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# IDENTIFICATION OF A 120-kDa PROTEIN ASSOCIATED WITH AROMATIC HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR¶

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**The aromatic hydrocarbon receptor nuclear translocator (ARNT) is a basic helix-loop-helix-PAS protein which forms a heterodimer with aromatic hydrocarbon receptor (AHR), this heterodimer mediating the signal transduction in response to the various xenobiotics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and directly interacting with target genes by binding to xenobiotic responsive elements. An anti-ARNT antibody was raised in rabbits against the bacterially expressed ARNT of amino acids 21-328 from the N-terminal. Using this antibody, besides ARNT itself, we detected at least one protein, 120 kDa, in the immunoprecipitate of anti-ARNT antibodies in HepG2 cells as well as in Hepa-1 cells. However, this protein is not present in the immunoprecipitate of the anti-AHR antisera nor in that of the preimmune sera of the rabbits used for the immunization.** 

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The abbreviations used are: a.a., amino acids; mAHR, mouse aryl hydrocarbon receptor; hARNT, human Ah receptor nuclear translocator; bHLH, basic helix-loop-helix; GST, glutathione S-transferase; PAS, Per-Ah receptor/ ARNT-Sim; HSP90, 90 kDa heat shock protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive elements.

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Aromatic hydrocarbon receptor nuclear translocator (ARNT)[1] belongs to a family of proteins which also comprises of its dimerization partner, the Ah receptor (AHR), and the *Drosophila* proteins PER and SIM (1-5) and referred to as the PAS protein family, as shown by a region containing 200 amino acids with high sequence similarities. The conserved features of the bHLH domain are a basic region required for specific DNA binding and two putative amphipathic α–helixes, separated by a variable loop, which are required for dimerization (6). The PAS domain of PER has been shown to mediate heterodimerization between certain members of the PAS family (7). For the Ah receptor, the PAS domain appears to play a role with ARNT in heterodimerization, in interactions with heat shock protein 90 (HSP90), and in ligand binding (8,9,10). In the absence of ligand, the Ah receptor is located in the cytosol associated with HSP90 (8,9,10). Treatment with ligands initiates the conversion of the receptor to a nuclear form and results in its translocation into the nucleus and its heterodimerization with ARNT (11). The resultant AHR-ARNT-ligand heterocomplex binds to xenobiotic responsive elements (XRE) upstream of dioxin responsive genes, such as cytochrome P450 1A1 (CYP1A1), resulting in increased initiation rates of target gene transcription (12,13). The XRE-binding properties of the AHR-ARNT heterocomplex are thought to mediate the biological effects of exposure of the enviormental contaminant TCDD or other halogenated planar aromatic hydrocarbons. Among the biochemical and toxicological effects attributable to TCDD exposure of rodents are cleft palate, the wasting syndrome, thymic atrophy, immune toxicity, and site specific tumor promotion (14).

Although there are striking structural similarities between the Ah receptor and ARNT, ARNT does not bind to HSP90, nor to any ligand. Much is still unknown about the higher order structure of ARNT and how ARNT interacts with other proteins to regulate its activity prior to ligand activation. One way to approach these questions is to isolate ARNT from cell extracts and examine the protein associated with it. To this end, an anti-ARNT antibody was raised in rabbits against bacterially expressed ARNT. By using this antibody, we demonstrated that ARNT exists as a heterodimer in non-activated cells.

## Materials and Methods

### Materials

Alkaline phosphatase conjugated goat anti-rabbit IgG was obtained from Promega (Madison, WI, U.S.A.). Protein A Sepharose and Glutathione Sepharose 4B were purchased from Pharmacia, Biotech AB (Uppsala, Sweden). Ni-NTA-agarose was purchased from Qiagen Inc. (Chatsworth,CA, U.S.A.). Dulbecco's minimum essential medium and fetal calf serum were from Flow Laboratories (Irvine, Scotland). TCDD was purchased from the Cambridge Isotope Laboratories (Cambridge, MA., U.S.A.). The Hepa-1 cell line was obtained from Dr. Sogawa of Tohoku University and the HepG2 cell line from Dr. Hori of Saga School of Medicine.

### Methods

*Cell culture and preparation of extracts:* Hepa-1 and HepG2 cells were cultured as described previously (15), with minor modification. Briefly, cells were grown in

Dulbecco's minimum essential medium containing 10% fetal calf serum,100 IU/ml penicillin and 0.1% streptomycin at 37°C in 95% air and 5% $CO_2$. Treatment of cells with TCDD was carried out at about 80% cell confluency. For TCDD treatment, either dimethyl sulfoxide as a control or 2 nM TCDD solubilized in dimethyl sulfoxide was added to the medium and growth was allowed to continue for 24 hours. Confluent cells were collected from tissue culture plates by scraping in phosphate buffered saline and centrifuged at 1000 x g. Pelleted cells were resuspended in 10 mM HEPES, pH 7.4. After swelling for 10 minutes on ice, the cells were collected by centrifugation as before and resuspended in 5 volumes of HEDG (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol). Cells were again collected by centrifugation, and the pellet was resuspended in an equal volume of HEDG and broken with 15 strokes in a Dounce homogenizer, using a tight pestle. The cell homogenates were centrifuged at 1000 x g for 15 minutes, and the supernatant was removed and recentrifuged at 105,000 x g for 1 hour. The clear supernatant as a cytosolic extract was stored at -80°C for future study.

Cell lysates were prepared essentially as cytosolic extracts; HEDG buffer containing 400 mM KCl was used instead of HEDG buffer.

*Construction of GST-ARNTexpression vector:* To generate the vector pGEX2T.hARNT (21-328 a.a.), the nucleotide region of 63-1574 of hARNT cDNA from HepG2 was amplified using the polymerase chain reaction (PCR) with the following primers:

ARNT-1; 5'-GTGGATCCGCGACTACTGCCAACCCCGA-3',
ARNT-2; 5'-ATGGATCCGTGGAAAGCTGCTCACGAA-3',
ARNT-3; 5'-TGGCAGCACACTCTATGATC-3', and
ARNT-4; 5'-CTGTTGCTGCTGCCTGGGTG-3'.

To make cloning easier, the *BamH 1* site was introduced in the primer ARNT-1 (Underlined sequences). Using standard cloning strategy, PCR products were cloned into pUC19 vector and the cloned product was digested with *BamH1* and *Stu1* to yield a fragment of nucleotide region 63-984 that encodes amino acid residues 21-328 of hARNT. Digested fragmens were ligated into pGEX2T which had been digested with *BamH1 and Sma1*. This construct was mapped by restriction digestion and sequenced.

The expression and purification of GST fusion protein was essentially as described by Guan and Dixon (16).

*Construction of $(His)_6$ tag Ah receptor expression vector:* To generate the vector pRSETA.mAHR (9-418 a.a.), the nucleotide region of 27-1516 of mAHR cDNA from C57BL/6J was amplified using the polymerase chain reaction with the following primers:

AHR-1: 5'-GTGGATCCGCTGAAGGAATT-3',
AHR-2: 3'-TAGGTACGACGTCTGTATGA-5',
AHR-3: 5'-AAGGGCAGCTTATTCTGGGC-3', and
AHR-4: 3'-CACTCCGACGCGACTTTGTA-5',

*BamH1* site was introduced in the primer AHR-1 (underlined sequences). The PCR products were cloned into pUC19, which had been digested with *BamH1* to yield a fragment of nucleotide region 27-1254 that encodes amino acid residues 9 to 418 of mAHR. The resulting fragment was ligated into pRSETA which had been digested with *BamHI*. This construct was mapped by restriction digestion and the junction at the 5' and 3' ends were sequenced. The fusion protein was expressed and purified from inclusion bodies according to the manufacturer's instructions (Invitrogen,CA, U.S.A.).

*Immunization of rabbits and purification of antibodies:* New Zealand white rabbits were immunized with recombinant GST-hARNT (21-328 a.a) and $(His)_6$-mAHR (9-418 a.a.) according to the following schedule. The first intramuscular injection of fusion

proteins (500 μg) emulsified with complete Freund's adjuvant (1:1, v/v) was followed by two subcutaneous injections of each fusion protein emulsified with incomplete adjuvant. The animals were bled 7 days after each booster immunization and the titer of the sera was determined by ELISA.

Purified GST-hARNT (21-328 a.a.) fusion protein was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The anti-hARNT sera was passed through the column twice. The column was washed with the washing buffer (0.1 M sodium acetate, pH 8.6, 0.5 M NaCl) and the bound fractions was eluted with elution buffer (0.05 M glycine.Cl, pH 2.3, 0.15 M NaCl). The antibody containing fractions were pooled and dialyzed against PBS, concentrated using an Amicon microconcentrator, and kept at -20°C for future study.

*Immunoblot:* Hepa-1 cell lysates were resolved on 10% SDS-PAGE and electrotransferred onto nitrocellulose membrane. The membrane was incubated with 0.1% Ponceau S to visualize the molecular weight markers, washed in distilled water, and blocked with 5% nonfat dry milk in TBS (10 mM Tris.Cl, pH 8.0., 150 mM NaCl) for 2 hours. The membrane was probed with 1μg/ml of affinity purified anti-ARNT antibody in TBS-T (0.5% Tween 20 in TBS) for 4 hours. Antigen-antibody complexes were detected with goat anti-rabbit antibody conjugated with alkaline phosphatase, using nitroblue tetrazolium and 5-bromo-4- chloro-3-indolyl phosphate as substrates.

*Immunoprecipitation:* Typically, 200 μg of appropriate lysates were diluted in immunoprecipitation buffer ( 50 mM Tris.Cl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 0.5% Nonidet P40, 5 μg/ml each of leupeptin, pepstatin, antipain, elastatinal, and chymostatin) to 1.2 ml. The lysates were precleared with Protein A Sepharose on ice for 2 hours. The precleared supernatant was mixed with 2 μg of affinity purified anti-ARNT antibodies; in the case of anti-mAHR sera or preimmune sera, 2 μl was used. After 2-3 hours of incubation on ice, antigen-antibodies complexes were collected by the addition of 50 μl of 50 % slurry of Protein A Sepharose. The immune complexes were washed four times with immunoprecipitation reaction buffer. The resulting immunoprecipitate was boiled with 25 μl of SDS-PAGE sample buffer for 7 minutes, resolved by 7.5 % SDS-PAGE, and visualized by silver staining.

Anti-ARNT immunocomplexes from HepG2 cytosols were resuspended in 2X SDS-PAGE sample buffer and boiled for 5 minutes. The supernatant was diluted 10 fold in immunoprecipitation buffer and reprecipitated with 2μg anti-ARNT.

## Results and Discussion

In an effort to obtain an ARNT specific antisera that could be useful in both immunoblot analysis and immunoprecipitation experiments, we raised rabbit antisera against a recombinant GST.hARNT (21-328 a.a.). To characterize the affinity purified antibody, we performed immunoblot analysis. The antibody recognized the recombinant GST.hARNT (21-328 a.a.), but not the bacterially expressed GST alone (data not shown). The presence of a single band about 95 kDa in size in Hepa-1 cell lysates indicate that the affinity purified antibody can detect the endogenous ARNT (Fig. 1). This is consistent with the known size of the ARNT protein (17,18). We adsorbed the antisera against the *E.coli* extract containing the GST. However, this absorption had no effect, i.e., both the absorbed and non absorbed antisera showed the same specificity against the cell extracts.

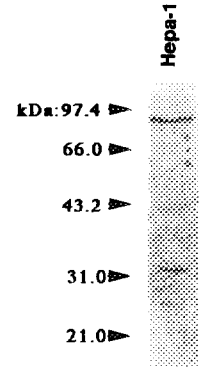


**Fig.1. Immunoblot:** Hepa-1 cell lysates (200 μg) were run on 10% SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted with anti-ARNT antibody as described under "materials and methods". The arrowhead indicates the relative position of ARNT protein. The asterisk indicates the possible proteolytic degradation product of ARNT.

To examine whether ARNT existed free in non-activated target cells or was associated with other protein(s), we performed immunoprecipitation of Hepa-1 cell lysates. It was possible that ARNT was stably associated with other protein(s) in a manner similar to the Ah receptor. Initial experiments using cell lysates of Hepa-1 revealed that in addition to about 95 kDa of ARNT, at least one additional polypeptide (approximately 120 kDa) was present in the anti-ARNT reactions but not in the control reactions in which preimmune sera of the same rabbit used for antibody production was employed. However, we found that crude cell lysates contain numerous proteins that bind directly to Protein A Sepharose, thus making detailed analysis and interpretation difficult. To avoid this problem, we used HepG2 cytosolic extracts and as expected, we found that the 120 kDa protein was also associated with ARNT (Fig. 2A, Lanes 4-6). Non-specifically bound protein was minimized using cytosolic extracts. Although ARNT is a nuclear protein, during the subcellular fractionation it appeared in the cytosol (19). The 120 kDa protein was also detected in anti-ARNT immunopreciptate from Hepa-1 cytosolic extracts (Fig. 2B).

When anti-ARNT immunocomplexes were dissociated in SDS and reprecipitated with the same antibodies, 120 kDa protein was not detectable in the repreciptated ARNT complex, suggesting that antibodies to ARNT do not directly cross-react with 120 kDa protein under these conditions (Fig. 2, Lane 7).

To clarify that the Ah receptor is not the 120 kDa protein identified in present study, we performed Immunoprecipitation experiments using anti-Ah receptor antisera. Since the Ah receptor was detected as about 110 kDa molecular weight protein (Fig. 2A, Lanes 1-3), it is highly unlikely that the Ah receptor could be the 120 kDa protein associated with ARNT, and it would seem that 120 kDa protein may be a unique protein. To further characterize this complex, we used TCDD treated cytosolic extracts

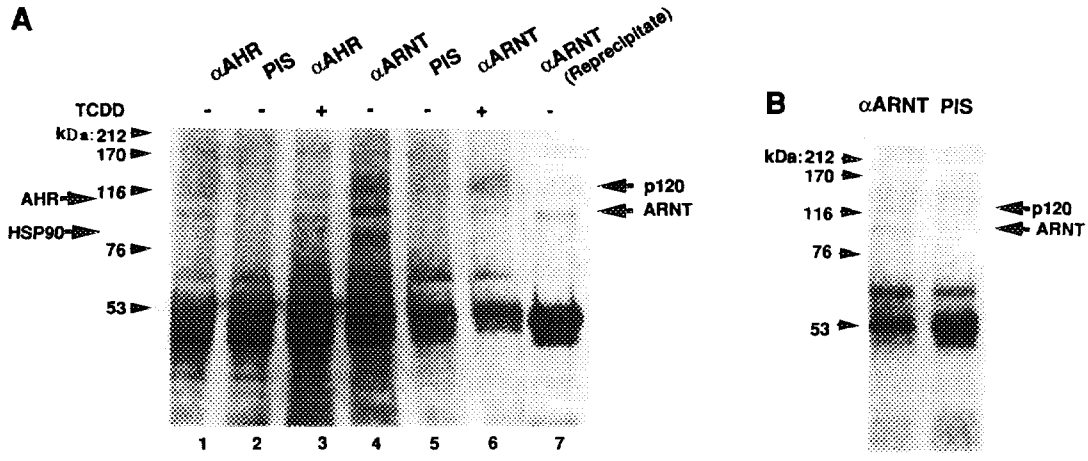


**Fig.2A. Immunoprecipitation using HepG2 cytosolic extracts:** Cytosolic extracts (200 µg ) from TCDD-treated and non-treated samples were co-immunoprecipitated with antibodies as indicated at the top of the each lane. In lane 7, HepG2 cytosolic extracts were first immunoprecipitated with anti-ARNT antibody as described above. The resulting immunocomplex was boiled in the presence of SDS-PAGE sample buffer to dissociate protein complexes, and the denatured proteins were diluted with *immunoprecipitation buffer for reimmunoprecipitation* with anti-ARNT antibody. The resulting immunocomplexes were analyzed by 7.5% SDS-PAGE and visualized by silver staining. (+) indicates TCDD treated and (-) indicates non-treated samples. Arrowheads on the right side indicate the ARNT associated factors and arrowheads on the left indicate the AHR associated factors. Small arrowheads indicate the relative position of the molecular weight markers. PIS; Preimmune sera of rabbit used for immunization of (His)6-AHR and GST-ARNT, α-ARNT; anti-ARNT, α-AHR; anti-Ah receptor antibody, p120; 120 kDa protein, AHR; Ah receptor, HSP90; Heat shock protein 90, ARNT; AHR nuclear translocator protein.

**Fig.2B. Immunoprecipitation using Hepa-1 cytosolic extracts:** *Immunoprecipitation experiments* were done using Hepa-1 cytosolic extracts described essentially as in legend to Fig.2A.

*for immunoprecipitation experiments.* In TCDD treated cytosolic extracts, there was a decrease of both ARNT and 120 kDa protein in the immunoprecipitate (Fig. 2, lane 6). One possible explanation for this finding could be that most of the ARNT remains in the nucleus as a member of the DNA bound form of the Ah receptor while little amount of ARNT and 120 kDa protein complex is distributed into the cytosol during cell fractionation. This notion is supported by the observation of Poland *et. al.* (19), who found that the quantity of ARNT in the cytosol decreased after TCDD treatment.

Whitlock and colleagues reported that about 110 kDa protein is present in the DNA bound form of the Ah receptor in the rat (20). However, there has been no report on the direct interactions between the Ah receptor and any other cellular protein of such a high molecular weight, except ARNT. The 120 kDa protein found in this study could be a component of the DNA bound form of the Ah receptor. The differences in molecular weight observed between two experiments, that of the Whitlock group and that of the present study, could be due to the species differences. This study also

established that 120 kDa protein directly interacts with ARNT rather than with the Ah receptor itself. By analogy with other transcription factors (21,22), 120 kDa protein could be act as a coactivator and serves as a contact between the XRE-bound complexes and general transcription factors.

One of the characteristics of the bHLH family of proteins is the formation of a variety of homo- and hetero-oligomers, resulting in multiprotein complexes with diverse DNA binding properties and biological functions (23). A different type of ARNT heterodimer may lead to functionally distinct ARNT actions, some being Ah receptor dependent and others not. A complete elucidation of the functions of 120 kDa protein awaits the molecular cloning of the gene encoding it.

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