Identification of the Ah Receptor Nuclear Translocator Protein (Arnt) as a Component of the DNA Binding Form of the Ah Receptor

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The Ah (dioxin) receptor binds a number of widely disseminated environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons, and mediates their carcinogenic effects. The ligand-bound receptor activates *Cyp1a1* gene transcription through interaction with specific DNA sequences, termed xenobiotic responsive elements (XREs). The Ah receptor nuclear translocator protein (Arnt) is required for Ah receptor function. Arnt is now shown to be a structural component of the XRE binding form of the Ah receptor. Furthermore, Arnt and the ligand-binding subunit of the receptor were extracted as a complex from the nuclei of cells treated with ligand. Arnt contains a basic helix-loop-helix motif, which may be responsible for interacting with both the XRE and the ligand-binding subunit.

The Ah receptor is involved in the induction of cytochrome P450IA1, cytochrome P450IA2, and several other enzymes that participate in xenobiotic metabolism. The two P450 cytochromes are important in the activation of polycyclic aromatic hydrocarbons (found in cigarette smoke and smog) and certain heterocyclic amines (found in cooked meat) to carcinogenic intermediates (*1*). The pathogenicity of many polychlorinated aromatic compounds, such as TCDD, also depends on their ability to bind to the Ah receptor. However, polychlorinated aromatic compounds are metabolized poorly, and the mechanism whereby the Ah receptor mediates their pathological effects is unknown (*2*).

The ligand-free form of the Ah receptor is found in the cytosol after conventional subcellular fractionation. This form of the receptor is a multimeric complex of ~280 kD that contains the 95-kD ligand-binding subunit and the 90-kD heat shock protein (Hsp90) (*3*, *4*). After cells are incubated with ligand, the ligand-binding subunit is found in the nucleus. Therefore, binding of ligand leads to nuclear translocation of the ligand-binding subunit. The ligand-binding subunit is extracted from nuclei in a complex of ~176 kD that does not contain Hsp90 (*5–7*). The Ah receptor resembles the glucocorticoid receptor (*6*, *8*), and this has led to the suggestion that the ligand-binding subunit of the Ah receptor may be a member of the steroid-thyroid hormone receptor superfamily. Transcriptional activation of the P450IA1 gene (*Cyp1a1*) by ligands of the receptor depends on binding of the ligand-bound receptor to DNA sequences in the upstream regulatory region of the gene, known as XREs or dioxin-responsive enhancers (DREs) (*9–11*). Evidence has suggested that the XRE-binding form of the receptor contains, besides the ligand-binding subunit, another protein that binds directly to DNA (*12*).

Mutants of the mouse hepatoma cell line Hepa-1 that are defective in induction of P450IA1 have been isolated. Some of these mutants are defective in Ah receptor function, and these have been assigned to three complementation groups (*13–15*). In mutants of group C (C^- mutants), the ligand-binding subunit of the Ah receptor is present in normal concentrations but is unable to translocate to the nucleus after binding ligand (*15*). We obtained transfectants of a C^- mutant in which nuclear translocation was restored by treating the mutant with human genomic DNA. From such a transfectant, we isolated a portion of the putative human C gene [also called the Ah receptor nuclear translocator gene (*arnt*)]. This genomic fragment was used to isolate a full-length cDNA that encoded Arnt. The *arnt* cDNA restored Ah receptor function on transfection into the C^- mutant (*16*). We sought here to determine the role of Arnt in Ah receptor function.

We prepared antiserum in rabbits to a bacterially expressed fusion protein consisting of *Staphylococcal* protein A and a portion of human Arnt. This antiserum (anti-Arnt) was specific for Arnt, as demonstrated by the following experiments. We obtained high-level expression of both full-length and a truncated Arnt by transiently transfecting COS-7 cells with plasmids pBM5-NEO-M1-1 and pBM5-NEO-M3-1 (*16*), respectively. The latter plasmid contains an *arnt* cDNA insert that is missing the nucleotides corresponding to the 163 COOH-terminal amino acids of Arnt. Anti-Arnt precipitated proteins from the transfectants that were of the size predicted for the cDNA fragments they contained (87 kD for M1-1 and 69 kD for M3-1) (Fig. 1A). Incubation of anti-Arnt with a β-galactosidase (β-gal)–Arnt fusion protein prevented immunoprecipitation of these proteins. No effect was observed when anti-Arnt was incubated with a fusion protein generated from a construct containing the β-*gal* gene linked to the *arnt* gene in the antisense orientation. Other less intense protein bands produced in extracts from cells transfected with Arnt (Fig. 1A) probably resulted from degradation of the Arnt protein or the utilization of cryptic promoters in the plasmids. The weak protein band from COS-7 cells transfected with the parental plasmid (pBM5-NEO) corresponds to the endogenous Arnt protein. The immunoglobulin G (IgG) fraction of the antiserum also precipitated an 87-kD protein from the cytosol and nuclei of Hepa-1 cells (Fig. 1B) and human LS180 cells (*17*), which is the size predicted from the human cDNA coding sequence. Competition with the β-gal–Arnt sense fusion protein, but not with the antisense fusion protein, prevented immunoprecipitation of the 87-kD protein from the Hepa-1 cytosol. In addition to the 87-kD proteins, several proteins of less than 39 kD were detected in the immunoprecipitates of Hepa-1 cells. However, these smaller proteins were also observed when preimmune IgG, and even no IgG, was used in the precipitation reaction (*17*), and they are nonspecific artifacts.

We used the antibodies to Arnt to determine whether Arnt is a structural component of the XRE-binding form of the Ah receptor. The Ah receptor was extracted with high salt from nuclei of Hepa-1 cells grown in the presence of TCDD. When the nuclear extract was incubated with a ^{32}P-labeled double-stranded oligonucleotide that contained the mouse XRE1 sequence, a retarded complex was observed after electrophoresis on a nondenaturing polyacrylamide gel (Fig. 2). The complex was eliminated if a 100-fold excess of unlabeled XRE1 oligonucleotide was included in the incubation, but not if the competitor oligonucleotide contained two nucleotide substitutions in the core sequence of XRE1 that eliminate binding by the Ah receptor (*18*). Furthermore, the retarded complex was barely discernible with an extract from Hepa-1 cells grown in the absence of TCDD and was not obtained with an extract prepared from the C^- mutant c4 grown with TCDD. These observations identify this band as corresponding to the XRE–Ah receptor complex. Another major XRE-protein complex, which had a faster

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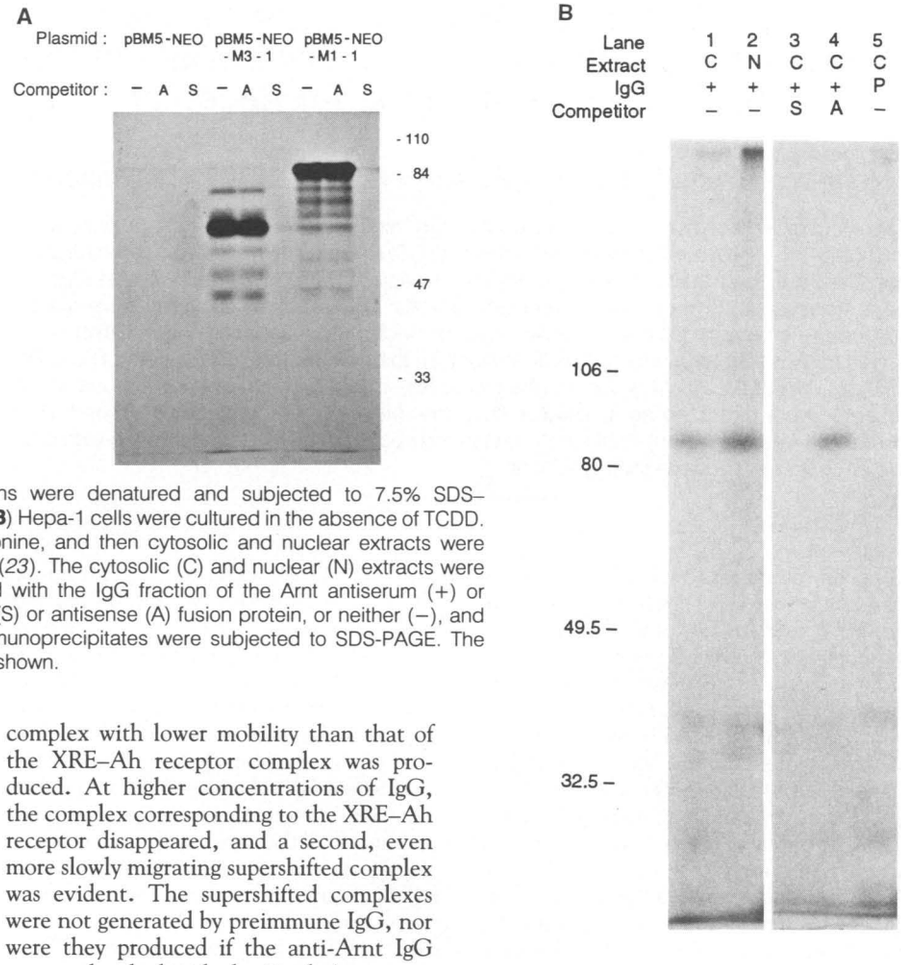

Fig. 1. Immunoprecipitation of Arnt by anti-Arnt sera. An antiserum to a *Staphylococcal* protein A–Arnt fusion protein containing Arnt amino acids 399 to 777 was raised in rabbits, and the corresponding IgG function was purified (*23*). The preimmune serum and corresponding IgG fraction were obtained from the same rabbit before immunization. (**A**) The COS-7 cells were transfected with pBM5-NEO, pBM5-NEO-M3-1, or pBM5-NEO-M1-1 with calcium phosphate (*16*). Three days later the cells were metabolically labeled with ^{35}S-labeled methionine for 75 min. Whole-cell extracts were denatured and volumes of the extracts that contained the same amount of radioactivity were mixed with an equal amount (on a protein basis) of the lysate that contained the β-gal–Arnt fusion protein (S), the corresponding antisense fusion protein (A), or neither (–) (*23*). The extracts were treated with anti-Arnt serum and then with protein A–agarose beads. The immunoprecipitated proteins were denatured and subjected to 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). (**B**) Hepa-1 cells were cultured in the absence of TCDD. They were metabolically labeled with [^{35}S]methionine, and then cytosolic and nuclear extracts were prepared and subjected to denaturing conditions (*23*). The cytosolic (C) and nuclear (N) extracts were adjusted to the same counts per minute, treated with the IgG fraction of the Arnt antiserum (+) or preimmune IgG (P), in the presence of the sense (S) or antisense (A) fusion protein, or neither (–), and subsequently with protein A–agarose beads. Immunoprecipitates were subjected to SDS-PAGE. The positions of protein standards (in kilodaltons) are shown.

migration than the XRE–Ah receptor complex, was also observed. However, this complex was obtained with extracts from c4 cells and Hepa-1 cells grown in the absence of TCDD and is therefore not Ah receptor–dependent. It may correspond to one of the constitutive XRE-binding proteins previously detected in Hepa-1 nuclei (*19*).

When a nuclear extract from Hepa-1 cells grown with TCDD was incubated with the ^{32}P-labeled XRE1 probe and then treated with anti-Arnt IgG, a "super-shifted" complex with lower mobility than that of the XRE–Ah receptor complex was produced. At higher concentrations of IgG, the complex corresponding to the XRE–Ah receptor disappeared, and a second, even more slowly migrating supershifted complex was evident. The supershifted complexes were not generated by preimmune IgG, nor were they produced if the anti-Arnt IgG was preabsorbed with the β-gal–Arnt sense protein. Preabsorption by extracts prepared from bacteria expressing the β-gal–*arnt* antisense construct had no effect on the formation of the supershifted bands. These results demonstrate that the supershifted complexes are caused by interaction between anti-Arnt IgG and the Ah receptor–XRE complex. The second supershifted band observed with higher concentrations of anti-Arnt IgG may correspond to complexes that contained more than one anti-Arnt IgG molecule. It was observed that higher concentrations of protein in the gel retardation assay nonspecifically reduced the amounts of XRE-protein complexes produced. Thus, the amounts of both the XRE–Ah receptor complex and the XRE–constitutive protein complex were diminished at the highest concentrations of anti-Arnt IgG and also when both IgG and the β-gal–Arnt fusion proteins were present in the incubation mixture for the assay.

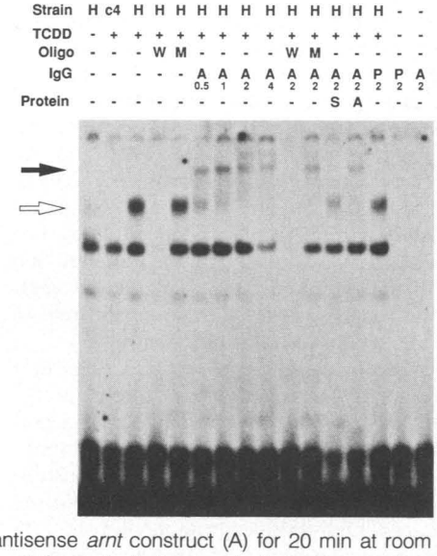

Fig. 2. Effect of Arnt antibodies on the electrophoretic migration of the XRE–Ah receptor complex. The synthetic oligonucleotide 5′-TCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′, which contained the sequence from nt –999 to nt –979 of the mouse *Cyp1a1* gene and encompassed XRE1, was annealed to the complementary 8-nt primer 5′-TGAGCTCG-3′, and the primer was extended and labeled with deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), thymidine triphosphate (TTP), and α^{32}P-labeled deoxyadenosine triphosphate (dATP). The wild-type (W) and mutant (M) competitors (the latter containing two nucleotide substitutions in the core of the XRE) are as described (*16*). Nuclear extracts were prepared (*24*) from Hepa-1 cells (H) cultured in the presence (+) or absence (–) of 3 nM TCDD for 90 min or from c4 cells cultured in TCDD. Nuclear extracts (5 μg of protein) were incubated with labeled probe and lysate (2 μg of protein) from bacteria expressing either the pTZ18U-sense *arnt* construct (S) or the pTZ18U-antisense *arnt* construct (A) for 20 min at room temperature as described (*11*). Anti-Arnt IgG (A, at the indicated amounts in micrograms) or preimmune IgG (P, at 2 μg) was added, and incubation was continued for a further 20 min at room temperature. The extracts were then subjected to nondenaturing polyacrylamide gel electrophoresis. The open arrow indicates the XRE–Ah receptor complex. The closed arrow indicates the supershifted complex.

It has been shown that the XRE–Ah receptor complex contains the ligand-binding subunit of the receptor (*10, 11*). Our results demonstrate that Arnt is also a component of this complex. Because Arnt is required for Ah receptor binding to the XRE (*20*), as well as for ligand-dependent nuclear translocation of the Ah receptor, it is probable that Arnt corresponds to the

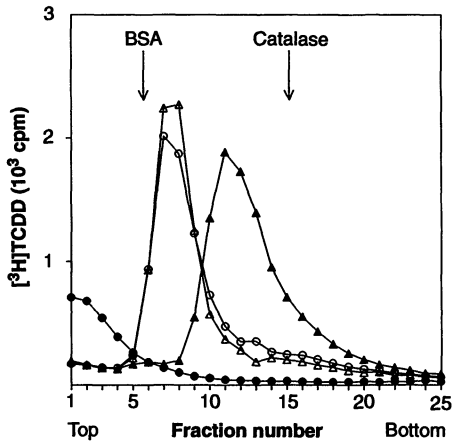

Fig. 3. Effect of Arnt antibodies on the sedimentation rate of the nuclear form of the Ah receptor. Hepa-1 cells were cultured with [^{3}H]TCDD (40 Ci/mmol) (3 nM), or [^{3}H]TCDD (3 nM) plus unlabeled TCDD (200 nM) at 37°C for 90 min. The nuclear fractions were prepared as described (*23*), except that protease inhibitors were not included. Analysis by sucrose density centrifugation was performed as previously described (*14*). Where indicated, anti-Arnt IgG or preimmune IgG was added at a ratio of one part IgG to five parts nuclear extract (protein: protein), and the extracts were incubated for 1 hour at 4°C before being loaded onto the gradients. Each gradient contained 1.4 mg of nuclear protein. The ^{14}C-labeled bovine serum albumin (4.4*S*) and ^{14}C-labeled catalase (11.3*S*) were included in gradients as sedimentation markers. Extracts from cells cultured with [^{3}H]TCDD and treated with anti-Arnt IgG (closed triangles); preimmune IgG (open circles); or neither (open triangles). Extract from cells cultured with [^{3}H]TCDD plus a 67-fold excess of unlabeled TCDD (closed circles).

second DNA binding protein previously detected in this complex (*12*). Nuclear and cytosolic extracts were prepared from COS-7 cells transfected with pBM5-NEO-M1-1 and grown with TCDD. The amounts of each XRE-protein complex detected by gel retardation analysis in these extracts were no greater than the amounts in the corresponding extracts prepared from untransfected COS-7 cells (*21*). This suggests that Arnt on its own is unable to bind the XRE.

When Hepa-1 cells were incubated in vivo with ^{3}H-labeled TCDD, and nuclear extracts were subjected to sucrose gradient centrifugation, a peak of radioactivity sedimented at about 6*S* (Fig. 3). No peaks were observed when the cells were incubated in the presence of an excess of unlabeled TCDD. These results identify the peak as corresponding to the Ah receptor (*5*, *6*). Addition of anti-Arnt IgG to the nuclear extract caused the receptor to sediment more rapidly in the gradient (Fig. 3). Furthermore, as the amount of antibody was progressively increased, there was a progressive increase in the sedimentation rate of the receptor (*17*). Preimmune IgG had no effect on the sedimentation rate of the nuclear receptor. Thus, Arnt and the ligand-binding subunit remain associated even when they are extracted from nuclei and released from DNA. Their sizes suggest that the form of the Ah receptor that can be extracted from nuclei, which is about 176 kD (*5*, *6*), is a heterodimer of Arnt (87 kD) (*16*) and the ligand-binding subunit (95 kD) (*4*).

The human Arnt protein contains a basic helix-loop-helix (bHLH) motif (*16*). Such motifs are found in transcription factors that bind DNA as homo- or heterodimers. The bHLH domain is responsible for both dimerization and DNA binding (*22*). It seems likely that the bHLH motif of Arnt is responsible for its association with the ligand-binding subunit of the Ah receptor and also participates in binding of Arnt to the XRE. This suggests that the ligand-binding subunit will also have a bHLH motif, and thus will bind DNA through this domain. Because members of the steroid-thyroid hormone receptor superfamily bind DNA through zinc finger domains, the Ah receptor will probably prove not to be a member of this superfamily.

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23. We generated the *Staphylococcal* protein A–Arnt fusion protein by ligating the Eco RI fragment of the human *arnt* cDNA containing nucleotides (nts) 1247 to 2384 into pRIT-31 and expressing the recombinant protein in *Escherichia coli*. We purified the fusion protein by IgG-Sepharose affinity chromatography and used it to immunize a rabbit according to procedures approved by the UCLA Animal Research Committee. We purified the IgG fraction from the resulting antiserum by protein A–agarose affinity chromatography. We metabolically labeled the cells by culturing them for 30 min in methionine- and serum-free medium and then for a further 75 min in a fresh aliquot of the same medium supplemented with trans-^{35}S-label (ICN) (400 μCi per dish). We prepared whole-cell extracts from COS-7 cells and the transfectants by boiling the cells for 5 min in denaturing buffer [100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.5% SDS, 0.1% sodium azide, 10 mM sodium phosphate (pH 7.5)], chilling, and adding aprotinin (40 trypsin-inhibiting units per milliliter), leupeptin (100 μM), and phenylmethylsulfonyl fluoride (PMSF) (200 μM) as protease inhibitors. To prepare cytosolic and nuclear extracts from metabolically labeled Hepa-1 cells, we incubated the cells in HED buffer [25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol (pH 7.6)] that contained aprotinin, leupeptin, and PMSF for 30 min, and then we homogenized and centrifuged them. Glycerol was added to the cytosolic supernatant to a final concentration of 10%. We extracted the nuclear pellet in HEDGK (HED buffer that contained 0.4 M KCl and 10% glycerol) with protease inhibitors, and we used the supernatant obtained on centrifugation of this extract as the nuclear extract. The extracts were then boiled in denaturing buffer. Bacterial lysates containing β-gal–Arnt sense and antisense fusion proteins were obtained from bacteria harboring plasmid pTZ18U into which the above *arnt* cDNA fragment had been inserted in the sense orientation relative to the *lacZ* gene or harboring a corresponding pTZ18U derivative containing the *arnt* fragment in the antisense orientation.
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