The molecular basis for differential dioxin sensitivity in birds: Role of the aryl hydrocarbon receptor

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related halogenated aromatic hydrocarbons (HAHs) are highly toxic to most vertebrate animals, but there are dramatic differences in sensitivity among species and strains. Aquatic birds including the common tern (Sterna hirundo) are highly exposed to HAHs in the environment, but are up to 250-fold less sensitive to these compounds than the typical avian model, the domestic chicken (Gallus gallus). The mechanism of HAH toxicity involves altered gene expression subsequent to activation of the aryl hydrocarbon receptor (AHR), a basic helix–loop–helix-PAS transcription factor. AHR polymorphisms underlie mouse strain differences in sensitivity to HAHs and polynuclear aromatic hydrocarbons, but the role of the AHR in species differences in HAH sensitivity is not well understood. Here, we show that although chicken and tern AHRs both exhibit specific binding of [3H]TCDD, the tern AHR has a lower binding affinity and exhibits a reduced ability to support TCDD-dependent transactivation as compared to AHRs from chicken or mouse. We further show through use of chimeric AHR proteins and site-directed mutagenesis that the difference between the chicken and tern AHRs resides in the ligand-binding domain and that two amino acids (Val-325 and Ala-381) are responsible for the reduced activity of the tern AHR. Other avian species with reduced sensitivity to HAHs also possess these residues. These studies provide a molecular understanding of species differences in sensitivity to dioxin-like compounds and suggest an approach to using the AHR as a marker of dioxin susceptibility in wildlife.

Results

Chicken and Tern AHRs Share High Sequence Identity and Exhibit Specific Binding of TCDD. We obtained full-length cDNA sequences for chicken and tern AHRs, which encode proteins of 858 aa (96.2 kDa) and 859 aa (96.3 kDa), respectively. The chicken AHR cDNA sequence matches that reported recently (29). The amino acid sequences of the chicken and tern AHRs are highly similar, with 92% identity overall (Fig. 5, which is published as supporting information on the PNAS web site). They share especially high sequence identity (98%) in the region encompassing amino acids 235–402 of the chicken AHR (tern amino acids 236–403), which corresponds to the LBD (amino acids 230–397) of the mouse AHR (30). The chicken and tern AHRs both belong to the AHR1 clade (4), and thus are orthologs of the human AHR.

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; HAP, hydroxylapatite; HAH, halogenated aromatic hydrocarbon; AHR, aryl hydrocarbon receptor; LBD, ligand-binding domain; IVTT, in vitro transcription and translation; VS, velocity sedimentation; UPL, unprogrammed lysate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF192502 and AF192503).

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Full-length chicken and tern AHR proteins synthesized by in vitro transcription and translation (IVTT) were expressed at similar levels (Fig. 1A). The ability of the in vitro-expressed chicken and tern AHRs to bind [3H]TCDD was assessed by using a velocity sedimentation (VS) assay (31) that has proven valuable in characterizing IVTT-expressed AHRs from a variety of species (32–35). Chicken and tern AHRs both exhibited specific binding of [3H]TCDD (2 nM) (Fig. 1B), but specific binding by the tern AHR was only 25% of that measured with the chicken AHR, even at a high concentration of [3H]TCDD (10 nM) (Fig. 1C). Varying the incubation time between 2 and 18 h had no effect on the amount of specific binding (Fig. 6, which is published as supporting information on the PNAS web site). Thus, the chicken and tern AHRs differ in their ability to bind [3H]TCDD.

Chicken and Tern AHRs Differ in TCDD-Binding Affinities and Transactivation Potential. To compare the TCDD-binding affinities of the chicken and tern AHRs, we first performed saturation binding analysis on in vitro synthesized AHR proteins using [3H]TCDD and the hydroxylapatite (HAP) adsorption assay (36). The mouse AHR (2) was used as a positive control. Both the mouse and chicken AHRs exhibited high-affinity specific binding, with \( K_D \) values of 2.39 ± 0.80 and 1.49 ± 0.78 nM, respectively (Fig. 2A). In contrast, specific binding of [3H]TCDD by the tern AHR was undetectable (<1 fmol, compared to 124 ± 8 fmol for mouse AHR and 56 ± 23 fmol for chicken AHR) (Fig. 2B).
This result suggests that the tern AHR-TCDD complex may be sensitive to the washes used to remove nonspecifically bound [3H]TCDD from HAP, as found for the human AHR (34, 37). Therefore, we performed saturation binding analysis using the gentler VS assay. Both chicken and tern AHRs exhibited saturable specific binding to [3H]TCDD (Fig. 7, which is published as supporting information on the PNAS web site), with $K_D$ values of 0.52 nM (chicken) and 3.73 nM (tern), reflecting a 7-fold difference in TCDD-binding affinity between the two species (Fig. 2B). In addition, these experiments confirmed the reduced [3H]TCDD-binding capacity of the tern AHR as compared to the chicken AHR. Together, these analyses reveal that the in vitro-expressed chicken and tern AHRs differ in three ways: the tern AHR has a lower TCDD-binding affinity, a lower TCDD-binding capacity, and its complex with TCDD is more easily disrupted in the HAP assay.

In light of the substantial difference in the [3H]TCDD-binding properties of the chicken and tern AHRs, we compared their abilities to activate transcription of an AHRE-containing reporter gene in a transient transfection assay. COS-7 cells were transfected with AHR constructs and treated with DMSO or TCDD (10 nM), and luciferase activities were measured.

**Fig. 3.** Construction and functional analysis of chimeric AHRs. (A) Diagram of chimeric constructs. The DNA-binding domain (DBD), LBD, and transactivation domain (TAD) of chicken and tern AHRs were used to generate all possible chimeric constructs. Numbers indicate amino acid differences between chicken and tern AHRs. Abbreviations indicate source of domains from chicken (C) or tern (T) AHR. (B) IVTT of AHRs. AHRs were expressed by IVTT, incubated with 2 nM [3H]TCDD for 18 h at 4°C, and analyzed by VS. (C) Specific binding of [3H]TCDD to in vitro-expressed chimeric AHRs. AHR proteins were synthesized by IVTT, incubated with 2 nM [3H]TCDD for 18 h at 4°C, and analyzed by VS. (D) Specific binding of [3H]TCDD to chimeric AHR proteins, calculated from fractions 10–20 of the curves in C. (E) Transactivation by chicken, tern, and the chimeric AHR proteins. COS-7 cells were transfected with AHR constructs and treated with DMSO or TCDD (10 nM), and luciferase activities were measured.

Chicken AHR LBD Confers High-Affinity TCDD-Binding to Tern AHR. To determine the relative roles of the separate AHR functional domains in the different properties of chicken and tern AHRs, we made six chimeric constructs by swapping the three functional domains (DNA-binding/dimerization, ligand-binding, and transactivation) of these AHRs (Fig. 3A). The chicken and tern AHRs differ by 8, 3, and 52 aa, respectively, in these domains. The chimeric and wild-type AHRs were expressed in vitro (Fig. 3B) and their ability to bind [3H]TCDD was measured by VS, using a concentration of [3H]TCDD (2 nM) that would distinguish between the high-affinity and low-affinity binding forms of avian AHRs (Fig. 2B). The three chimeric constructs containing the chicken LBD exhibited substantial [3H]TCDD binding compared to that of the wild-type chicken AHR (Fig. 3C and D). The remaining three chimeric AHRs, all of which possessed the tern LBD, had very low [3H]TCDD binding, similar to that of the wild-type tern AHR. Consistent with the in vitro [3H]TCDD binding results, the chicken AHR and the three chimeric constructs with the chicken LBD supported TCDD-inducible luciferase expression after transient transfection into COS-7 cells, whereas the tern AHR and the other three chimeric constructs did not (Fig. 3E). Thus, the LBD is responsible for the distinct

reporter gene, even at 10 nM TCDD, which produces maximal activation of mouse and chicken AHRs (Fig. 2C). These results show that the reduced activity inferred from assays with in vitro-expressed tern AHR also is evident when this AHR is expressed in a cellular context.
functional properties of the chicken and tern AHRs both in vitro and in whole cells.

Val-325 and Ala-381 in the Tern LBD Are Responsible for Altered Ligand-Binding and Transactivation. The LBDs of chicken and tern AHRs differ at three amino acid residues: Ala-257, Ile-324, and Ser-380 in the chicken AHR are Thr-258, Val-325, and Ala-381 in the tern AHR. To assess the contribution of each of these amino acids to the properties of the two AHRs, each of the corresponding residues in the tern AHR was changed to its chicken counterpart via site-directed mutagenesis (Fig. 4A). The resulting tern AHR constructs (T258A, V325I, and A381S) were expressed in vitro (Fig. 4B) and tested for [3H]TCDD binding and for their ability to transactivate the luciferase reporter gene. As we saw earlier, the specific binding of [3H]TCDD to wild-type tern AHR was ≈20% of that seen with the chicken AHR (Fig. 4 C and D). Specific binding of [3H]TCDD to the tern AHR mutant T258A was low and similar to that of the wild-type tern AHR. In contrast, tern AHR mutants V325I and A381S exhibited levels of [3H]TCDD binding approaching those of the wild-type chicken AHR (Fig. 4 C and D). Consistent with this, the V325I and A381S mutants were able to activate luciferase transcription in response to TCDD, whereas the T258A mutant could not (Fig. 4E). Together, these results provide consistent evidence that the reduced ability of the tern AHR to bind [3H]TCDD and activate transcription is the result of the Val-325 and Ala-381 residues in its LBD.

Discussion
Vertebrate animals display a range of sensitivities to effects of HAHs; aquatic birds, in particular, have greatly reduced sensitivity as compared to typical laboratory models (20–24). We show here that the reduced HAH sensitivity of common terns as compared to chickens is associated with differences in functional properties of the tern AHR, including lower TCDD-binding affinity and binding capacity and reduced ability to activate transcription in cell culture. We show also that this variation in AHR function can be ascribed to two specific amino acid differences within the LBD of this protein.

Structural and Functional Differences Between Chicken and Tern AHRs. Chicken and tern AHRs share a high degree of sequence conservation (see Supporting Text, which is published as supporting information on the PNAS web site). Nevertheless, they exhibit important functional differences. The difference in TCDD-binding affinity between chicken and tern AHRs (7-fold) is similar to that observed in previous studies of the mouse Ahrb and Ahrd variants (11, 41) and in comparisons of the mouse Ahrb and human AHR (34, 41, 42). The tern AHR and human AHR share functional characteristics, including a reduced ligand-binding affinity as well as lability of the AHR-TCDD complex under in vitro conditions (37, 43).

The reduced ability of the tern AHR to bind [3H]TCDD and activate transcription as compared to the chicken AHR is a result of two amino acid differences. Independent V325I and A381S substitutions in the tern AHR converted the ligand-binding and transactivation abilities of the tern AHR to those of a chicken AHR, strongly suggesting that both of these positions contribute to the difference in function between chicken and tern AHRs. Interestingly, Ala-381 in the tern AHR is homologous to (i.e., at the equivalent position as) Ala-375 of the high-affinity mouse Ahb protein and Val-375 and Val-380 of the lower-affinity mouse Ahb and human AHR proteins, respectively (Fig. 4A), which is published as supporting information on the PNAS web
site, and Supporting Text). Previous studies have shown that this residue is a key determinant of ligand binding in mammalian AHRs (11, 41, 42, 44). Our results indicate that this position is functionally important in diverse vertebrate AHRs. Although the tern AHR possesses the residue (Ala) that is associated with high-affinity binding in mammalian AHRs, the function of the tern AHR is deficient in comparison to that of the chicken AHR, which has a Ser in this position. This residue forms part of the antiparallel β-sheet in the ligand-binding pocket of the AHR, as inferred from a recent AHR homology model (45, 46). According to this structural model, the reduction in binding affinity of AHRs bearing Val at this position is the result of the larger side-chain [Val(CH(CH3)2)] as compared to Ala ([CH3]), which hinders access to the binding cavity (45). Our results show that the presence of serine (CH3OH) in this position, as found in the chicken AHR, appears to enhance the binding affinity and/or capacity, perhaps by stabilizing the ligand-receptor interaction through hydrogen-bonding interactions with the dioxygen bridge of TCDD. Regardless of the mechanism, the residues Val → Ala → Ser at this position appear to form a series of substitutions leading to increasing stability of TCDD interaction with the AHR. Whether a similar pattern of increased binding will be true also for other AHR ligands remains to be determined.

Role of AHR in Differential Sensitivity to Dioxin-Like Compounds. The difference in sensitivity to effects of HAHs between chickens and terns has been demonstrated both in vivo and in cultured cells (23, 24, 28). To what extent do the functional differences shown here for these AHRs expressed in vitro explain the species-specific in vivo sensitivity? Although in vitro assay systems such as reticulocyte lysate do not completely replicate the normal cellular context in which these AHRs function, several observations suggest that the functional differences measured in vitro are likely to be relevant in vivo. (i) The reduced binding affinity and capacity of the tern AHR in vitro are reflected in its reduced ability to activate transcription in response to TCDD in whole cells (Fig. 2C). (ii) Differences in ligand-binding affinity among in vitro-expressed AHRs from mouse strains and humans reflect in vivo differences (11, 16, 41, 47, 48). (iii) Two previous studies (25, 26) using avian tissue cytosols showed differences in AHR binding properties that correspond to differences in sensitivity to HAHs. Although neither study involved terns, the results are consistent with the idea that differences in AHR binding properties among bird species occur within a native tissue context. Thus, the functional differences in chicken and tern AHRs demonstrated here in vitro are likely to contribute to the differential in vivo sensitivity of these species.

Whether the 7-fold difference in binding affinity between chicken and tern AHRs can fully explain the 80- to 250-fold difference in species sensitivity is not clear. Because the biological potency of AHR agonists is not a linear function of their receptor-binding affinity (49), modest differences in affinity for TCDD could result in larger differences in sensitivity in vivo, as seen in mice expressing the human AHR (16) and in Xenopus laevis (50). Alternatively, other factors such as differences in AHR expression could be important determinants of in vivo sensitivity, as suggested by studies in Ahr congenic mice (47), mice hypomorphic at the Ahr locus (51), and transgenic mice expressing the human AHR (16). Other properties of the AHR, as well as other physiological or biochemical differences between chickens and terns, may also contribute to the differential in vivo sensitivity (see Supporting Text).

A lack of knowledge about species differences in sensitivity is a major source of uncertainty in assessing the effects of HAHs in humans and wildlife. A molecular understanding of the role of the AHR in such differences will contribute to ecological and human risk assessments by providing a mechanistic foundation for extrapolation among species, facilitating predictions of those species that may be most at risk. Sequencing and in vitro functional analysis of AHRs or AHR LBDs could supplement current approaches involving in vivo exposures (20, 24) or use of cultured hepatocytes (21, 22) to assess species differences in AHR sensitivity. Consistent with this, the AHR sequences of other bird species that exhibit reduced sensitivity to HAHs show amino acid differences like those of the tern AHR (Fig. 8B and Supporting Text).

Materials and Methods
cDNA Cloning and Sequence Analysis. Hepatic RNA was isolated from white leghorn chicken embryos and tern chicks, and initial RT-PCR products were obtained as described (52). Chicken and tern liver cDNAs were synthesized by using a Marathon cDNA Amplification kit (Clontech). 5’ and 3’ RACE were performed by using oligonucleotide primers (see Supporting Text) designed to recognize both chicken and tern sequences, coupled with adaptor primers. Gene-specific primers were B-31R and B-52R for 5’ RACE and B-21F and B-25F for 3’ RACE. All PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced at the University of Maine Sequencing Facility (Orono, ME).

Expression Constructs. Full-length chicken and tern AHR cDNAs were amplified with primers Bkoz and Bterm. A Kozak consensus sequence was incorporated into the 5’ primer Bkoz. Several clones were sequenced to ensure accuracy. Clones were digested with Sall and XbaI at the primer sites and ligated into pcDNA 3.1/Zeo(+) vector (Invitrogen) cut with Xhol and XbaI to create pcDNA-GgAHR (chicken) and pcDNA-ShAHR (tern). To construct the chimeric chicken/tern expression plasmids, six fragments corresponding to the DNA-binding (chicken amino acids 1–242, tern amino acids 1–243), ligand-binding (chicken amino acids 243–390, tern amino acids 244–391), and transactivation (chicken amino acids 391–858, tern amino acids 392–859) domains were amplified by PCR using Bkoz/NcoR, NcoF/NarR, and NarF/Bterm primers, respectively. Ncol and NarI restriction sites were introduced via the primers, allowing ligation of chicken and tern fragments to each other to create six chimeric AHR cDNAs. These were ligated into the NotI/XbaI sites of the pcDNA 3.1/Zeo(+) vector. Chimeric constructs were verified by sequencing. Other expression constructs used were: mouse AHR (pSPORTMAHR; C. Bradfield, University of Wisconsin, Madison) (2), killifish Arnt2 (pcDNA-ARNT2) (53), and pGudLuc6.1 (M. Denison, University of California, Davis) (34).

Site-Directed Mutagenesis. We used a QuikChange XL Site-directed mutagenesis kit (Stratagene) to change three amino acid residues in the tern LBD. The pcDNA-tern AHR construct was the PCR template, and the primers were f325mt, r325mt, f381mt, and r381mt. Mutated constructs were sequenced completely.

In Vitro Protein Synthesis and Ligand-Binding Assay by Velocity Sedimentation. Trt-QtK Coupled Reticulocyte Lysate Systems (Promega) were used to synthesize [35S]methionine-labeled or unlabeled proteins. [35S]Methionine-labeled IVTT reactions (5 μl) were subjected to SDS/PAGE, followed by fluorography. [35S]-labeled proteins were quantified by scintillation counting of excited gel fragments.

AHR ligand-binding was assayed by using unlabeled proteins by VS on sucrose gradients in a vertical tube rotor as described earlier (31, 32). IVTT reactions (50 μl) were diluted 1:1 with MEEDMG buffer (32) and incubated overnight at 4°C with various concentrations of 2,3,7,8-tetrachloro[1,6-3H]dibenzo-p-dioxin ([3H]TCDD; 35 Ci/mmol; Chemsyn Science Laboratories, Lenexa, KS; 1 Ci = 37 GBq). NSB was determined by

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parallel incubations of TnT lysate without AHR [unprogrammed lysate (UPL)]; UPL lacks specific binding when assayed in the presence and absence of excess 2,3,7,8-tetrachlorodibenzo-p-dioxin (9, 32, 34).

**Saturation Binding Analysis.** Saturation binding was performed by using two methods, which are outlined here and described in detail in Supporting Text. The initial analyses used a modification of an HAP adsorption assay (36). AHR proteins were synthesized by IVTT, diluted in MEEDG buffer, and incubated with [3H]TCDD for 22 h at 4°C. After the incubation, 200 μl of resuspended HAP was added to each tube, tubes were incubated on ice, the HAP was washed, and radioactivity was measured. In the second method, saturation binding was performed by using VS on sucrose gradients (32) essentially as described above. Saturation binding curves are plotted as “free” [3H]TCDD (nM) vs. specifically bound [3H]TCDD after subtraction of NSB (binding of [3H]TCDD to UPL) (34).

**Cell Culture, Transfection, and Luciferase Assays.** COS-7 cells were from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (Sigma) with 10% FCS (Sigma) at 37°C under 5% CO2. Cells were plated at 3 × 10⁴ cells per well in 48-well plates. Transfections were carried out 24 h after plating in triplicate wells. DNA and Lipofectamine 2000 reagent (Life Technologies) were each diluted in serum-free DMEM. For each well, a total of ~300 ng of DNA was complexed with 1 μl of Lipofectamine 2000. The mixture was then added to cells in DMEM with serum. Cells were dosed 5 h after transfection with either DMSO or TCDD (10 nM) at 0.5% final DMSO concentration. *Renilla* luciferase (pRL-TK; Promega) was used as the transfection control. Transfected DNA amounts were 5 ng each of chicken or tern AHR construct, 50 ng of pSPORTMAHR (mouse AHR), 50 ng of pcDNA-ARNT2, 20 ng of pGudLuc 6.1, and 3 ng of pRL-TK. The total amount of transfected DNA was kept constant by addition of empty pcDNA vector. Cells were lysed 18 h after dosing, and luminescence was measured by using the Dual Luciferase Assay kit (Promega) in a TD 20/20 Luminometer (Turner Designs). Final luminescence values are expressed as a ratio of the firefly luciferase units to the *Renilla* luciferase units.

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