that this novel protein inhibits AhR-dependent transactivation of the XRE-driven gene, and thus designated it AhR repressor (AhRR) [10].

Recently, AhRR orthologs have been reported in human [11,12], rat [11,13], and fish [14,15] and their genes have three conserved XRE sequences in the 5′-flanking promoter region. Accordingly, it has been reported that the AhRR expression is activated by the AhR/Arnt heterodimer in some cell lines [11,12] and multiple tissues of various species [10,12–14], indicating that AhRR participates in a negative feedback loop in the AhR signaling pathway [10,14,15].

Although the precise mechanism of inhibitory activity of AhRR remains to be elucidated, it has been proposed that AhRR competes with AhR for heterodimerization with Arnt and binding to the XRE sequence, a property that is likely to be mediated by N-terminal bHLH-PAS domains of these proteins [10].

In this study, we report that AhRR has a transcriptional repression domain within its C-terminal region, which exhibits a trichostatin A (TSA)-sensitive HDAC activity. By Cytotrap yeast two-hybrid screening with the C-terminal sequence of AhRR used as bait, we isolated Ankyrin-repeat protein2 (ANKRA2) as a binding partner to the AhRR C-terminal sequence.

Materials and methods

Plasmids. Fragments for AhRR(1–701 a.a.), AhRR(1–342 a.a.), AhRR(342–701 a.a.), AhRR(342–478 a.a.), AhRR(478–701 a.a.), AhRR(478–555 a.a.), AhRR(555–701 a.a.) were excised from pBOS-AhRR [10] and cloned into the pBOSGAL4DBD vector [16]. pG3TK-Luc was produced by inserting three copies of the GAL4 binding site excised from pG5EC and TK promoter sequence excised from pBSCAT2 into the XhoI site of pGL3 vector (Clontech). pBOSHA-AhRR was constructed as follows: pBOST7HA vector was constructed by inserting the blunt-ended BglII/PstI fragment of pGADT7 vector containing T7 promoter and HA epitope Tag into blunt-ended BamHI site of pEFBOS vector [17]. The EcoRI/SalI fragment excised from pBSKAhRR (Mimura, unpublished data) was inserted into the EcoRI and SalI site of pBOST7HA. pBOS-FLAG-ANKRA2 was constructed as follows: pBOST7FLAG vector was constructed by inserting the fragment containing the T7 promoter and FLAG epitope Tag cleaved from pGADT7FLAG vector into blunt-ended BamHI site of pEFBOS vector. Mouse ANKRA2 cDNA was amplified with a pair of primers, 5′-CatcgatACATGGCTACATCTGCAAAT-3′ and 5′-CggatccTCACTCCCTGATGTTCTGAA-3′ as the 5′ and 3′ primers, respectively. The amplified cDNA fragment was digested with ClaI and BamHI, and inserted into the ClaI and BamHI site of pBOST7FLAG. Expression plasmids encoding HDAC4-FLAG and HDAC5-FLAG were kindly provided by Dr. Stuart L. Schreiber (Harvard University, MA, USA).

Antibody production. Recombinant glutathione S-transferase (GST)-tagged mouse AhRR (342–701 a.a.) and recombinant maltose-binding protein (MBP)-tagged mouse AhRR (342–701 a.a.) were expressed in *Escherichia coli* and purified with Glutathione Sepharose 4B (Amersham Biosciences) and amylose resin (New England Biolabs), respectively, according to the manufacturer's protocols. Polyclonal rabbit antisera were raised against the recombinant GST-AhRR (342–701 a.a.) and further affinity-purified with the recombinant MBP-AhRR (342–701 a.a.) (Hokudo Inc., Japan).

Cell culture. Mouse embryonic fibroblast (MEF) cells were isolated from C57B/6J mice. COS-7, MEF, and Hepa-1c1c7 (Hepa-1) cells were maintained in high glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen) under 5.0% CO_2 at 37 °C.

Luciferase assay. Hepa-1 cells (5.0×10^4 cells/well) were grown in 24-well dishes for 24 h and were transfected with the expression plasmids indicated in the figure legends, pG3TK-Luc and the expression plasmids for sea pansy luciferase as an internal control using Lipofectamine™ (Invitrogen). Forty-eight hours after transfection, the cells were harvested and luciferase was quantified by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols. For control of transfection efficiency, firefly luciferase activity was normalized to cotransfected sea pansy luciferase activity as a standard.

Coimmunoprecipitation and immunoblot analysis. Cell lysates from the transfected COS-7 and MEF cells were prepared as described and used for immunoblot analysis either directly or after immunoprecipitation. Immunoprecipitation with anti-Flag M2 agarose (Sigma) or anti-HA agarose (Sigma) was performed for 12 h and the immunoprecipitates were washed according to the published procedure for immunoblot analysis. Immunoblot analysis was performed as described [7] using anti-FLAG (Sigma), anti-HA (Sigma), anti-Tubulin (Sigma), and anti AhRR antibodies.

RNA interference experiments. The siRNAs for mouse ANKRA2 or mouse AhRR were designed and synthesized by B-Bridge International Inc. The coding sequences were: ANKRA2, (5′-AGGAAAAGGUCGAG AAAGUdTdT-3′) and AhRR, (5′-GGAAAGGCCUUGUGGCUAAdT dT-3′). Hepa-1 cells or MEF cells (5.0×10^4 cells/well) were transfected with siRNA for ANKRA2 (20 pmol) or AhRR (50 pmol) with or without expression plasmids by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Quantification of mRNA. Total RNAs are extracted from cells using Isogen (Nippon Gene, Tokyo) and reverse transcribed by Superscript II (Invitrogen). Real-time PCR was carried out in ABI PRISM 7700 sequence detection system using the following primer sets: ANKRA2, forward (5′-TCTACCACACCTCTGTTAGC-3′) and reverse (5′-GCA CTTTCTCGACCTTTTCC-3′); AhRR, forward (5′-GCTTTCTGTCCT GCGCCTC-3′) and reverse (5′-TCCTTCCTGCACGGGGAAC-3′); CY P1A1, forward (5′-GGACATTTGAGAAGGGCCAC-3′) and reverse (5′-CGTCCAGCTTCCTGTCCTGA-3′); actin, forward (5′-GACAGGAT GCAGAAGGAGAT-3′) and reverse (5′-TTGCTGATCCACATCTGC TG-3′).

Cytotrap yeast two-hybrid assay. The CytoTrap™ (Stratagene) yeast screening was performed with a murine thymus cDNA library (Stratagene) and pSos-AhRR (342–701) as prey and bait, respectively, according to the manufacturer's instructions.

Results

Functional characterization of the AhRR transcriptional repression domain

We previously reported that AhRR inhibits the transcription activity of Arnt [10]. In order to confirm transcriptional repression activity and to localize the transcriptional repression domain of AhRR, we fused a series of AhRR deletion mutants to the GAL4 DNA binding domain (GAL) (Fig. 1A). These fragments were transfected into Hepa-1 cells, along with a luciferase reporter gene driven by three GAL4 binding sites and the TK promoter (Fig. 1A). The luciferase activity driven by the TK promoter was repressed 5.2-fold by the transfection with plasmid, GAL4DBD-AhRR (1–701) (Fig. 1B). Plasmids encoding the fusion proteins, GAL4DBD-AhRR (342–701), (478–701), and (555–701) also repressed luciferase expression 5.6-, 4.9- and 3.7-fold, respectively, while plasmids of AhRR (1–342), AhRR (342–478), and AhRR

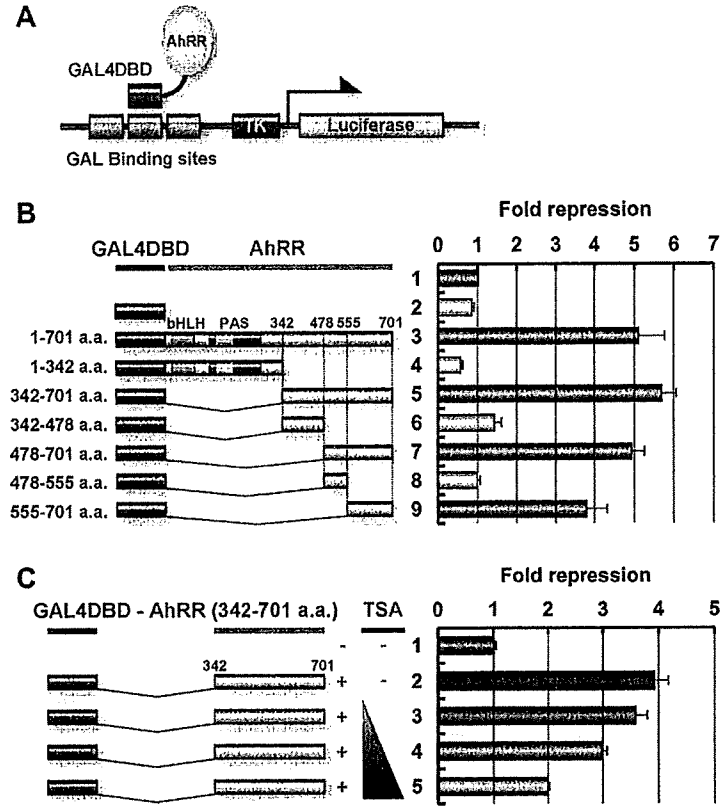

Fig. 1. The carboxy-terminal half of AhRR shows a repressor activity, which is sensitive to the HDAC inhibitor, TSA. (A) Schematic representation of luciferase reporter assay. (B) Transcriptional repression activity of AhRR. Hepa-1 cells were transiently transfected with the expression plasmids of GAL4DBD-AhRR and GAL-TK-Luc reporter gene containing three GAL binding sites. Cell extracts were prepared 48 h after transfection and used for luciferase assays. The fold repression is relative to the reporter gene alone. (C) Effects of TSA on AhRR-mediated transcriptional repression. The transfections were performed as described in (B), Cells were treated with increasing amounts (2, 4, and 8 ng/ml) of TSA 24 h after transfection and then, 12 h later, whole cell extracts were prepared and used for luciferase assays.

(478–555) did not significantly repress luciferase expression. These data localized a core region needed for transcriptional repression by AhRR to the sequence of amino acid 555–701.

To investigate how the repression activity of the AhRR fragment (342–701) functions in the TK promoter-driven reporter system, we used the HDAC inhibitor, TSA, which reversed the repression of reporter gene expression by the AhRR fragment (342–701) in a dose-dependent manner (Fig. 1C). These results suggest that the repression activity of the AhRR C-terminus is due to an HDAC activity. Since the C-terminal sequence of AhRR is well conserved among multiple mammalian species (Supplementary Fig. S1), we next searched for transcriptional corepressor, which interact with the AhRR C-terminus.

Isolation of ANKRA2 as a factor interacting with AhRR and interaction of AhRR with ANKRA2 and HDAC4 or HDAC5

To isolate a corepressor of AhRR, we performed a Cytotrap yeast two-hybrid screen with the C-terminal fragment of AhRR (342–701) used as bait (Fig. 2A) and isolated several clones including Dhx8, EB1, EB3, p21, Prostaglandin E receptor, EGF-containing fibrin-like extracellular matrix protein1, and ANKRA2. We chose ANKRA2 for further work in this paper, because ANKRA2 is reported to interact with HDAC4 and HDAC5 [18]. Recently, its mammalian paralogue, RFXANK has also been reported to interact with HDAC4 and HDAC5 [18] and to repress MHC class II promoter activation through association with HDAC4 and HDAC5 [19]. Taken together, these results suggest a potential role of ANKRA2 as mediator in transcriptional repression. ANKRA2 is a protein of 312 amino acids with consecutive 3 ankyrin repeats and the cDNA encoded a C-terminal fragment, amino acid 117–312 (Fig. 2A).

To address whether a physical interaction occurs between AhRR and ANKRA2, whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 were used for *in vivo* coimmunoprecipitation studies. As expected, when the cell extracts were immunoprecipitated with an anti-FLAG antibody, AhRR was coimmunoprecipitated

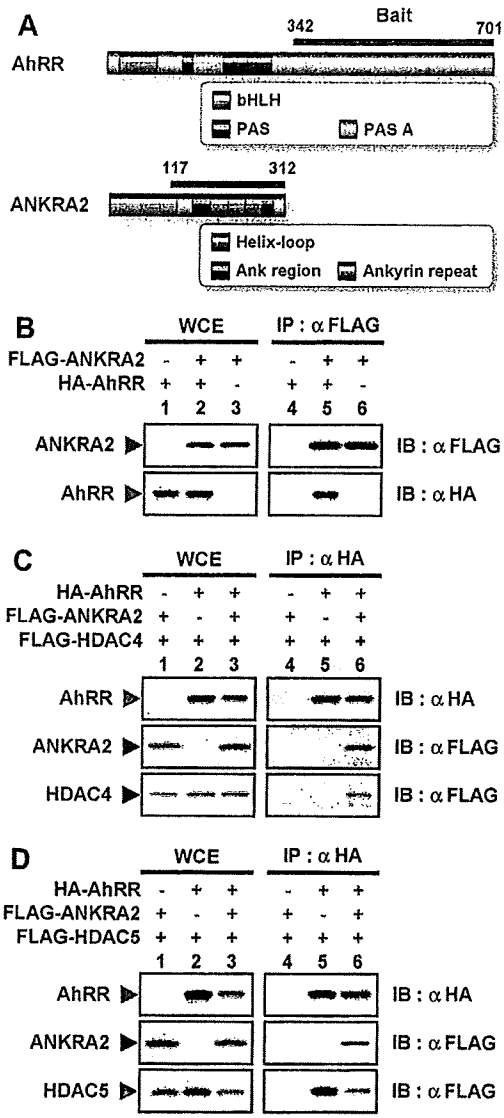

Fig. 2. Physical interaction of AhRR with ANKRA2 and HDAC4 or HDAC5. (A) Schematic diagrams of AhRR and ANKRA2. The carboxy-terminal half of AhRR was used as bait in a yeast two-hybrid screen. The AhRR bait interacted with a fragment (117–312 amino acid) of ANKRA2. ANKRA2 is a protein of 312 amino acids that contains an amino-terminal helix-loop-helix domain and four ankyrin repeats at the C terminus. (B) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR and FLAG-tagged ANKRA2. Whole cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-FLAG antibodies. Immunoprecipitates were analyzed by immunoblot with the indicated antibodies. Crude lysates were analyzed by immunoblot to control protein expressions (WCE). (C) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR, FLAG-tagged ANKRA2 and either FLAG-tagged HDAC4 or HDAC5. Cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-HA antibodies. Proteins were analyzed by immunoblot as described above.

with ANKRA2 (Fig. 2B, lane 5). We next asked if HDAC4 and HDAC5 also interact with AhRR. Whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 together with either FLAG-tagged HDAC4 or FLAG-tagged HDAC5 were immunoprecipitated with an anti-HA antibody. HDAC4 was coimmunoprecipitated with AhRR only when ANKRA2 was cotransfected (Fig. 2C, lane 6). On the other hand, HDAC5 was also coimmunoprecipitated with AhRR even in the absence of ANKRA2, indicating that HDAC5 interacts with AhRR either directly or through ANKRA2 (Fig. 2D, lanes 5 and 6).

Depletion of ANKRA2 by siRNA attenuates transcriptional repression activity of AhRR

If ANKRA2 is a corepressor for AhRR, downregulation of the endogenous level of ANKRA2 by siRNA against ANKRA2 should reverse the transcriptional repression activity of AhRR. To confirm the requirement of ANKRA2 for the repression activity of AhRR, Hepa-1 cells were transiently transfected with expression plasmids for GAL-AhRR together with ANKRA2 siRNA or control siRNA. As shown in Fig. 3A, ANKRA2 siRNA transfection significantly reduced the expression of ANKRA2 mRNA. As expected, when the cells were treated with ANKRA2 siRNA, the repression activity of AhRR was significantly reversed as compared with that observed in cells treated with control siRNA (Fig. 3B).

To further investigate whether ANKRA2 is required for the endogenous AhRR/Arnt heterodimer to repress the XRE-driven transcription of endogenous target genes such as *CYP1A1*, we conducted RNA interference experiments in MEF cells. When MEF cells were transfected with ANKRA2 siRNA, endogenous ANKRA2 mRNA levels were significantly lowered both in the presence or absence of an AhR ligand, 3MC (Fig. 3C). Under normal conditions, CYP1A1 mRNA expression was barely detectable, but knockdown of ANKRA2 by ANKRA2 siRNA caused a small, but significant increase in basal CYP1A1 mRNA levels (Fig. 3D, lane 1). When treated with 3MC for 6 h, MEF cells inducibly expressed CYP1A1 mRNA to a similar level in the presence and absence of ANKRA2 siRNA (Fig. 3D, lane 2). These results suggest that in the silent state of MEF cells, the AhRR-ANKRA2 suppressor complex is involved in silencing the CYP1A1 basal expression, while under inducing conditions, ANKRA2 does not greatly affect the inducible expression of *CYP1A1* gene.

To confirm that the AhRR–ANKRA2 repressor complex silences the transcription of endogenous CYP1A1 in MEF cells, we used siRNA against AhRR. As previously reported in many other cell lines and tissues, AhRR mRNA and protein was constitutively expressed in MEF cells under normal conditions and was further enhanced in response to 3MC (Fig. 3E, lanes 1 and 2, and F). When MEF cells were transfected with AhRR siRNA, both mRNA and protein levels of AhRR were markedly downregulated (Fig. 3E, lanes 1 and 3, and F). In agreement with the result of the ANKRA2 siRNA experiment, treatment of AhRR siRNA increased CYP1A1 mRNA under normal

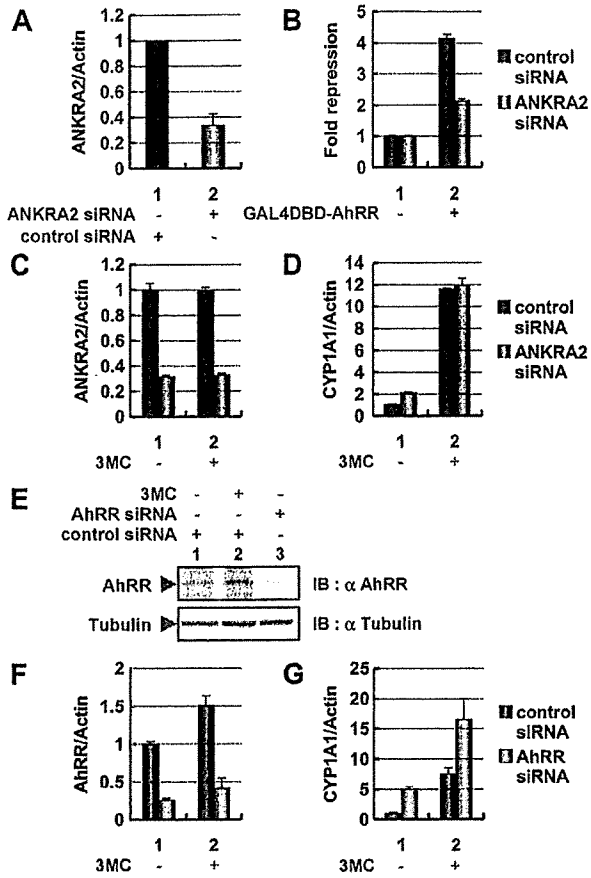

Fig. 3. Effects of ANKRA2 and AhRR siRNAs on the transcriptional repression activity of AhRR. (A) Hepa-1 cells were transiently transfected with ANKRA2 or control siRNA and 48 h after transfection, RNA were prepared from the transfected cells. ANKRA2 mRNA was quantified by RT-PCR method. (B) Hepa-1 cells were transiently transfected with GAL4DBD-AhRR and a reporter gene of GAL-TK-Luc, along with ANKRA2 siRNA or control siRNA. Cell extracts were prepared 48 h after transfection and used for luciferase assays. (C–G) MEF cells were transiently transfected with ANKRA2 siRNA (C and D) or AhRR siRNA (E–G) or control siRNA. After 48 h of transfection, cells were treated with 2 μM of 3MC or Me_2SO and then, 24 h later, cell extracts were prepared and indicated mRNA expression level was quantified using real time RT-PCR (C, D, E, and F) or immunoblot analysis (E).

conditions to even a higher extent than ANKRA2 siRNA treatment (Fig. 3G, lane 1). Interestingly, AhRR siRNA also further increased the induced expression of CYP1A1 mRNA in response to 3MC, as compared with the expression in cells treated with control siRNA, suggesting the existence of an ANKRA2-independent repression mechanism by AhRR (Fig. 3G, lane 2). As reported previously, AhRR may prevent AhR from forming a heterodimer with Arnt in a competitive manner, thereby blocking the binding of AhR to the XRE sequence in the absence of ANKRA2. Taken together, these results indicate that in a silent state of CYP1A1 expression under normal conditions, AhRR represses the expression of *CYP1A1* gene by binding the XRE sequence and recruiting ANKRA2, HDAC4 and/or HDAC5.

Discussion

We previously reported that AhRR repressed AhR transcription activity by competing with Arnt in AhR/Arnt heterodimerization, as well as by binding the XRE sequence in the promoter of CYP1A1 gene. Since Arnt has a weak transcription activity at its C-terminal end, it is possible that AhRR could have some inhibitory activity to compensate for Arnt transcription activity. In this report, we first demonstrated that the C-terminus (555–701 a.a.) of AhRR shows a transcription inhibitory activity which was sensitive to the HDAC inhibitor, TSA, suggesting that AhRR which binds the XRE sequence recruits a HDAC protein. To isolate factors that interact with the C-terminal sequence of AhRR, we utilized the Cytotrap yeast two-hybrid screening method using the C-terminal sequence of AhRR as bait, resulting in isolation of a fragment (113–312 a.a.) of ANKRA2 consisting of 312 amino acids and containing three ankyrin (ANK) repeats. The isolated ANKRA2 fragment was shown to physically interact with AhRR (Fig. 2B). ANK repeats are one of the most common protein sequence motifs mediating protein–protein interactions, but they have not been clarified to bind any specific amino acid sequence or structure. Rather, they are thought to bind a variety of proteins through adaptive alterations in their binding surface features and in the domain size of the ANK repeat by sequence duplication or deletion [20]. Recent studies have demonstrated that ANKRA2 also interacts with megalin [21] and the α-subunit of rat large-conductance Ca^{2+}-activated K^+ channel (rSlo) [22]. According to Rader et al. [21], the C-terminus (177–312 a.a.) of ANKRA2 interacts with a proline-rich motif (PXXPXXP) within the 19 amino acid sequence of the magalin tail, and Lim and Park [22] have shown that 52–150 a.a. of ANKRA2 interacts with the C-terminal end (1119–1210 a.a.) of rSlo Channel. Since there is no apparent sequence similarity in the ANKRA2-interacting domains of megalin, rSlo and AhRR, the precise molecular mechanisms how ANKRA2 interacts with these proteins remain to be investigated.

Our coimmunoprecpitation experiments have revealed that HDAC4 interacts with AhRR in an ANKRA2-dependent manner. HDAC5 may interact with AhRR either directly or via ANKRA2, when the previous report is taken into account [18]. It remains to be studied how HDAC4 and HDAC5 are recruited on the surface of the AhRR and ANKA2 complex, reciprocally or simultaneously. ANKRA2 is also abundantly and ubiquitously expressed in various tissues of mice [21] and cultured cells such as Hepa-1, HeLa and MEF cells used in this study (data not shown). For functional analysis of ANKRA2 and AhRR, we used siRNA to knock down gene expres-

sion. In Hepa-1 cells, repression of TK promoter-driven luciferase activity by GAL4DBD-AhRR was reversed by the addition of ANKRA2 siRNA, indicating that the repression activity of AhRR required ANKRA2. Treatment of MEF cells with ANKRA2 siRNA significantly activated the expression of *CYP1A1* under normal conditions. In contrast, the siRNA treatment had apparently no effect on the enhanced CYP1A1 expression in response to the inducer. These results suggest that under normal conditions, a silent state of *CYP1A1* gene expression is not merely due to the lack of a transcription activator, but resulted from negative regulation by a heterodimer of AhRR and Arnt, which recruits ANKRA2. This observation was substantiated by the experiments using AhRR siRNA, which significantly enhanced the expression of *CYP1A1* gene under normal conditions to even a higher level than did ANKRA2 siRNA under the non-inducing conditions. The greater effect of AhRR siRNA on CYP1A1 expression becomes more pronounced under inducing conditions. These observations could be explained by a two step inhibitory mechanism. First, AhRR inhibits the transcription activity of AhR in an ANKRA2-independent manner, by competing with AhR for forming a heterodimer with Arnt and binding the XRE sequence, as reported previously. Next, AhRR bound to the XRE sequence recruits ANKRA2 and HDAC4 and/or HDAC5 for more efficient repression. In the presence of the inducer 3MC, AhRR synthesis is accelerated so that the AhRR siRNA treatment displays a greater effect on the inducible expression of CYP1A1 than ANKRA2 siRNA. Recently, we have found that the silent state of CYP1A1 is actually negatively regulated by the AhRR system in macrophages (unpublished data). We will be investigating how this silencing mechanism involving AhRR and ANKRA2 functions in different cell types and how it affects target genes other than *CYP1A1* under normal conditions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.131.

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Aryl Hydrocarbon Receptor Plays a Significant Role in Mediating Airborne Particulate-Induced Carcinogenesis in Mice

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Urban particulate air pollution is associated with an increased incidence of cancers, and especially lung cancer. Organic extracts of airborne particulate matter (APM) cause cancer in mice, and PAHs adsorbed to APM are associated with particle-induced carcinogenesis. PAHs are agonists for AhR and are predominantly responsible for lung cancer through induction of highly carcinogenic metabolites. PAH metabolization requires CYP1A1 induction through activation of AhR, and therefore we hypothesized that carcinogenesis due to PAHs in APM would be reduced in AhR−/− mice. To examine this hypothesis, we performed a long-term continuous-application study of carcinogenesis in AhR−/− mice using airborne particulate extract (APE) of APM collected in Sapporo. Tumor development (squamous cell carcinoma) occurred in 8 of 17 AhR+/+ mice (47%), but no tumors were found in AhR−/− mice, and CYP1A1 was induced in AhR+/+ mice but not in AhR−/− mice. These results demonstrate that AhR plays a significant role in APE-induced carcinogenesis in AhR+/+ mice and CYP1A1 activation of carcinogenic PAHs is also of importance. Therefore, measurement of CYP1A1 induction in vitro may be useful for assessment of APM-induced carcinogenesis in humans. We also show that PAH-like compounds are major contributors to AhR-mediated carcinogenesis, whereas TCDD and related compounds make a smaller contribution.

Introduction

Urban air particulate matter (APM) are mutagenic in short-term genetic bioassays (*1*, *2*), and many exhibit carcinogenic activity in vitro and in vivo (*3*). Epidemiological studies show that urban particulate air pollution is a risk factor for lung cancer (*4*, *5*). However, the biological mechanisms underlying APM carcinogenicity remain unknown.

Among the numerous genotoxic and carcinogenic compounds adsorbed onto urban APM, polycyclic aromatic hydrocarbons (PAHs) are the most prominent because of their known carcinogenic and/or mutagenic properties (*6*, *7*). The carcinogenicity of PAHs occurs through metabolic activation by cytochrome P450 and epoxide hydrolase, which results in formation of highly carcinogenic diol-epoxide metabolites that form DNA adducts that initiate the carcinogenic process (*8*).

Among the cytochrome P450s, CYP1A1 and CYP1B1 have important roles in metabolic activation of carcinogenic PAHs (*9*). Induction of drug-metabolizing enzymes including CYP1A1 and CYP1B1 by PAHs and other environmental contaminants is mediated by a ubiquitous intracellular receptor called the aryl hydrocarbon receptor (AhR) (*10*). AhR is a ligand-activated transcription factor that occurs in many cells and tissues and mediates PAH-induced toxicity, teratogenicity, and carcinogenicity (*11*). Increased expression of AhR occurs in human lung carcinoma compared to normal human lung tissues (*12*). APM extracts induce CYP1A1 and CYP1B1 in the human lung-derived cell line CL5 (*13*) and show significant AhR-mediated activity in vitro in ethoxyresorufin-*O*-deethylase (EROD) induction and in an AhR luciferase reporter system (*14*).

Previously, we have shown that the skin carcinogenicity of BaP, a prototypical PAH, is lost in AhR-deficient (AhR−/−) mice, suggesting that AhR-mediated induction of CYP1A1 is important in BaP-induced skin carcinogenesis in mouse (*15*). We also reported that the skin carcinogenicity of dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), a powerful carcinogenic PAH (*16*), was dramatically suppressed in AhR−/− mice, suggesting that the AhR-induced CYP1A1 expression may correlate with susceptibility to DB[*a*,*l*]P carcinogenesis (*17*). These findings imply that AhR-mediated induction of P450s including CYP1A1 is important in activation of PAHs in mouse carcinogenesis.

For evaluating health risks, an understanding of the role of AhR in carcinogenesis caused by environmental mixtures is of importance since people are exposed to such mixtures in daily life. To date, there is no direct proof that AhR plays a significant role in vivo as a mediator of carcinogenesis of environmental mixtures, including APM. However, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as well as PAHs can bind to AhR to elicit induction of P450s, and urban APM is a complex mixture of substances such as PAHs, TCDD, and related compounds. Therefore, the immunotoxic and carcinogenic reactions elicited by PAHs and TCDD in APM may be mediated by AhR.

The main objective of this study was to examine whether AhR signaling has a net potentiating effect on APM carcinogenicity in mice. Skin tumorgenesis was investigated by long-term treatment with an APM extract (airborne particulate extract; APE) collected by hi-volume samplers, through topical application to the skin of wild type AhR+/+ and AhR−/− mice. A second aim was to evaluate the AhR-mediated biological activity of APE and to differentiate the effect on this activity of PAHs from that of TCDD and related compounds. For this purpose, AhR-mediated activity was determined with crude and cleaned APE, using a reporter-gene assay based on chemically activated luciferase expression (the CALUX assay).

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Materials and Methods

Air Particulate Samples. APM samples were collected on the roof of the Hokkaido Research Institute of Environmental Sciences, which is situated in a residential area about 2 km from the center of Sapporo and entirely surrounded by fields and grounds and not affected directly by vehicle exhaust PM. APM was collected on glass or tissue quartz-filters using high-volume samplers at a rate of 80 m^3/h during the cold season (October–March) from 1973 to 1986. Exposed filters were replaced with new filters daily. A total of 910 24-h filter samples were obtained by filtering 1 770 000 m^3 of air. After weighing the filter samples, they were placed in plastic sacks, vacuum sealed, and maintained in the refrigerator room at a constant −20 °C. The extraction procedure was conducted immediately before the chemical analysis, in vitro bioassay, and the beginning of the animal experimentation (April 2001). To ensure storage of filter samples was suitable, comparative studies were made for the 19-year period. During the storage of the filter samples from 1988 to 2007, no significant changes in mutagenic activity and PAHs concentration were detected. Thus, we presume that there was little or no degradation of the stored samples over time (1973–2001).

Extraction of Organic Matter. Organic material was extracted from the filter samples by ultrasonication using dichloromethane. After filtration through paper to remove undissolved matter, the APE samples were combined. A portion was dried, dissolved in hexane or dimethyl sulfoxide, and used for analysis of PAHs and TCDD and related compounds or for mutagenicity testing and the CALUX assay, respectively. Another portion of APE was used in a skin-painting experiment.

Chemical Analysis and Mutagenicity Test of APE. Sixteen PAHs including six carcinogenic PAHs according to IARC evaluation in APE (Table 1) were analyzed on an Agilent 6890 gas chromatograph (GC) with a 5973 mass spectrometer (MSD) using selected ion monitoring. A DB-5 MS column (J&W Scientific; 30 m × 0.25 mm i.d.; film thickness, 0.25 μm) was used to separate the PAHs. Dioxin-like compounds (17 polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzo-*p*-furans (PCDFs)) and 12 PCBs were analyzed by high-resolution gas chromatography-high-resolution mass spectrometry (HRGC-HRMS). A toxicity equivalent (Eq) value for each compound was calculated using the WHO-toxicity equivalent factor (TEF). The total Eq concentration based on HRGC-HRMS analysis (Chemical TCDDEq) for APE was yielded by summation of the calculated Eq concentration for each dioxin-like compound. Mutagenicity of APE was examined by preincubation in the Ames mutagenicity test (*18*) using *Salmonella typhimurium* TA98 and TA100 with (+S9 mix) and without metabolic activation (−S9 mix).

Determination of AhR Mediated Activity of APE. AhR-mediated activity of APE was determined by the CALUX assay, which is based on a genetically engineered rat H4IIE hepatoma cell line with an AhR-controlled firefly luciferase reporter gene construct for detection of CYP1A1-inducing compounds in APE. The assays were performed in 96-well plates as described previously (*19*). Briefly, 24 h after seeding the cells were dosed with crude APE or cleaned APE. Using a sulfuric acid silica column, the APE was cleaned by removing PAHs and PAH-related compounds (PAH-like compounds), including PAHs, nitroarenes, aza-arenes, aminoarenes, methyl-
arenes, etc. The exposure time was either 3 h to measure most compounds (especially PAHs) or 24 h to measure TCDD and related compounds, which are resistant to biotransformation in the cells. The final results are expressed as toxicity equivalents (Eq) in the CALUX assay for BaP (CALUX BaPEq) or TCDD (CALUX TCDDEq), based on the CALUX assay concentration–response curve of BaP or TCDD.

Detection of CYP1A1 by RT-PCR. APE (6.4 mg) was applied to the shaved back of mice once a week for 4 weeks. Six days after the last application, aliquots of RNA (1 μg) were extracted from the dorsal skin of control and APE-treated mice of the two genotypes, and the expression of CYP1A1 mRNA was determined by RT-PCR.

Preparation of Sample Extracts for skin Painting. The mutagenic activities in TA100 with S9 mix per unit of APE and BaP were 4840 and 310 000 revertants/mg, respectively. The amount of extract equivalent to mutagenicity of 1 mg of BaP in the Ames assay was 64 mg (310 000 ÷ 4840 = 64) of APE. Extract for skin painting was adjusted to a concentration equivalent to the mutagenic toxicity equivalent of BaP (M BaPEq) 100 μg (= APE 6.4 mg)/200 μL of acetone.

Animals Procedures. AhR−/− mice were developed by Mimura et al. (*20*). AhR+/+ (n = 17) and AhR−/− (n = 15) female mice aged 6–8 weeks old were used in the study. All mice were genotyped by PCR screening of DNA from the tip (*15, 20*). The animals were housed in clean racks in a filtered-air environment under controlled conditions of temperature (22 ± 1°C), relative humidity (50 ± 5%), and a 12-h light-dark cycle. Sterilized diets and water were available ad libitum throughout the study.

Treatment and Tumor Induction. The dorsal skin of AhR+/+ and AhR−/− mice was shaved 2 days before treatment. Acetone suspensions of APE at 32 mg/mL (equivalent to 500 μg/mL M BaPEq) prepared from the combined APE were epicutaneously dropped onto the shaved backs in a volume of 200 μL. A single application of 200 μL containing 6.4 mg of APE is equivalent to 100 μg M BaPEq. This dose of APE was chosen with toxicity in mind and to obtain data for comparison with the results of the continuous application test (*15*), in which 200 μg of BaP was used once a week. After the application, the mice were restrained until the acetone had completely evaporated. Treatment was repeated continuously once a week until a skin tumor was detected. Animals were inspected weekly for tumor development, and the numbers of skin tumor lesions of larger than 2 mm were counted. The mice were sacrificed and dissected in the 58th week. The main organs were fixed in 10% neutral-buffered formalin, embedded in paraffin as tissue slices, and sectioned and stained with hematoxylin and eosin for microscopic histopathological evaluation.

Statistical Analysis. The statistical significance of the difference in tumor incidence between AhR+/+ and AhR−/− mice was evaluated by Student t-test. A p value of less than 0.05 was considered significant.

Results

PAH Concentrations in APE. Quantitative chemical analysis of APE showed the presence of many carcinogenic PAHs, including BaP, dibenzo[*a,h*]anthracene (DahA), and indeno-[1,2,3-*cd*]pyrene (IND) (Table 1). Measured PAH concentrations were corrected for biological activity and expressed as BaP toxicity equivalent (BaPEq) concentration. The BaPEq concentration for each PAH was calculated by multiplying the PAH concentration by the corresponding TEF (*21*), using the TEFs given by Nisbet and LaGoy (*22*).

The BaPEq concentration and the relative contribution to carcinogenic activity of each PAH, expressed as a percentage of the total BaPEq concentration of the mixture, and the measured air concentration of each PAH are given in Table 1. Benzo[*b+j*]fluoranthene (BbjF) had the highest measured air concentration, followed by chrysene (Chr), benzo[*ghi*]-perylene (BghiP), IND, BaP, and BaA. A total BaPEq concentration of 10.2 ng/m^3 was calculated for APE (Table 1), and the BaPEq concentration of 5.6 ng/m^3 for BaP accounted for 55% of the total; therefore, BaP contributed most to the total calculated BaPEq concentration, in agreement with literature data (*23, 24*).

TABLE 1. Measured and TEF[a]-Adjusted (BaPEq) PAH Concentrations in APE

compound (abbreviation)	TEF[a]	measured concentration ng/m³, air	measured concentration (ng/mg, APE)	BaPEq concentration ng/m³, air	BaPEq concentration (ng/mg, APE)	rel contribution in BaPEq (%)
naphthalene (Naph)	0.001	0.23	(25)	0.0002	(0.025)	0.0
acenaphthylene (Aceny)	0.001	0.11	(12)	0.0001	(0.012)	0.0
fluorene (Flu)	0.001	0.13	(14)	0.0001	(0.014)	0.0
phenanthrene (Phen)	0.001	1.6	(173)	0.0016	(0.173)	0.0
anthracene (Ant)	0.01	0.38	(41)	0.0038	(0.41)	0.0
fluoranthene (Fluor)	0.001	4.2	(453)	0.0042	(0.45)	0.0
pyrene (Pyr)	0.001	4.6	(496)	0.0046	(0.50)	0.0
benzo[*a*]anthracene (BaA)[b]	0.1	5.5	(593)	0.55	(59)	5.4
chrycene (Chr)	0.01	9.1	(982)	0.091	(9.8)	0.9
benzo[*b*+*j*]fluoranthene (BjbF)[b]	0.1	11	(1187)	1.1	(119)	10.8
benzo[*k*]fluoranthene (BkF)[b]	0.1	4.6	(496)	0.46	(50)	4.5
benzo[*a*]pyrene (BaP)[b]	1	5.6	(604)	5.6	(604)	54.8
indeno[1,2,3-*cd*]pyrene (IND)[b]	0.1	6.2	(669)	0.62	(67)	6.1
dibenzo[*a*,*h*]anthracene (DahA)[b]	1	1.7	(183)	1.7	(183)	16.6
benzo[*ghi*]perylene (BghiP)	0.01	8.7	(939)	0.087	(9.4)	0.9
total				10.2	(1103.0)	100

[a] Data from ref *22*. [b] Probably or possibly carcinogenic to humans according to IARC evaluation.

TABLE 2. CALUX BaPEq, CALUX TCDDEq, and Chemical TCDDEq Concentrations in Crude or Cleaned APE

	CALUX BaPEq[a] ng/m³, air (ng/mg, APE)	CALUX TCDDEq[b] pg/m³, air (ng/mg, APE)	chemical TCDDEq[c] pg-TEQ/m³, air (pg-TEQ/mg, APE)	
crude APE	979 (105634)	21 (2266)		
cleaned APE	7.87 (849)	0.613 (66)		
				0.13 (14)
			PCDDs TEQ	0.036 (3.9)
			PCDFs TEQ	0.091 (9.8)
			Co-PCBs TEQ	0.0072 (0.78)

[a] CALUX BaPEq : BaP equivalent based on CALUX assay using a BaP standard curve. [b] CALUX TCDDEq : TCDD equivalent based on CALUX assay using a TCDD standard curve. [c] Chemical TCDDEq :TCDD equivalent based on HRGC-HRMS analysis.

Mutagenic Activity of APE. The TA100 strain was more sensitive to the mutagens in APE than the TA98 strain, both with and without metabolic activation. Addition of S9 produced an increase in mutagenic response in both strains, which indicates the presence of promutagens in APE (Data are not shown.).

AhR-Mediated Activity (CALUX Assay) and TCDD Concentration in APE. Luciferase expression induced by crude and cleaned APE was transformed into BaP or TCDD equivalent concentration using a BaP or TCDD standard curve based on response in the CALUX assay after a 3-h or 24-h exposure time. These data (CALUX BaPEq, CALUX TCDDEq) and the results of HRGC-HRMS analysis of TCDD and related compounds in cleaned APE (Chemical TCDDEq) are shown in Table 2. The CALUX BaPEq concentration of crude APE was 979 ng/m³ air, or 105634 ng/mg APE, and the measured BaP concentration (5.6 ng/m³) (Table 1) accounted for only 0.57% of CALUX BaPEq. The CALUX BaPEq of cleaned APE (7.87 ng/m³) accounted for only 0.80% of CALUX BaPEq for crude APE, suggesting that most of the CALUX BaPEq for crude APE was derived from PAH-like compounds in APE. Therefore, the small amount of CALUX BaPEq derived from TCDD and related compounds in cleaned APE could be ignored. Similarly, CALUX TCDDEq for cleaned APE (0.613 pg/m³) accounted for only 2.9% of CALUX TCDDEq for crude APE, showing that the contribution of TCDD and related compounds to CALUX TCDDEq for crude APE was very small. The concentration of TCDD and related compounds (Chemical TCDDEq) in the HRGC-HRMS analysis of cleaned APE was 0.13 pg/m³, and the relative rate of luciferase induction of TCDD for BaP (=1) after a 3-h exposure was 3.85×10^4. Based on these numbers, it was calculated that 0.13 pg/m³ of Chemical TCDDEq would be equivalent to 5.01 pg/m³ of CALUX BaPEq and that the contribution of this value to CALUX BaPEq for crude APE was as low as 0.51%. The contribution of 0.13 pg/m³ of Chemical TCDDEq to CALUX TCDDEq for crude APE was also low (0.62%).

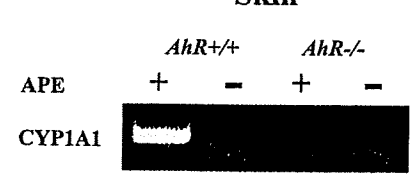

FIGURE 1. CYP1A1 gene expression in the skin of AhR+/+ and AhR−/− mice with and without APE treatment.

Induction of CYP1A1 by APE. Expression of CYP1A1 in the skin of AhR+/+ and AhR−/− mice was investigated using RT-PCR. Following APE treatment, CYP1A1 was induced in AhR+/+ mice but not in AhR−/− mice. No induction of CYP1A1 was apparent without APE treatment, regardless of the genotype (Figure 1). Therefore, the results show that APE induces CYP1A1 through an AhR-dependent pathway.

Tumor Incidence in Mice. APE suspended in acetone was continuously applied once weekly to the dorsal skin of 17 female AhR+/+ mice and 15 female AhR−/− mice. No tumors and hypertrophic changes were observed by the naked eye in AhR−/− mice, whereas AhR+/+ mice showed gradual depilation and inflammatory changes in the skin. In AhR+/+ mice, the first subcutaneous tumor appeared 29 weeks after initiation of treatment, and tumors were present in 3 mice after 41 weeks and 5 mice after 49 weeks. After 58 weeks, 8 of the 17 mice (47%) had papillomatous tumors of larger than 2 mm. Of the 8 induced tumors, 6 were solitary, but multiple tumors occurred in two mice: one having two and one having three small papillomas. In tissue examination under a microscope, it was found that all of the 2-mm or larger tumors in AhR+/+ mice were squamous cell carci-

TABLE 3. Incidence of Skin Tumors Induced in Two Mouse Genotypes after Repeated Application of APE

	AhR genotype	
	+/+	−/−
no. of mice	17	15
squamous cell carcinoma	8	0
papilloma	0	0
keratocanthoma	0	0
total no. of tumor-bearing mice (%)	8 (47%)	0 (0%)

noma, which showed infiltrative growth into muscular tissues in parts of the whole tumor (histopathological image). In addition, erosion and bleeding, which were thought to be caused by extract toxicity, were observed in the anal region of a few AhR+/+ mice. In AhR−/− mice, no tumors developed in the experimental period of 58 weeks, giving a statistically significant difference in tumor incidence between AhR+/+ and AhR−/− mice (8/17 vs 0/15, $p < 0.01$; Table 3). The gross appearance of back skins in AhR+/+ and AhR−/− mice after 58 weeks following repeated application of APE is illustrated in Figure 2. No tumors were evident in internal organs, and there were no other remarkable side effects or observations.

Discussion

PAHs in APE are important environmental carcinogens that pass through the cell membrane and bind to AhR, leading to induction of drug-metabolizing enzymes such as CYP1A1. PAHs are metabolically activated by these enzymes and transformed to DNA-binding carcinogenic substances. Therefore, carcinogenesis caused by PAHs in APE should be decreased in AhR−/− mice, since transactivation of the drug-metabolizing enzymes will not occur. A study in AhR−/− mice showed that acute toxicity or teratogenicity of dioxin is AhR-dependent (*20*, *25*), and our previous study of BaP in AhR−/− mice suggested that induction of CYP1A1 via AhR and metabolic activation of BaP by this enzyme are important in BaP carcinogenesis (*15*). Therefore, the current study was performed to examine the potential AhR dependence of carcinogenesis caused by APE derived from APM, an environmental mixture of compounds collected in Sapporo, in AhR+/+ and AhR−/− mice.

Tumor Incidence and CYP1A1 Expression in AhR+/+ Mice. APE treatment induced CYP1A1 gene expression in AhR+/+ mice and tumors were observed in 47% of these mice over 58 weeks. In contrast, CYP1A1 was not induced, and tumor formation was completely suppressed in AhR−/− mice. These results provide strong support for the hypothesis that the carcinogenic action of APE is mediated primarily by AhR. The primary route of metabolic activation of PAHs involves induction of CYP1A1 mediated by AhR (*10*), which leads to enhanced turnover of PAHs and increased production of highly carcinogenic metabolites. The expression of CYP1A1 in the skin of APE-treated AhR+/+ mice (Figure 1) is in agreement with our earlier study showing CYP1A1 gene expression induced by BaP (*15*). Therefore, it is likely that AhR plays an important role in conversion of APE into carcinogenic compounds through induction of CYP1A1. In the present study, Ahr −/− mice were resistant to APE-induced skin carcinogenesis as well as BaP. On the other hand, there has been a report indicating that Ahr −/− mice were not less susceptible to BaP induced adduct formation when BaP was administered orally (*26*). In addition, the toxicity of BaP is augmented in AhR nonresponsive (*27*) and CYP knockout mice (*28*). Further long-term carcinogenesis studies using oral administration are needed to address the paradoxical effect regarding carcinogenicity and genotoxicity.

Following APE application to skin, tumors occurred in 47% of AhR+/+ mice over about 14 months but not at all in AhR−/− mice, suggesting that APE causes AhR-dependent carcinogenesis. In an application test performed with BaP only, tumors developed in 94% of AhR+/+ mice in 6 months with administration of 200 μg of BaP per week (*15*), compared to 100 μg of APE M BaPEq in the current study. We note that the correlation between mutagenesis and carcinogenesis is complex (*29*), and use of the value of M BaPEq dose, instead of BaP dose, may not necessarily be appropriate; thus, care should be taken regarding interpretation of data using the applied amount of APE based on M BaPEq, as discussed below.

Contribution of PAHs to the Carcinogenic Effect. The contribution of BaP alone to the total BaPEq concentration of APE was 55% (Table 1). BaP is the most studied PAH compound and is thought to be representative of the 16 PAHs in the APE in this study; however, BaP is just one of at least 100 PAHs that have been identified in APM and just one of many carcinogenic compounds in the atmosphere. In our previous application test using only BaP, the applied amount was 200 μg. Since the 6.4 mg dose of APE per week in the current study contained only 3.87 μg BaP, the tumor incidence in the APE-treated AhR+/+ mice does not appear to be solely due to BaP in the mixture. The contribution of BaP to total carcinogenesis capacity is reported to be 6−7.4% and 2.4% for gasoline-powered vehicles (*30*, *31*) and 1.4% for flue gas-condensed substances in coal-heating furnaces (*32*). Based on these data, the contribution of BaP to APE carcinogenesis is estimated to be about 5% or lower.

The contribution to the total carcinogenicity of the PAH fraction comprising compounds with 3, 4, or more rings has been estimated to be 84−91% and 81% for gasoline-engine exhaust (*30*, *31*), and the PAH fraction seems to contribute predominantly to the total carcinogenicity of diesel exhaust (*33*) and hard-coal combustion flue gas condensate (*32*). The major sources of air pollution in Sapporo from 1973 to 1986 were gasoline-powered vehicles, diesel-powered vehicles, and coal firing for home heating, suggesting that most carcinogenesis caused by APE is due to PAHs from such pollution sources. However, the contribution of carcinogenic compounds in APM other than PAHs, such as nitroarenes (NO_2-PAHs), aza-arenes, and polycyclic aromatic compounds (*34*), should also be taken into account. Continuous application of gas condensed substances from home heating furnace to

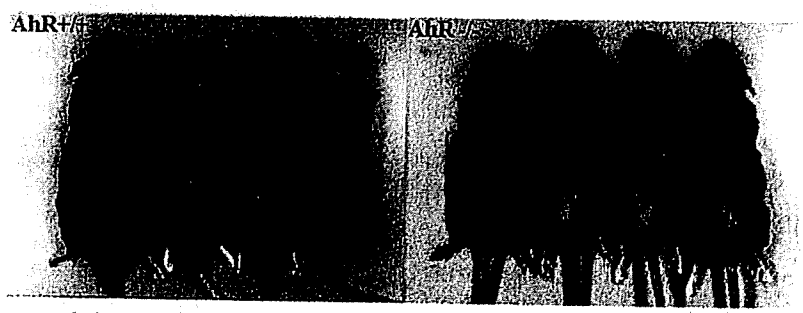

FIGURE 2. Gross appearance of skin tumors in AhR+/+ and AhR−/− mice after repeated application of APE.

the back of mice has shown that fractions including nitroarenes and aza-arenes account for only 4–7% of total carcinogenesis capacity (*35*). However, nitroarenes are an important subgroup of PAHs found in extracts from diesel and gasoline engine exhausts. 3-Nitrobenzanthrone (3-NBA) is an extremely potent mutagen and suspected human carcinogen that is one of several nitroarenes identified in urban PM. Recently, it has been reported that CYP1A1/2 could play an important role in the oxidative metabolism of 3-NBA and the main metabolite of 3-NBA, 3-aminobenzanthrone, to reactive DNA adducts, thereby enhancing their own genotoxic potential (*36, 37*). Thus, for accurate risk assessment of nitroarenes including 3-NBA, further studies on the carcinogenic effect after metabolic activation by P450s are required.

BaPEq Concentration. The carcinogenic potency of a PAH can be assessed based on its BaPEq concentration, and the total BaPEq concentration in mixtures is obtained from the sum of the BaPEq concentrations of components, assuming additive carcinogenic effects in the mixture. BaP had the highest contribution to the total calculated BaPEq concentration (55%), indicating the importance of BaP as a surrogate compound for PAHs mixtures in air; BaP contributions of 42–50% and 50–67% in urban air have also been reported (*23, 24*).

AhR Mediated Activity (CALUX BaPEq and CALUX TCDDEq). Evaluation of AhR-mediated activity of urban APM is important toxicologically for characterization of its carcinogenic potential. In the current study, AhR-mediated activity of APE was determined using the CALUX assay, for crude APE and cleaned APE, to differentiate the contribution to this activity of PAH-like compounds and TCDD and related compounds. A significant induction of AhR-mediated activity was observed for crude extract, expressed as CALUX BaPEq 979 ng/m^3 and CALUX TCDDEq 21.0 pg/m^3. The health consequences of these data are unknown, but this activity might contribute to adverse health effects of APM. The health risks of APM including PAH like compounds and dioxins will be assessed in further in vivo studies.

The CALUX assay response of crude APE may be due to both easily biodegradable and persistent AhR agonists, such as PAHs and TCDD, respectively. To test whether TCDD and related compounds contribute to the AhR-mediated activity of crude APE, cleaned APE was tested in 3-h and 24-h CALUX assays. A reduction in AhR-mediated activity due to depletion of PAH-like compounds in cleaned APE was observed for both CALUX BaPEq and CALUX TCDDEq, suggesting that PAH-like compounds are responsible for most AhR-mediated activity in crude APE. As PAHs, PAH-related compounds may be able to bind to and activate AhR. These compounds may include possible human and/or animals carcinogens. The Chemical TCDDEq concentration of 0.13 pg/m^3 indicates that TCDD and related compounds make relatively small contributions to this activity.

Finally, Revel et al. have demonstrated in an animal model that a natural AhR antagonist, resveratrol, inhibits BaP-induced CYP1A1 enzyme activity and subsequent formation of DNA adducts (*38*). Given the importance of AhR in APM-induced carcinogenesis, a competitive AhR inhibitor may provide a chemopreventive effect against development of cancer. Therefore, long-term exposure to APM in mice would be useful for evaluation of prevention of cancer by AhR antagonists.

In conclusion, our results show that APE induces carcinogenesis in AhR+/+ mice and provide the first direct evidence that AhR plays an essential role in APE-induced carcinogenesis. Our data also indicate that PAH-like compounds are significant contributors to AhR-mediated activity, whereas TCDD and related compounds make an almost negligible contribution.

Acknowledgments

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Minireview

Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that functions as an intracellular mediator in the xenobiotic signaling pathway. Although a number of studies have examined AhR-mediated *CYP1A1* induction in detail, recent studies of AhR-null mice have revealed that AhR plays important regulatory roles in the normal homeostasis and development of animals. In this short review, we summarize the present state of knowledge about the molecular mechanisms of AhR-mediated CYP1 induction, and we also focus on recent advances in the study of the physiological functions of AhR.

Keywords: Cytochrome P450; CYP1A1; AhR; TCDD; Gene regulation; Nuclear-cytoplasmic transport; Carcinogenesis; Reproduction; Inflammation

Members of the cytochrome P450 (CYP)[1] superfamily of hemoproteins are found across a wide range of species from bacteria to vertebrates [1]. These iron-containing proteins catalyze the monooxygene reaction of various endogenous and exogenous substrates and are classified according to the similarity of their amino-acid sequences. There are 18 CYP mammalian gene families, and four of these families (1, 2, 3, and 4) mainly metabolize foreign compounds including drugs, food additives and environmental pollutants [2]. Members of these CYP families are often specifically induced in response to exposure to foreign chemicals [3].

The molecular mechanisms regulating the induction of CYP proteins have been extensively examined, and a number of different inducers, *cis*-acting DNA elements, *trans*-acting transcription factors, and coactivators have been identified, and these studies have been greatly facilitated by the relatively recent development of novel genetic techniques, such as gene-knockouts, chromatin immunoprecipitation (Chip) and small interfering RNA (siRNA). The induction of CYP1 family member expression is regulated by a heterodimer composed of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt), which contains a characteristic basic-helix–loop–helix (bHLH) and PER-Arnt-SIM (PAS) homology region [4,5]. In contrast, the expression of CYP2, 3, and 4 family members is regulated by the nuclear receptors CAR, PXR (SXR), and PPAR, respectively, all of which possess a nuclear receptor gene family (family NR1) zinc finger motif and form heterodimers with the

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[1] *Abbreviations used:* AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; Chip, chromatin immunoprecipitation; siRNA, small interfering RNA; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-helix–loop–helix; PAS, PER-Arnt-SIM; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NLS, nuclear localization signal; NES, nuclear export signals; RXR, retinoid X-receptor; XRE, xenobiotic responsive element; BTE, basic transcription element; AhRR, AhR repressor; LOH, loss of heterozygosity; PAHs, polycyclic hydrocarbons; CA-AhR, constitutively active form of AhR; SOCS-2, suppressor of cytokine signaling 2.

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retinoid X-receptor (RXR) [3]. In this short review, we summarize our present understanding of the molecular mechanisms controlling the expression of CYP1 family members mediated by AhR with an emphasis on recent studies examining the physiological function(s) of AhR. The mechanisms of NR1-induced expression of CYP2, 3, and 4 family members have been addressed in a number of excellent recent review articles [6–8] , and interested readers should refer to those works.

Molecular mechanisms of CYP1 induction mediated by AhR

Activation of AhR

It is well established that ligation and activation of AhR by endogenous or exogenous compounds such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) leads to nuclear transport and transcriptional activation. AhR contains a nuclear localization signal (NLS) in its bHLH region [9], and mice harboring a mutation in the conserved NLS are resistant to TCDD-induced toxicity [10], consistent with results seen in AhR-deficient mice. AhR contains nuclear export signals (NES) in both the bHLH [9] and PAS domains [11] and can shuttle between the cytoplasm and the nucleus. This process is inhibited by LMB, a specific inhibitor of CRM1-dependent nuclear export [12].

In the absence of ligands, AhR is associated with a cytoplasmic protein complex consisting of Hsp90, p23, and ARA9 (XAP2 or AIP). Hsp90 binding is thought to mask the AhR–NLS, and this interaction is essential for the cytoplasmic retention of AhR [13]. Overexpression of ARA9 increases the fraction of AhR found in the cytosol [14], and the LxxLL motif of the NR box in AhR, which was found to mediate protein–protein interactions of transcriptional cofactors with nuclear receptors, is also involved in the cytoplasmic retention of AhR by protein–protein interactions [15]. Additionally, protein kinase C-mediated NLS phosphorylation inhibits the ligand-dependent nuclear import of AhR. Taken together, these findings suggest that the regulation of AhR nuclear import has two distinct components: ligand binding initiates the interaction of the NLS with nuclear import components, and this is negatively regulated by NLS phosphorylation [16] (Fig. 1b).

Several studies have examined the ligand-independent activation of AhR. When several different cell lines, such as Hepa-1 and 10T1/2 fibroblast clonal sublines, are grown in suspension culture, AhR nuclear translocation and the induction of *CYP1A1* or *CYP1B1* mRNA occur in the absence of xenobiotic AhR ligands [17,18]. Additionally, preventing cell–cell interactions of the keratinocyte cell line HaCaT by growing at a low cell density or in Ca^{2+}-deficient S-MEM induces the nuclear accumulation of AhR and promotes the expression of a reporter gene driven by the xenobiotic responsive element (XRE) sequence [19]. In these cells, *Slug*, a member of the snail/slug family of zinc finger transcriptional repressors critical for epithelial–mesenchymal transitions, is transcriptionally activated following AhR nuclear translocation [20]. Furthermore, the second messenger cAMP, an endogenous mediator of hormone and neurotransmitter signaling, is also reported to activate AhR and lead to its nuclear translocation [21]. Finally, omeprazole, a benzimidazole derivative used clinically as an inhibitor of the gastric proton pump, induces CYP1A1 expression in an AhR-dependent manner, but it does not directly bind AhR [22,23]. Ligand-independent AhR activation suggests cross-talk between AhR and other signaling pathways, but further studies are needed to clarify the mechanisms regulating this pathway.

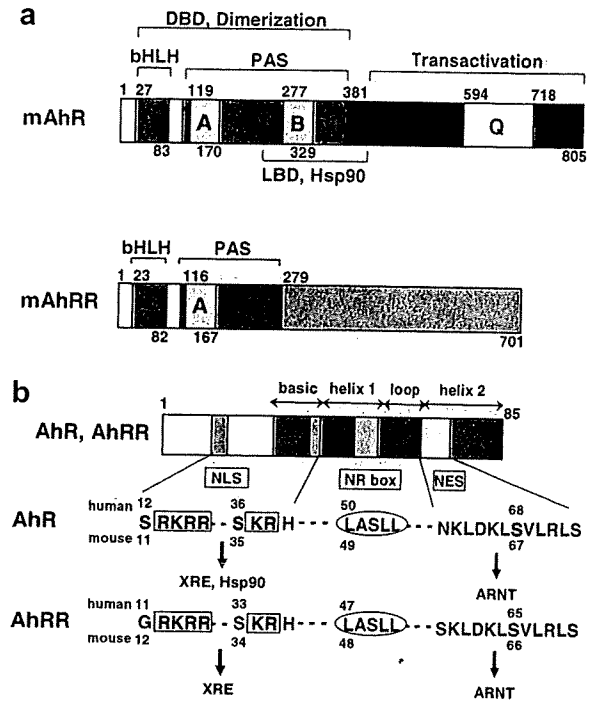

Fig. 1. (a) Schematic representation of functional domain of AhR and AhRR. A, B: weakly homologous repeated regions. Q: glutamine-rich transcription activation region. LBD, Hsp90 ligand binding and Hsp90 interaction domain. DBD DNA binding domain. (b) Schematic representation of the NH_2-terminal functional domains of AhR and AhRR in relation to signals for nuclear import or export. It is noted that a ligand-dependent nuclear import of AhR is inhibited by substitution of aspartic acid for Ser-12 or Ser-36 in human AhR [16], which mimics the negative charge of phosphorylation. On the other hand, a nuclear export of AhR is inhibited by substitution of Ser-68 in human AhR [19].

Transcriptional regulation of CYP1 genes by AhR

The TCDD-induced expression of *CYP1A1* is mediated through the XRE [24]. The core consensus sequence of XRE is 5′-TNGCGTG-3′, and this site is recognized by the AhR/Arnt heterodimer. Approximately 1 kb upstream of the *CYP1A1* gene, a cluster of XREs functions as an enhancer element, and a basic transcription element (BTE), a GC box sequence localized to the proximal

promoter of *CYP1A1*, is also required for the induction of *CYP1A1* [25].

Chromatin remodeling is initiated by liganded AhR/Arnt heterodimer binding to the XREs in the enhancer region, and this leads to increased DNAase sensitivity and the appearance of a DNAase hypersensitive site within 300 bp upstream of the transcription initiation site. BRG1, a component of the SWI/SNF ATP-dependent chromatin-modeling complex, is involved in the TCDD-dependent remodeling of the *CYP1A1* gene [26]. The AhR/Arnt heterodimer transactivates in conjunction with general transcription factors (GTFs) through interactions with coactivator proteins including CBP/p300, SRC-1, NCoA-2 and p/CIP, and the coactivator/corepressor protein RIP140 [27]. In addition, the TRAP/DRIP/ARC/Mediator complex must be recruited to the *CYP1A1* promoter to activate target gene expression in response to xenobiotic stress [28]. However, less is known about the factors regulating the induction of CYP1A2 expression, although the AhR/Arnt heterodimer is clearly required for this to occur. One study suggests that the AhR/Arnt heterodimer may function as a coactivator without directly binding the XRE. Instead, it may interact with other DNA-binding factors of a novel xenobiotic responsive element termed XREII to induce transcription activation [29].

The AhR repressor (AhRR) was identified as a negative regulator of AhR activity. AhRR contains both NLS and NES that are homologous to AhR (Fig. 1b), but AhRR is localized constitutively to the nucleus. Here, AhRR forms a heterodimer with Arnt, but XRE binding by the AhRR/Arnt heterodimer leads to transcriptional repression. Finally, AhRR expression is induced in an AhR-dependent manner, indicating that AhR and AhRR form a regulatory feedback loop [30] (Fig. 2).

Regulation of AhR protein stability

AhR is rapidly degraded both *in vivo* and *in vitro* following ligand binding, and several studies have examined the regulation of AhR degradation. When AhR was fused to the heterologous NLS of nucleoplasmin, it constitutively accumulated in the nucleus and was degraded in a 26S proteasome-dependent manner [31]. Conversely, when nuclear export of AhR was blocked by LMB, AhR accumulated in the nucleus following ligand binding and was not efficiently degraded. In this system, AhR degradation required both an NES and redistribution from the nucleus to the cytoplasm [32]. Although these data are hard to reconcile, a detailed understanding of the factors controlling the degradation of AhR is essential because this is an important component regulating AhR activity. Additionally, it is important to determine whether degradation of AhR is coordinated with cycles of transcriptional activation.

Functional role of AhR in physiology and toxicology

Over the past decade, many studies have examined AhR as a mediator of the adverse cellular response to environmental contaminants, such as TCDD and 3MC. However, the high degree of evolutionary conservation of AhR across a variety of animal species suggests AhR may possess xenobiotic-independent functions [33]. Indeed, a role

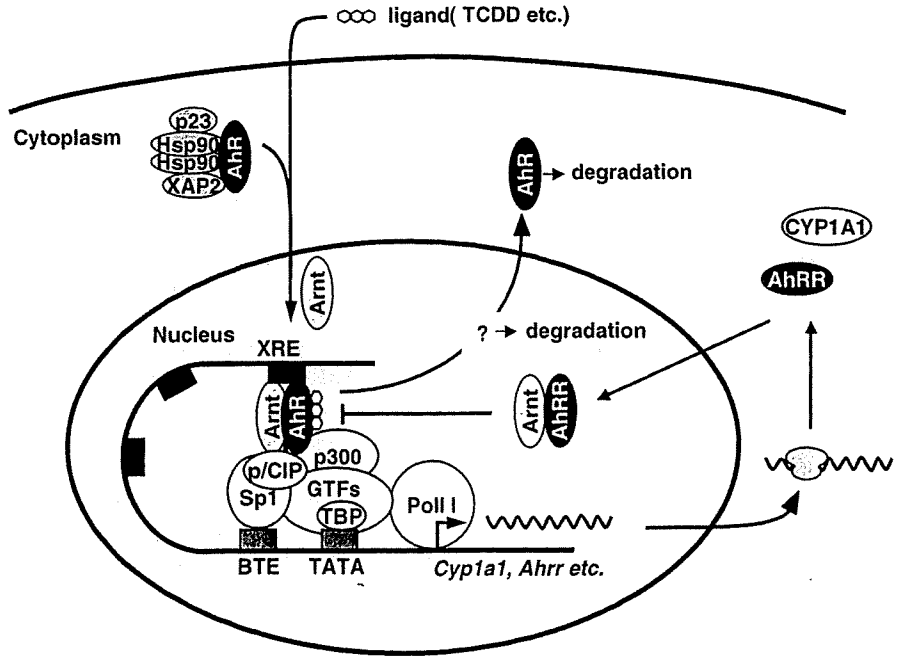

Fig. 2. A model of AhR signaling pathway [5].

for AhR in development was proposed based on the observed expression of AhR and Arnt during mouse embryogenesis [34]. In addition, activation of AhR has been linked to alterations in cell proliferation, apoptosis, adipose differentiation, tumor promotion, and immune and reproductive function. Consistent with these roles, several endogenous compounds, such as bilirubin [35], lipoxin A4 [36] and tryptophan derivatives including FICZ [37], have been isolated as potential natural ligands for AhR. Finally, the generation of AhR-null mice by three independent groups [38–40] has provided strong support for a variety of physiologic roles of AhR, e.g., homeostasis and development.

The role of AhR in carcinogenesis and teratogenesis

A number of papers have examined the role of AhR in regulating the cell cycle and proliferation. However, this remains controversial because studies have reached apparent conflicting conclusions as to whether AhR inhibits or promotes cell cycle progression. One recent report showed that AhR inhibited the growth of epithelial MCF-7 cells, but it promoted the proliferation of HepG2 hepatoma cells. Thus, the precise function of AhR in cell proliferation may differ in a cell type-dependent manner [41]. The constitutive expression of AhR induced tumors in the glandular part of the stomach [42] and increased hepatocarcinogenesis in transgenic B6C3F1 mice following a single injection of *N*-nitrosodiethylamine [43]. Thus, AhR may be oncogenic to varying degrees in different tissues. Consistent with this, AhR-null mice are resistant to benzo[*a*]pyrene-induced tumors [44], directly implicating AhR as a key factor in the development of environmental carcinogenesis. However, the role of AhR in the development of naturally occurring tumors remains largely unknown. In contrast, we recently identified a novel function for AhR as a tumor suppressor in colorectal carcinogenesis (manuscript in preparation).

A role for AhR in renal development has clearly been established. In wild-type mice, exposure to TCDD during development induces hydronephrosis, reduced kidney size, and some developmental renal disorders. In contrast, AhR-deficient mice are completely resistant to these TCDD-induced teratogenic effects [45]. Additionally, in humans with Wilms tumor, a form of renal cancer, there is a relatively high rate of loss of heterozygosity (LOH) at band 7p15-21. A minimal common region of LOH is located between markers *D7S517* and *D7S503* [46], and homozygous deletion of this region is frequently found in these tumors [47]. Interestingly, the *AhR* gene maps to this deleted region, suggesting that AhR may be a candidate for a Wilms tumor suppressor gene. Additionally, a recent paper showed that promoter hypermethylation is a novel epigenetic mechanism downregulating AhR activity in hematological malignancies such as ALL, and, in the patients studied, 33% exhibited some degree of AhR promoter hypermethylation [48].

The role of AhR in reproduction and vascular development

The fertility of AhR-null females is reduced, and the phenotype of these mice is similar to that seen with ARKO and ERα/ERβ double knockout mice. The litter size of AhR deficient mice was significantly decreased compared to wild-type mice [49], and this resulted from impaired folliculogenesis and ovulation in AhR deficient females [50]. An *in vitro* reporter gene assay and *in vivo* ChIP assay suggested that AhR synergistically cooperates with the orphan nuclear receptor Ad4BP/SF-1 to activate *CYP19* gene transcription in ovarian granulosa cells. CYP19 is thought to modulate ovarian estradiol concentrations and drive the estrus cycle. Thus, AhR plays a crucial role in female reproduction by regulating the expression of the ovarian P450 aromatase (CYP19), a key enzyme in estrogen synthesis.

Bradfield et al. [51,52] used a Cre-lox system to study AhR signaling in endothelial/hematopoietic cells, and AhR is necessary for the normal developmental closure of the ductus venosus. In mice unable to express AhR in hepatocytes, the patent ductus venosus results in massive portosystemic shunting of blood flow leading to a profound reduction in hepatocyte size. Although these studies clearly identified an important role for AhR in vascular development, the mechanisms of AhR action in this process remain largely unknown.

The role of AhR in inflammation and the immune system

Environmental exposure to polycyclic hydrocarbons (PAHs) may promote inflammatory and/or allergic disorders, and a role for AhR in inflammation has been suggested. Mice specifically expressing the constitutively active form of AhR (CA-AhR) in keratinocytes appeared normal at birth, but they developed severe skin lesions postnatally. These lesions histologically resembled atopic dermatitis, suggesting that the constitutive activation of the AhR signaling pathway is sufficient to trigger inflammatory skin lesions [53]. In contrast, lipoxins are eicosanoids with potent anti-inflammatory effects in many inflammatory diseases. Lipoxin A4 is a natural ligand for AhR, and it controls the migration of dendritic cells and production of interleukin-12 *in vivo*. Lipoxin A4 activates AhR and increases the expression of suppressor of cytokine signaling 2 (SOCS-2) [54]. Thus, the overwhelming activation of AhR may lead to dysregulated inflammation, but, under normal circumstances, AhR may play an anti-inflammatory role. Further studies are needed to clarify the molecular role of AhR in modulating inflammation.

Thymocyte development and T cell-dependent immune reactions are exquisitely sensitive to AhR-dependent TCDD toxicity. To better understand the role of AhR in T cell development and homeostasis, mice were generated transgenically expressing CA-AhR in T cells under the control of the CD2 promoter. AhR activation in T-lineage cells alone directly induced the thymocyte changes, and the

normal increase in splenocyte number following immunization did not occur in these mice. However, the number of resting splenocytes was not affected, suggesting that AhR functions in the growth of activated and proliferating T cells [55].

Using the B6-into-B6D2F1 model of acute graft-vs.-host disease, Kerkvliet et al. [56,57] showed that AhR activation in donor T cells generates a subpopulation of $CD4^+$ $CD25^+$hi T regulatory cells. They suggested that TCDD-mediated AhR activation preferentially activated these regulatory T cells which subsequently dampened the post-immunization T cell proliferation.

M50367, an orally active anti-allergy agent, is a ligand for AhR, and M50367 activation of AhR signaling skews the Th1/Th2 balance toward Th1 dominance, resulting in immunological responses with anti-allergic effects. This is completely abolished in AhR-null mice. Additionally, forced expression of a constitutively active form of AhR suppresses naïve Th cell differentiation into Th2 cells, demonstrating that AhR functions as a modulator of the *in vivo* Th1/Th2 balance through activation of AhR in naïve Th cells [58].

Taken together, these results suggest that AhR is intimately involved in a number of different aspects of immunological responses. The molecular mechanisms controlling AhR function in the immune system will be interesting to determine in future studies.

Conclusion

CYP1A1 is strongly induced by exogenous ligands such as TCDD, and a number of studies have examined the transcription factors and chromatin remodeling factors responsible for CYP1A1 induction. The genetic regulation of CYP1A1 expression is a good model system for the examination of the temporal and spatial recruitment of various transcription factors, nucleosomal remodeling factors and their interactions. Identifying the physiological functions of AhR, and clarifying the mechanisms responsible for its activation in both normal physiology and in response to xenobiotics will provide great insight into a variety of diverse cellular processes. Additionally, modulation of AhR signaling may be a good candidate for the development of therapies targeting endocrine or environmental diseases.

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LETTERS

Dioxin receptor is a ligand-dependent E3 ubiquitin ligase

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Fat-soluble ligands, including sex steroid hormones and environmental toxins, activate ligand-dependent DNA-sequence-specific transcriptional factors that transduce signals through target-gene-selective transcriptional regulation[1]. However, the mechanisms of cellular perception of fat-soluble ligand signals through other target-selective systems remain unclear. The ubiquitin–proteasome system regulates selective protein degradation, in which the E3 ubiquitin ligases determine target specificity[2–4]. Here we characterize a fat-soluble ligand-dependent ubiquitin ligase complex in human cell lines, in which dioxin receptor (AhR)[5–9] is integrated as a component of a novel cullin 4B ubiquitin ligase complex, $CUL4B^{AhR}$. Complex assembly and ubiquitin ligase activity of $CUL4B^{AhR}$ *in vitro* and *in vivo* are dependent on the AhR ligand. In the $CUL4B^{AhR}$ complex, ligand-activated AhR acts as a substrate-specific adaptor component that targets sex steroid receptors for degradation. Thus, our findings uncover a function for AhR as an atypical component of the ubiquitin ligase complex and demonstrate a non-genomic signalling pathway in which fat-soluble ligands regulate target-protein-selective degradation through a ubiquitin ligase complex.

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. This target selectivity depends on the recognition of specific DNA elements by sequence-specific transcription factors[1] and the recognition of degradation substrates by E3 ubiquitin ligases[2–4]. These transcription factors and ligases serve primarily as specific adaptors that subsequently recruit transcriptional co-regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. The selective biological effects of fat-soluble ligands have been reported to be mediated by two classes of sequence-specific transcription factors, nuclear receptors[1] and arylhydrocarbon receptor (AhR) belonging to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) family[5–9].

AhR ligands modulate oestrogen and sex hormone, signalling both positively and negatively[8,10–13]. Functional impairments of male and female reproductive organs in AhR-deficient mice indicate the possible importance of AhR in sex hormone signalling[10,14]. Different AhR agonists[9], including 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), modulate oestrogen-dependent oestrogen receptor (ER)-α transactivation through the association of activated AhR/Arnt with ER-α[15]. Similarly, the transcriptional activity of nuclear androgen receptor (AR) was modulated by association with activated AhR (Supplementary Fig. S2a). However, ligand-bound AhR did not block oestrogen-induced co-activator recruitment on the oestrogen-responsive promoter (Supplementary Fig. S2b). This implies another mode of function for ligand-activated AhR beyond transcriptional regulation.

On activation of AhR by 3MC, we observed that protein levels of endogenous ER-α (in mammary tumour MCF-7 cells), ER-β (in ovarian tumour KGN cells) and AR (in prostate cancer LNCaP cells) were drastically decreased (Fig. 1a–c, and Supplementary Fig. 3a) without a change in messenger RNA levels (data not shown), irrespective of the presence of their cognate hormones. Other AhR agonists[9] (namely β-naphthoflavone (β-NF), environmental toxins such as TCDD and benzo[a]pyrene, and the endogenous metabolite indirubin) were similarly effective in protein degradation for ER-α (Fig. 1b) and ER-β/AR (data not shown), in agreement with a previous report on downregulated levels of uterine ER-α protein in rats treated with TCDD[16]. An AhR partial agonist/antagonist α-naphthoflavone (α-NF) was unable to accelerate the degradation of either AhR or ER-α (Fig. 1b, and Supplementary Fig. S3b).

AhR ligand-induced degradation (Fig. 1a–c) and functional repression (Supplementary Fig. S2c, d) of sex steroid receptors were abrogated in the presence of a proteasome inhibitor MG132. Consistently, poly-ubiquitination of ER-α was promoted by the activated AhR regardless of the presence of oestrogen (Fig. 1d, and Supplementary Fig. S3c). Pulse-chase kinetic analysis indicated that 3MC-induced degradation of ER-α was coupled to that of AhR[8,17,18] (Supplementary Fig. S3d). Moreover, the self-ubiquitination activity of the ligand-bound AhR immunocomplex was detected in an E1/E2-dependent manner (Supplementary Fig. S3e). Together with 3MC-dependent recognition of sex steroid receptors by AhR[8,12,13,15], these properties of AhR resemble those of classical adaptor components of the E3 ubiquitin ligase complexes, such as F-box proteins[3] or von Hippel–Lindau protein[19]. We therefore reasoned that activated AhR might act as an E3 ubiquitin ligase complex component.

To address this idea, AhR-containing complexes were purified from HeLa cells expressing Flag–AhR treated with 3MC or α-NF[15,20]. AhR formed large complexes in the presence of 3MC (Supplementary Fig. S4a–c). Further purification revealed five major 3MC-dependent complexes containing AhR (Fig. 1e). Complexes A and C contained well-known co-activators TRAP220/DRIP205/Med220 and p300 (ref. 1) (Supplementary Fig. S4d, e). Endogenous ER-α was detected in complexes B and C; however, ubiquitinated components were seen only in complex B (Fig. 1f, g).

Complex B was composed of the ubiquitin ligase core components cullin 4B (CUL4B)[3,21,22], damaged-DNA-binding protein 1 (DDB1)[23–27] and Rbx1 (Roc1)[3], together with subunits of the proteasomal 19S regulatory particle (19S RP), Arnt and transducin-β-like 3 (TBL3) (Fig. 1h). These components eluted with AhR in the presence of 3MC but not in the presence of α-NF (Fig. 1i, and Supplementary Fig. S4f). Neither CUL4A nor known substrate-specific adaptor components of CUL4A, such as DDB2, CSA and DET1[23,24], were present

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in the AhR–CUL4B complex. As the cullin amino terminus binds adaptor components and the carboxy terminus interacts with an E2 enzyme-binding subunit Rbx1 (ref. 3), we performed tandem purification of the AhR–CUL4B complex with glutathione *S*-transferase (GST)-tagged CUL4B-N (N-terminal domain of CUL4B) and Flag–AhR. This led to the identification of a core complex consisting of five components: DDB1, AhR, Arnt, TBL3 and CUL4B (Fig. 1j). Together with Rbx1, this complex is denoted by $CUL4B^{AhR}$.

Immunoprecipitation of AhR together with endogenous CUL4B from MCF-7 and LNCaP cells was observed only in the presence of 3MC (Fig. 2a, b). Consistently, ligand-dependent co-localization of AhR with CUL4 was seen in MCF-7 cells (Fig. 2c). Whereas CUL4B seemed to act as a scaffold mediating DDB1–TBL3 and AhR–DDB1 interactions in $CUL4B^{AhR}$ (Fig. 2d, lane 4), ligand-activated AhR induced the assembly of complex components (Fig. 2d, lanes 1–3). DDB1 did not bridge CUL4B association with TBL3 or AhR, apparently because of the absence of the signature WDXR/DWD box[22,25–27] of either TBL3 or AhR, which is essential for DDB1 binding (Fig. 2d, lane 5, and Supplementary Fig. S5a). Consistently, specific and 3MC-dependent interaction of the conserved C-terminal acidic domain of AhR with the N-terminal region of CUL4B, but not with DDB1, was observed in a GST pulldown assay (Supplementary Figs S5b and S6). Because a constitutively active AhR mutant (AhRΔPASB)[9] interacted with CUL4B in the absence of ligand (Supplementary Fig. S5b), ligand-dependent structural alteration presumably induces AhR–CUL4B interaction. An AhR mutant lacking the CUL4B-binding

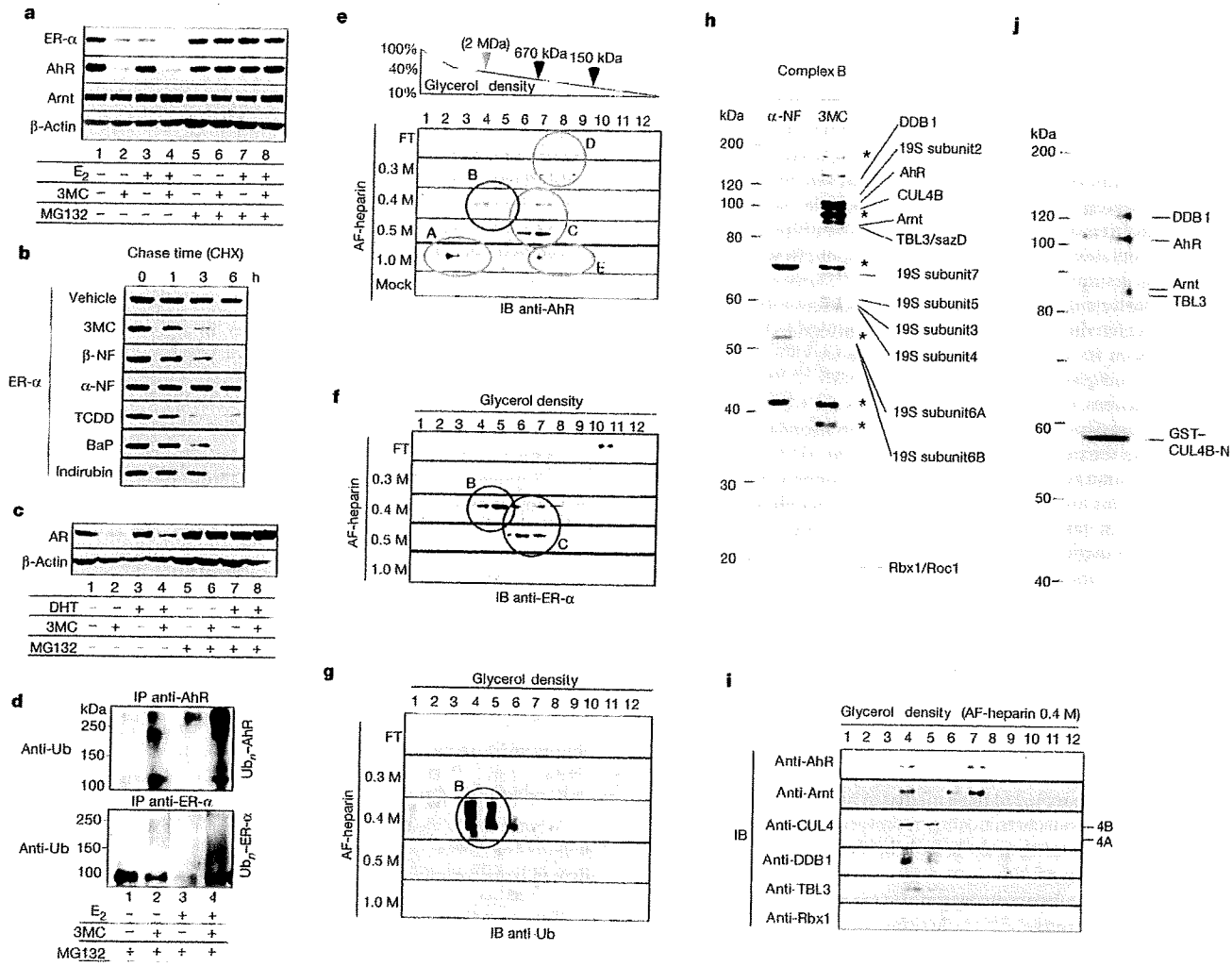

Figure 1 | Activated AhR acts as an E3 ubiquitin ligase. a–c, AhR-ligand-induced proteasomal degradation of ER-α (**a**, **b**) and AR (**c**). MCF-7 cells (**a**, **b**) and LNCaP cells (**c**) were incubated as indicated with E_2 (10 nM), DHT (10 nM) and/or 3MC (1 μM), β-NF (1 μM), benzo[a]pyrene (BaP; 100 nM), TCDD (10 nM), indirubin (10 nM) and α-NF (1 μM) in the presence or absence of MG132 (10 μM) and cycloheximide (CHX; 5 μM) for 3 h (**a**, **c**) or the indicated durations (**b**). Cell lysates were subjected to western blotting with specified antibodies. **d**, AhR-ligand-induced ubiquitination of ER-α. MCF-7 cells were incubated with the indicated ligands for 6 h. Western blots were subjected to dark exposure to detect poly-ubiquitinated forms of the receptors. IP, immunoprecipitation; Ub, ubiquitin. **e**, **f**, Biochemical separation and identification of AhR-associated complexes. Flag–AhR-associated proteins in the presence of 3MC or α-NF from HeLa cells stably expressing Flag–AhR were first fractionated by glycerol-density-gradient centrifugation (top, fractions 1–12), and then separated by Toyopearl AF-heparin column chromatography with the indicated KCl concentrations (FT, 1.0 M KCl). Samples from the 3MC-treated cells were resolved into five distinct complexes. IB, immunoblotting. **g**, Components of an AhR-associated complex are highly ubiquitinated. Western blots with anti-ubiquitin antibody. **h**, Identification of AhR-associated CUL4B ubiquitin ligase complex components. Components from complex B in **e** (fractions 4 and 5 from the glycerol-density-gradient centrifugation, eluted from an AF-heparin column at 0.4 M KCl) were resolved by SDS–PAGE, silver-stained and identified by matrix-assisted laser desorption ionization–time-of-flight MS analysis. **i**, Co-elution of the complex B components as a large complex. **j**, Association of activated AhR with the CUL4B complex. The $CUL4B^{AhR}$ complex from Flag–AhR-expressing HeLa cells treated with 3MC was affinity purified with GST-tagged N-terminal domain of CUL4B followed by anti-Flag antibody column fractionation.