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Molecular Basis for Differential Dioxin Sensitivity in Birds: Characterization of Avian AHR Isoforms

Eun-Young KIM[1], Hisato IWATA[1], Tomoko YASUI[1], Naomi INOUE[1], Jin-Seon LEE[1], Diana G. FRANKS[2], Sibel I. KARCHNER[2], Mark E. HAHN[2] and Shinsuke TANABE[1]

[1]*Center for Marine Environmental Studies (CMES), Ehime University, Japan*
[2]*Biology Department, Woods Hole Oceanographic Institution, Woods Hole, U.S.A.*

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Abstract—Our previous study demonstrated that avian species possesses two distinct aryl hydrocarbon receptors (AHR1 and AHR2). To elucidate the functional characterization of avian AHRs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) binding affinities and *in vitro* DRC-induced transcriptional activities of AHR isoforms from chicken (*Gallus gallus*), black-footed albatross (*Phoebastria nigripes*), common cormorant (*Phalacrocorax carbo*) and jungle crow (*Corvus macrorhynchos*) were investigated. All avian AHR isoforms exhibit specific binding affinity to [^{3}H]TCDD, as assessed by velocity sedimentation. An *in vitro* reporter gene transactivation assay revealed that AHR2s from chicken, albatross and cormorant had reduced transcriptional efficiencies to TCDD as compared to AHR1s from the corresponding species. Particularly, chicken AHR2 exhibited dramatically reduced transactivation. In contrast, the transcriptional ability of crow AHR2 was almost comparable to that of AHR1. One possible reason accounting for this difference in transactivation may be the structural difference in C-terminal halves.

Furthermore, tissue expression profiles of AHR isoform mRNAs in cormorant and crow revealed that AHR1s were expressed in most tissues, whereas AHR2s were detectable in limited tissues. However, comparison of hepatic mRNA expression levels of two AHR isoforms showed a species difference; AHR1 is dominant than AHR2 in cormorant, in contrast to crow, in which AHR2 is the major form. These results suggest that function of AHR isoforms mediating the response to DRC exposure is diverse even within avian species.

Keywords: aryl hydrocarbon receptor1 (AhR1), aryl hydrocarbon receptor 2 (AhR2), specific binding, transcriptional activation, birds

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand activated intracellular protein that is responsible for toxic effects of dioxins and its related compounds (DRCs). Although there are dramatic differences in sensitivity to dioxin toxicity among avian species, the information on molecular characterization of avian AhR is

limited. Our previous study demonstrated that avian species possesses two distinct aryl hydrocarbon receptors (AHR1 and AHR2). These novel AhRs were grouped in the same clade as fish AhR1 and AhR2 by phylogenetic analysis (Yasui *et al.*, 2004, 2007). In addition, our previous study also revealed that some avian species accumulated high levels of dioxins, including polychlorinated dibenzo-*p*-dioxins, furans and coplanar PCBs in the livers (Iwata *et al.*, 2001; Kubota *et al.*, 2004). These results provide a rationale for studying the molecular characteristics of the AhR pathway in avian species. In a recent study, the function of chicken and tern AhRs corresponding to mammal AhR and fish AhR1 were characterized (Karchner *et al.*, 2006). However, little is known about the structural and functional role of the novel avian AhR2 isoforms that we have identified. Here, we investigated the molecular evolution and structural and functional characteristics of multiple AhR isoforms in chicken (*Gallus gallus*), black-footed albatross (*Phoebastria nigripes*), common cormorant (*Phalacrocorax carbo*) and jungle crow (*Corvus macrorhynchos*).

MATERIALS AND METHODS

2,3,7,8-Tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin was obtained from Chemsyn Science Laboratories ([^{3}H]TCDD, 35 Ci/mmol, >99% radiochemical purity) (Lenexa, KS, USA) and EaglePicher^{TM} ([^{3}H]TCDD, 27.5 Ci/mmol, >97% radiochemical purity by HPLC) (Lenexa, KS, USA). [^{35}S]Methionine and methylated [methyl-^{14}C]ovalbumin were purchased from Amersham Biosciences and NEN Life Science Products Inc. (Boston, MA, USA), respectively. Methylated [methyl-^{14}C]catalase was synthesized by PerkinElmer (Boston, MA, USA).

Black-footed albatrosses were accidentally caught from the North Pacific in 1995. Common cormorants were captured from Lake Biwa, Japan in 2001–2003 under the license of Shiga Prefecture. Jungle crows were collected from Tokyo, Japan in 2002 and 2003. Total RNAs were isolated using Nucleic Acid Purification Kit (TOYOBO) and Nucleic Acid Purification System MagExtracter MFX-2100. Unlabeled or [^{35}S]methionine-labeled proteins were synthesized by TnT Quick Coupled Reticulocyte Lysate Systems following the manufacturer's instructions. Transfections were carried out in triplicate wells 20–24 hours after plating. DNA and Lipofectamine 2000 transfection reagent (Invitrogen) were diluted in serum-free Opti-MEM medium.

RESULTS AND DISCUSSION

Here, we reported the functional characterization of two distinct AhRs, designated AhR1 and AhR2, from four avian species, chicken, black-footed albatross, common cormorant and jungle crow. The comparative analysis of gene synteny among vertebrates indicates that avian AhR1 is an ortholog gene of mammalian AhR and fish AhR1, and avian AhR2 is a paralog gene of fish AhR2; the avian and fish AhR2 genes arose from independent differential chromosomal duplication following the tandem gene duplication in ancestral vertebrate.

Avian AhR proteins were synthesized by an *in vitro* transcription/translation

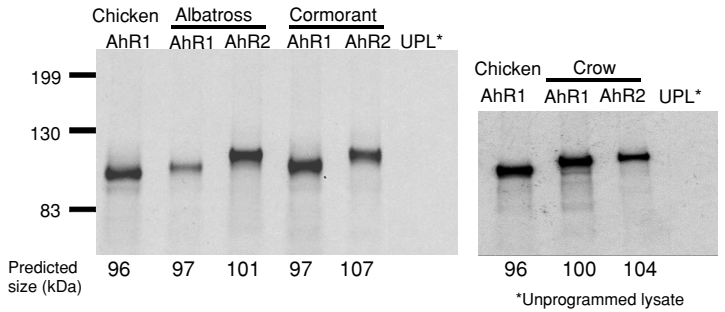

Fig. 1. Protein synthesis by *in vitro* transcription /translation system.

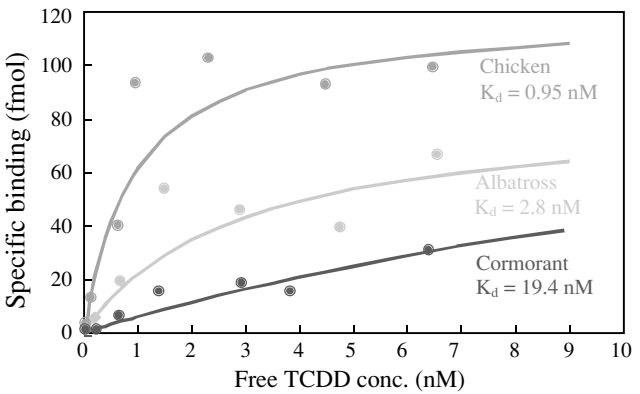

Fig. 2. Saturation binding of TCDD to avian AhR1s.

system to determine whether these AhR cDNAs encode functional proteins. Gel electrophoresis of the [^{35}S]methionine-labeled translation products of all AhR isoform cDNAs revealed single bands similar in their predicted molecular size (Fig. 1). To examine the ligand binding ability of albatross and cormorant AhRs, the *in vitro* translated AhRs were incubated with [^{3}H]TCDD and separated by velocity sedimentation on sucrose density gradients. Compared to the non-specific binding detected using *in vitro* translation products of an empty vector, a peak of [^{3}H]TCDD specific binding was observed for each avian AhR1. These results demonstrate that AhR1s of these species encode proteins that are capable of specific binding with TCDD. Furthermore, to compare the TCDD binding affinity of avian AhR1s, saturation binding analysis were performed with varing concentrations of [^{3}H]TCDD (Fig. 2). The Kd values for chicken, albatross and cormorant were estimated to be 0.95 nM, 2.8 nM and 19.4 nM, respectively. Thus chicken AhR1 would be much more sensitive to dioxin binding than others.

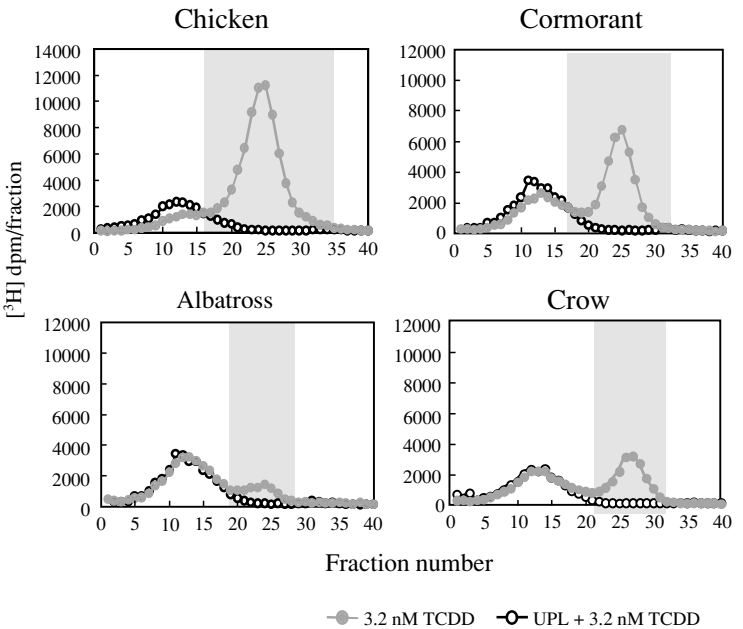

Fig. 3. Specific binding of [^{3}H]-TCDD to avian AhR2 isoforms. The non-specific binding of [^{3}H]TCDD using UPL was also assessed (open circles). Radioligand concentrations were verified by sampling each tube for total counts. Specific binding is estimated from the difference between total binding (radioligand only) and non-specific binding (radioligand binding to UPL).

Considering the two amino acid residues responsible for the differential binding affinity between chicken AhR (Ile^{324} and Ser^{380}) and tern AhR (Val^{325} and Ala^{381}) (Karchner *et al.*, 2006), the binding affinity of avian AhR1 was predicted to follow the order: chicken AhR (I-S type) > albatross AhR1 (I-A) > cormorant AhR1 (V-A). It is interesting that the Kd values for these AhR1s are in the same order as predicted from the identity of critical amino acid residues in ligand binding domain.

To examine the transactivation abilities of these avian AhR1s, a luciferase reporter gene vector was constructed using chicken CYP1A5 promoter region containing six XREs. Compared to the relative luciferase activity in "no AhR" transfected cells treated with TCDD, AhR1-transfected cells exposed to TCDD exhibited reporter gene activity in a dose-dependent manner. In addition, the EC_{50} values of TCDD for chicken, cormorant and crow AhR1s were estimated to be 0.03, 0.14 and 0.67 nM, respectively. These results showed that the ability of avian AhR1s to transactivate the luciferase reporter gene in the presence of TCDD mirrored the results of the TCDD-binding assay.

Compared to the non-specific binding, all avian AhR2s apparently exhibited a peak of [^{3}H]TCDD specific binding (Fig. 3). These results suggested that avian

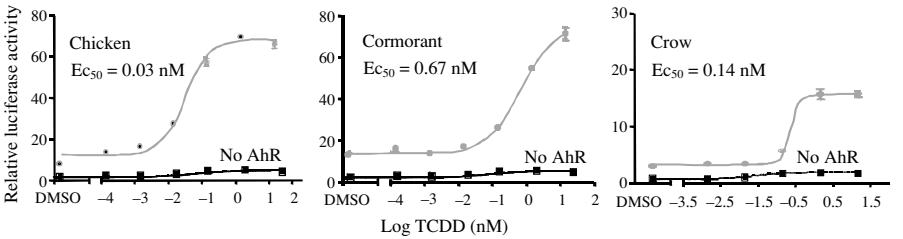

Fig. 4. Transcriptional activation by avian AhR1s. COS-7 cells were transfected with expression constructs for the cormorant Arnt1 (50 ng), pGL3-ccCYP1A5 (a luciferase reporter construct under the control of cormorant CYP1A5 promoter sequence), pRL-TK (transfection control) and each AhR expression construct (5 ng). "No AhR" indicates that cells were transfected only with the reporter construct and the transfection control. The cells were exposed to dimethyl sulfoxide or TCDD. The luciferase activity was quantified as the firefly luciferase activity relative to the transfection control *Renilla* luciferase.

AhR2s can also bind to TCDD, as shown in avian AhR1s. However, in spite of the differences in relative peak areas for avian AhR2, the critical amino acids in ligand binding domain showed the same type in cormorant, albatross and crow, except for chicken.

Compared to "no AhR"-transfected cells treated with TCDD, avian AhR2-transfected cells exposed to TCDD had only slightly increased luciferase expression in cormorant, albatross and chicken. These results suggest that avian AhR2s are responsive to TCDD, but may be less efficacious at activating target gene transcription than avian AhR1s. Alternatively, avian AhR2 isoforms may be expressed at a lower level in COS-7 cells, or they may regulate the transcription of a distinct set of target genes as compared to AhR1s. On the other hand, the transcriptional activity of crow AhR2 was almost comparable to that of AhR1. One possible cause of the difference in transactivation may be due to the structural difference in transactivation regions between AhR1 and AhR2. To examine the transactivation abilities of these avian AhR1s, a luciferase reporter gene vector was constructed using chicken CYP1A5 promoter region containing six XREs. Compared to the relative luciferase activity in "no AhR" transfected cells treated with TCDD, AhR1-transfected cells exposed to TCDD exhibited reporter gene activity in a dose-dependent manner (Fig. 4). In addition, the EC_{50} values of TCDD for chicken, crow and cormorant AhR1s were estimated to be 0.03, 0.14 and 0.67 nM, respectively. These results showed that the ability of avian AhR1s to transactivate the luciferase reporter gene in the presence of TCDD mirrored the results of the TCDD-binding assay.

Levels of AhR1 mRNA expression were similar in most tissues except muscle and pancreas, in which mRNA levels were low. On the other hand, AhR2 mRNA was mainly expressed in the liver, and was detectable in gonad, brain and intestine, suggesting that each AhR isoform may play a distinct role. The absolute contents of both AhR transcripts were determined using real-time RT-PCR in the

livers of cormorants and crows. AhR1 is dominant isoform in cormorant, whereas AhR2 is a major form in crow, indicating that different AhR isoforms may play the dominant role in the response to TCDD.

All together these results, the present study demonstrated that first, the transactivation properties and relative binding ability to TCDD of avian AhR1 may be predictable from the critical amino acid residues in the ligand binding domain. Secondary, the AhR2s in cormorant, albatross and chicken may be less functional and regulate the transcription of target gene in a different manner from AhR1 isoform, whereas crow AhR2 may play the dominant role in TCDD response. Finally, the function of avian AhR isoforms mediating the response to TCDD exposure may be diverse even within bird species.

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E.-Y. Kim (e-mail: eykim@agr.ehime-u.ac.jp), H. Iwata, T. Yasui, N. Inoue, J.-S. Lee, D. G. Franks, S. I. Karchner, M. E. Hahn, S. Tanabe