IDENTIFICATION AND CHARACTERIZATION OF MULTIPLE DNA BINDING FORMS OF THE Ah RECEPTOR COMPLEX FROM DIFFERENT SPECIES

K. Tullis[1], M. S. Denison[1] and H. I. Swanson[2]. [1]Department of Environmental Toxicology, University of California, Davis, CA 95616 and [2]Department of Pharmacology and Toxicology, Northwestern University, Chicago, IL 60611

1. 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 rant, 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.

2. Methods and Materials

Preparation of Cytosol and Nuclear Extracts: Hepatic cytosol from male Hartley guinea pigs (250-300g), male Sprague-Dawley rats (150g) and male Balb/c, C3H/HeN and C57BL/6N mice (20g) (obtained from Charles River Laboratories, Wilmington, DE 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 (Indiana University School of Medicine, Indianapolis) 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., Los Angeles, CA)) and precipitated UV-crosslinked [^{32}P]-DRE-protein complexes were resolved by SDS-polyacrylamide gel electrophoresis.

3. Results

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 (Fig. 1). The 247 kDa complex appears to contain two distinct protein-DNA complexes of approximately 232 and 256 kDa and represents two proteins covalently crosslinked to a single DRE oligonucleotide, while the 97, 105, and 115 kDa complexes represent single protein-DRE crosslinks.

UV-crosslinking to DRE oligonucleotides containing variable numbers of BrdU residues revealed that the 105 kDa protein, identified as the AhR ligand binding subunit by photoaffinity labeling with a radioiodinated AhR agonist,[9] crosslinks to the DRE core consensus (5'-GCGTG-3'); the 97 and 115 kDa non-ligand binding proteins differentially crosslink immediately 5'-ward of the core (Fig. 2). Immunoprecipitation of native transformed TCDD:AhR complexes with an anti-arnt polyclonal antibody and SDS-PAGE analysis (data not shown), demonstrated that only the 105 and 97 kDa complexes were precipitated. These results imply binding of the 105 and 97 kDa protein complexes; the lack of precipitation of the 115 kDa complex may be due to the fact that it does not bind the arnt protein. Resuspension of SDS-treated immunoprecipitated complexes with buffer

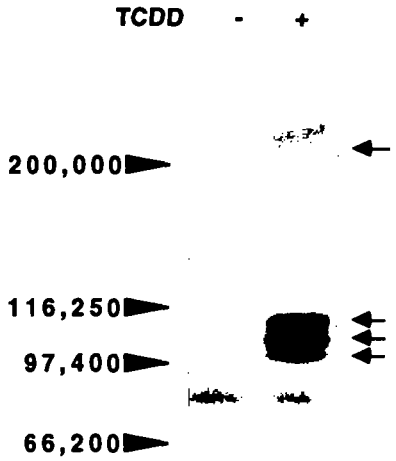

Figure 1. TCDD-inducible, UV-crosslinking of the transformed guinea pig hepatic cytosolic TCDD:AhR complex to DRE-containing DNA.

followed by reimmunoprecipitation of the denatured complexes with the arnt antibody and SDS-PAGE analysis, revealed only the 97kDa crosslinked complex (data not shown) and suggest that it represent the arnt protein. Overall, these results not only demonstrate that the arnt protein (97kDa) binds to the AhR ligand binding subunit (105kDa) but that it fails to bind to, or immunoprecipitate, the 115 kDa complex. Additional UV-crosslinking studies (data not shown) have demonstrated the presence of all three complexes in nucleus of TCDD-treated cells in culture (GPC16 and MLE/BV). These results combined with our previous UV crosslinking results not only suggest that the critical protein-DNA contacts which occur between the AhR complex and the DRE are made primarily by the ligand-binding subunit but they indicate that the AhR complex exists as two distinct heteromeric DNA-binding forms, containing one 105 kDa ligand-binding subunit and either arnt (97kDa) or the 115kDa AhR factor (ARF).[9] Given that both of these complexes can bind to DNA, these results would also be consistent with the hypothesis that ARF substitutes for arnt in the DNA binding complex.

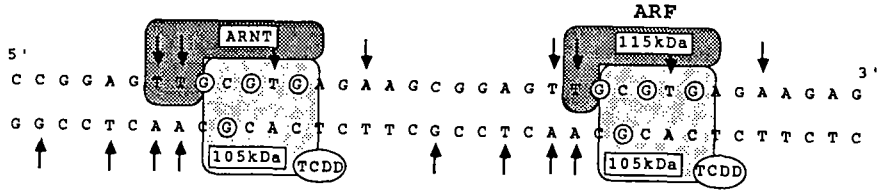

Figure 2. Model for the specific interaction of subunits contained within the two transformed TCDD:AhR complexes with the DRE derived from UV-crosslinking analysis. The position of the AhR, arnt and Ah Receptor Factor (ARF) is indicated. The arrows indicate the positions at which BrdU residues were incorporated.

4. Conclusions

The results of our studies are consistent with the hypothesis that two distinct DNA binding forms of the AhR exist and that they are found in the nucleus of cells *in vivo*. Interestingly, although these three DRE binding protein subunits have been identified in guinea pig, rat, human, and two strains of mice, only two DRE-binding proteins have been identified in C57BL mouse liver and hepa1 cells in culture (data not shown). The reason for this obvious species difference in protein subunit composition of the transformed AhR complex is not clear, but it does not appear to adversely affect AhR functionality. Given the presence of a basic helix-loop-helix motif in the AhR that appears to be involved in its dimerization arnt[3,4], we would propose that the ARF protein contains a similar protein motif that would allow it to dimerize with liganded AhR to produce a DNA binding form of the AhR complex. Whether these two DNA binding forms are both transcriptionally active remains to be confirmed, however, the presence of these multiple complexes in a variety of species and tissues suggests that both of these complexes are physiologically relevant.

5. References

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