinity association with at least one nuclear protein, the ARNT (Ah receptor nuclear translocator) protein.[2-5] Once complexed with ARNT, the complex gains the ability to bind to DNA specifically and with high affinity and it is then considered to be in its transformed (nuclear) form. Whether additional proteins are present within the transformed complex remains to be determined. Following transformation, biochemical and genetic evidence indicates that the binding of these transformed complexes to specific DNA sequences, termed dioxin responsive elements (DREs), adjacent to the cytochrome P4501A1 (CYP1A1) gene leads to DNA bending, chromatin disruption, increased promoter accessibility and increased rates of transcription initiation of the CYP1A1 gene with the subsequent accumulation of P4501A1-specific mRNA.[2,4,6]

One focus of our research has been a detailed analysis of the specific interaction of transformed TCDD:AhR complexes with DRE-containing DNA. Utilizing a sensitive gel retardation assay, we have demonstrated that hepatic cytosolic AhR from a wide variety of species can be transformed *in vitro* to a form which can bind to a DRE-containing oligonucleotide.[7,8] Site directed mutagenesis and competitive gel retardation analysis to identify those nucleotides important for TCDD:AhR:DRE complex formation; a putative TCDD:AhR DNA-binding consensus sequence of GCGTGNNA/TNNNC/G was derived from these studies. More recently, we have examined the specific proteins subunits of the transformed guinea pig hepatic cytosolic TCDD:AhR complex which directly interact with the DRE by covalently UV-crosslinking of the proteins to DRE oligonucleotides substituted with variable numbers of bromodeoxyuridine (BrdU) residues.[9] These studies revealed the involvement of at least three separate protein subunits (97, 105 and 115 kDa) in the DNA binding of transformed TCDD:AhR complex and they suggest the existence of two distinct heteromeric transformed AhR complexes, each containing at least one 105 kDa ligand-binding subunit and either one 115 or one 97 kDa non-ligand-binding subunit. Here we have extended our previous analysis of these multiple DNA binding of transformed TCDD:AhR complexes.

3. Methods and Materials

Preparation of Cytosol and Nuclear Extracts: Hepatic cytosol from male Hartley guinea pigs (250-300g) (obtained from Simonsen, Gilroy, CA USA) was prepared as previously described.[8] Nuclear extracts from cells in culture incubated in the absence or presence of TCDD (1nM) were prepared as described.[6]

Cell Cultures: Mouse hepatoma (Hepa1c1c7) cells (Hepa1) were obtained from J. P. Whitlock, Jr. (Stanford University) and were grown as previously described.[6] Mouse hepatoma (MLE/BV) cells, derived from a spontaneously immortalized C3H/HeN mouse liver cell, were obtained from B. V. Madhukar (Michigan State University) and were grown as described for the Hepa1 cells. Guinea pig intestinal adenocarcinoma (GPC16) were obtained from the American Type Culture Collection (Rockville, MD) and maintained according to their cell-type specific culture protocols.

AhR Ligand and DNA Binding Analysis: Gel retardation and UV-crosslinking analysis of cytosolic and nuclear proteins were carried out as previously described.[8,9] UV-crosslinked protein-DNA complexes were immunoprecipitation using an anti-arnt antibody (provided by Dr. O. Hankinson (Univ. of Calif. at Los Angeles, USA)) or anti-AhR antibody (provided by Dr. R. Pollenz, Medical University of South Carolina, USA) and precipitated UV-crosslinked [^{32}P]-DRE-protein complexes were resolved by SDS-polyacrylamide gel electrophoresis.

4. Results and Discussion

Guinea pig hepatic AhR complex was transformed *in vitro* to its DNA binding form by incubation with TCDD, dioxin and transformed TCDD:AhR complex was covalently crosslinked by

UV-irradiation to a BrdU-substituted DRE-containing oligonucleotide.[9] Denaturing gel electrophoresis and autoradiography identified four TCDD-inducible protein-DNA complexes, with molecular weights of approximately 97, 105, 115, and a somewhat broader complex at 247 kDa as we have previously observed. UV-Crosslinking experiments (Figure 1) have also revealed that the DNA binding form(s) of the AhR from a variety of species (rat, mouse (C3H and Balb/c) and human (data not shown)) also contains three distinct DNA-binding proteins of similar molecular weight, while that from C57 mouse only contained two DNA binding proteins (with apparent molecular weights of 100 and 106 kDa). These multiple crosslinked protein-DNA complexes have also been identified in a variety of different tissues. Additional experiments which have demonstrated the presence of these multiple TCDD-inducible crosslinked complexes in nuclear extracts from TCDD-treated C3H-derived mouse hepatoma and guinea pig intestinal epithelial cell lines, indicate that these complexes and multiple protein subunits exist in intact cells.

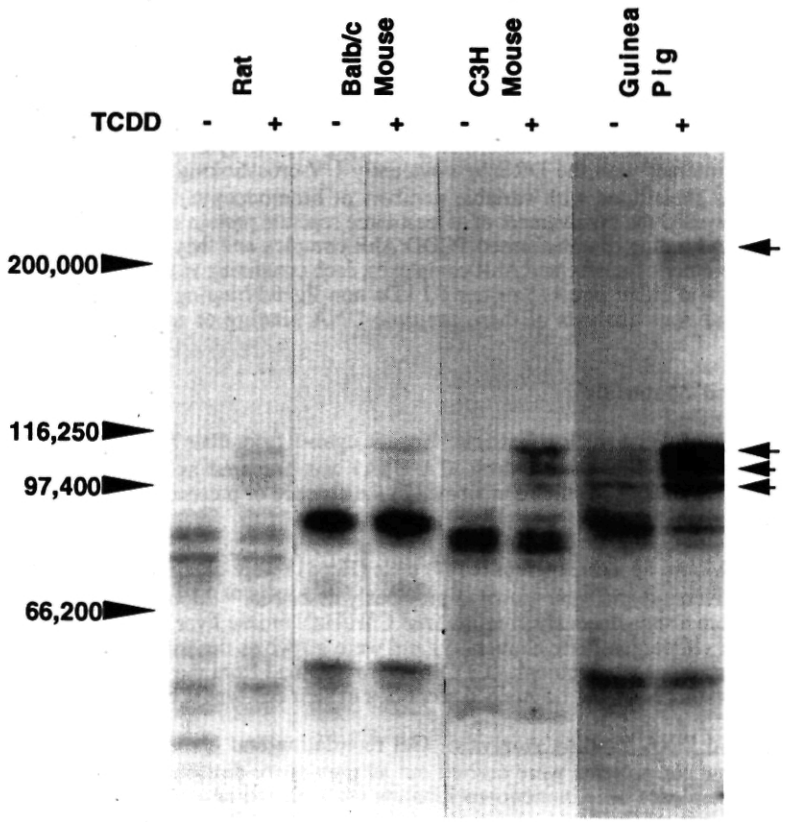

Figure 1. TCDD-inducible, UV-crosslinking of transformed hepatic TCDD:AhR complex from a variety of species to a DRE-containing DNA oligonucleotide. Hepatic cytosol from the indicated species, incubated in the absence (-TCDD) or presence (+TCDD) of 20 nM TCDD, was crosslinked to [^{32}P]-BrdU-substituted DRE oligonucleotide. Protein-DNA complexes were denatured, separated by SDS-PAGE and visualized by autoradiography. The arrows indicate the positions of the TCDD-inducible protein-DNA complexes and the protein standards are indicated.

To identify the individual TCDD-inducible DNA binding proteins, anti-AhR and -ARNT antibodies were used in immunoprecipitation and Western blot experiments. UV-crosslinked guinea pig hepatic TCDD:AhR:DRE complexes were denatured in SDS, diluted with buffer and protein-DNA complexes immunoprecipitated and resolved by SDS-PAGE. These experiments identified the 97 kDa protein-DNA complex as containing the ARNT protein and the 105 kDa protein-DNA complex as containing the AhR. These results were also supported by Western blot analysis of cross-linked complexes. The 115 kDa protein-DNA complex was not immunoprecipitated by either antibody, nor was it recognized by a variety of AhR or ARNT antibodies directed against various regions of these proteins. These results demonstrate that the 115 kDa protein-DNA complex contains a unique protein which is associated with the DNA binding form of the AhR. Given the presence of a basic helix-loop-helix motif and PAAS domain in the AhR that appears to play a role in protein dimerization[3,4], we would propose that this Ah receptor factor (ARF) contains a similar protein motif that would allow it to dimerize with liganded AhR to produce a DNA binding form of the AhR complex. Although the identity of the 115 kDa ARF and the functional activity the multiple AhR complexes remains to be determined, their presence in intact cells and in a variety of species and tissues would support their role in the ability of TCDD to activate gene expression. Studies are currently underway to isolate and purify ARF.

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5. References

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