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cDNA cloning and characterization of an aryl hydrocarbon receptor from the harbor seal (*Phoca vitulina*): a biomarker of dioxin susceptibility?☆

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs) are found at high concentrations in some marine mammals. Species differences in sensitivity to TCDD and PHAHs are a major limitation in assessing the ecological risk to these animals. Harbor seals accumulate high levels of PHAHs and are thought to be highly sensitive to the toxic effects of these compounds. To investigate the mechanistic basis for PHAH toxicity in harbor seals (*Phoca vitulina*), we sought to characterize the aryl hydrocarbon receptor (AHR), an intracellular protein that is responsible for PHAH effects. Here we report the cDNA cloning and characterization of a harbor seal AHR. The harbor seal AHR cDNA has an open reading frame of 2529 nucleotides that encodes a protein of 843 amino acids with a predicted molecular mass of 94.6 kDa. The harbor seal AHR protein possesses basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains. It is most closely related to the beluga AHR (82%) and human AHR (79%) in overall amino acid identity, indicating a high degree of conservation of AHR structure between terrestrial and some marine mammals. The ligand binding properties of the harbor seal AHR were determined using protein synthesized by in vitro transcription and translation from the cloned cDNA. Velocity sedimentation analysis on sucrose gradients showed that the harbor seal AHR exhibits specific binding of [^{3}H]TCDD. The [^{3}H]TCDD-binding affinity of the harbor seal AHR was compared with that of the AHR from a dioxin-sensitive mouse strain (C57BL/6) using a hydroxylapatite assay. The equilibrium dissociation constants of seal and mouse AHRs were 0.93 ± 0.19 and 1.70 ± 0.26 nM, respectively. Thus, the harbor seal AHR bound TCDD with an affinity that was at least as high as that of the mouse AHR, suggesting that this seal species may be sensitive to PHAH effects. The characteristics of the AHR potentially can be used as a biomarker of susceptibility to dioxin-like compounds, contributing to the assessment of the risk of these compounds to marine mammals and other protected animals.

Keywords: Aryl hydrocarbon receptor; TCDD; In vitro expression; Seal; Pinniped; Species-specific; Susceptibility; Dissociation constant

☆ The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank database under accession number AB056700.

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1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs) are widespread environmental toxicants that have generated serious concern because of their ubiquitous distribution, toxicity, and bioaccumulation potential (Jensen, 1987; Tanabe et al., 1994). As apex consumers, marine mammals such as seals and odontocete whales often accumulate relatively high levels of these and other lipophilic pollutants in their tissues (Tanabe et al., 1987; Bergek et al., 1992; Hutchinson and Simmonds, 1994; Norstrom and Muir, 1994; Colborn and Smolen, 1996; O'Shea, 1999). In the last 40 years, several episodes of marine mammal mortality and morbidity have occurred worldwide (Addison, 1989; Colborn and Smolen, 1996). Chemical pollutants including PHAHs have been suggested as causal agents or contributing factors in some of these episodes (Tanabe et al., 1994; Deguise et al., 1995; Colborn and Smolen, 1996; Ross, 2000; Van Loveren et al., 2000). However, the role of these chemicals in marine mammal disease remains uncertain and controversial (Addison, 1989; O'Shea, 2000a,b; Ross et al., 2000). Contributing to this uncertainty are the dramatic differences in sensitivity to PHAHs that exist among vertebrate species (Poland and Knutson, 1982; Pohjanvirta and Tuomisto, 1994), coupled with the lack of direct information concerning the sensitivity of marine mammals to TCDD or other PHAHs (Marine Mammal Commission, 1999).

The harbor seal is a widely distributed pinniped, inhabiting temperate and sub-arctic coastal areas on both sides of the North Atlantic and North Pacific Oceans. Harbor seals are thought to be one of the most sensitive species to infectious and chemical disease, as suggested by recent episodes of mass mortality. Since 1988, almost 18 000 harbor seals have died in the North and Baltic seas (Dietz et al., 1989; Osterhaus, 1989); smaller episodes of seal mortality have also occurred in North America (Duignan et al., 1995). It has been suggested that the effects of the viral epizootic affecting harbor seals may have been worsened by impairment of the seal's immune system by environmental pollutants (de Swart et al., 1995; Ross et al., 1995, 1996b, 2000; Van Loveren et al., 2000). Harbor seals inhabiting polluted coastal areas are known to accumulate high levels of dioxin-like chemicals. For example, the relatively high concentrations of PCBs (85–701 μg/g wet wt.) found in harbor seal blubber in the German and Dutch Wadden Sea (Reijnders, 1980; Addison, 1989; Lukas et al., 1990; Storr-Hansen and Spliid, 1993) have been implicated in the mass mortalities noted above (de Swart et al., 1996; Ross et al., 1996a), as well as, impaired reproductive success (Helle et al., 1976; Reijnders, 1986) and other effects (Brouwer et al., 1989). Concentrations of PCBs and other contaminants in North American seals are somewhat lower, but remain a cause for concern (Hong et al., 1993; Lake et al., 1995; Hong et al., 1996; Simms et al., 2000). The accumulation and potential impact of dioxin-like compounds in harbor seals demonstrate the need to better understand the mechanistic basis of PHAH susceptibility in these and other pinnipeds.

Exposure to TCDD, the most potent dioxin, produces a wide range of species-specific toxic and biological effects, including cancer, hepato- and immuno-toxicity, teratogenesis, and lethality (reviewed in Poland and Knutson, 1982; Pohjanvirta and Tuomisto, 1994). Most of these effects are mediated by an intracellular protein, the aryl hydrocarbon receptor (AHR), to which these chemicals bind with high affinity (Poland et al., 1976). The AHR is a member of a family of transcriptional regulatory proteins that contain the basic-helix-loop-helix/Per-ARNT (AHR nuclear translocator)-Sim (bHLH-PAS) domains (reviewed in Schmidt and Bradfield, 1996; Hahn, 1998a). The unliganded AHR exists as a complex that includes two molecules of a 90 kDa heat-shock protein (hsp90) and hepatitis B virus X-associated protein 2 (XAP2; also called Ara9 and AIP) (Meyer et al., 1998). Binding of ligand to the AHR leads to dissociation of the hsp90 complex. Transformation to the DNA-binding form requires association of the ligand-bound AHR with ARNT, another protein in the bHLH-PAS family (Hoffman et al., 1991; Reyes et al., 1992). Activa-

tion of gene transcription by transformed AHR complexes occurs through interaction with xenobiotic-responsive enhancers (XREs) located in the promoter of CYP1A and other TCDD-responsive genes. TCDD toxicity is thought to be the result of altered gene expression regulated by the AHR-ARNT dimeric complex and/or other changes caused by the ligand-activated AHR (Schmidt and Bradfield, 1996). Studies in AHR-null mice have shown that the AHR is essential for the acute toxicity and teratogenicity of TCDD (Fernandez-Salguero et al., 1996; Mimura et al., 1997; Peters et al., 1999).

Differences in AHR characteristics are known to underlie some of the species and strain differences in PHAH sensitivity. For example, mouse strains classified as 'responsive' (C57BL/6) and 'nonresponsive' (DBA/2) to PHAHs express distinct AHR alleles (Poland and Glover, 1990; Chang et al., 1993) and the 5–10-fold difference in the LD50 for TCDD sensitivity of the two strains can be explained by a similar difference in the dioxin-binding affinities of their respective AHR proteins (Okey et al., 1989; Poland et al., 1994). This suggests that AHR characteristics can be critical determinants of PHAH susceptibility. The sensitivity of marine mammals to PHAH is largely unknown and is not easily measured, since legal and ethical concerns preclude the direct testing of toxic chemicals on these animals. Alternative approaches are needed to assess their sensitivity for PHAH toxicity. We propose that the sensitivity of marine mammals to PHAH might be inferred by determining the characteristics of their AHRs using in vitro molecular and biochemical experiments that do not require the use of toxicity testing in whole animals (see also Karchner et al., 2000; Jensen and Hahn, 2001).

The objective of this study was to investigate the potential TCDD sensitivity of harbor seals by characterizing the AHR from this species. This was accomplished by the cDNA cloning, in vitro expression, and functional analysis of the harbor seal AHR. The ligand binding affinity of the seal AHR was examined by saturation binding using radiolabeled TCDD. The results suggest that the AHR may be a useful biomarker of potential susceptibility to PHAH toxicity in marine mammals.

2. Materials and methods

2.1. Materials

The mouse AHR expression vector (pSPORT-mAHR; Ah^{b-1} allele) (Burbach et al., 1992) was generously provided by Dr C. Bradfield (McArdle Center for Cancer Research, Madison WI). 2,3,7,8-Tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin (33 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was obtained from Ultra Scientific (Hope, RI). Methylated [methyl-^{14}C]ovalbumin was obtained from NEN Life Science Products, Inc. (Boston, MA). Methylated [methyl-^{14}C]catalase was synthesized as described earlier (Karchner et al., 1999).

2.2. Sample collection and RNA isolation

Harbor seal livers were obtained from animals that died or were euthanized for medical reasons during rehabilitation at The Marine Mammal Center (TMMC) in 1991. The samples were stored at −80 °C, with one brief accidental thaw, until used for RNA isolation. The animal used for the experiments reported here was MMC # HS-468 ('popcorn'; died 7 June 1991). Total RNA was isolated using RNA STAT-60 (Tel-Test B, Inc.). Poly(A)$^+$ RNA was purified with oligo(dT) spin columns (5 Prime-3 Prime, Inc).

2.3. RT-PCR and sequencing procedures

The seal AHR was cloned using a RT-PCR approach with degenerate primers that were designed as described earlier (Hahn and Karchner, 1995; Karchner and Hahn, 1996). One μg of poly(A)$^+$ RNA was reverse transcribed with random hexamers using the Gene-Amp RNA-PCR kit (Perkin-Elmer) following the manufacturer's directions. Primers were synthesized by National Biosciences, Inc or Life Technologies, Inc Primer sequences were: Qf, 5′-AACCCITCIAAGMG-

ICAYMG-3′; B2, 5′-gctctagaGCTCIRCYTCIG-TRTAICC-3′; HSL-3′4, 5′-ATTGATGCGGC-TAATGGGCTGCCAC; HSL-5′7, 5′-GCTGG-GGAGGACTGTAATGAAACATC-3′; Bel, 5′-CCCAAGCTTGGGCACCATGAACAGCAGC-AG-3′; and HSL-3′, 5′-GGGGTTGGAATTATA-GGAATCCACTGG-3′. After first strand cDNA synthesis, PCR amplification was performed using QF and B2 primers under the following conditions: 30 cycles of (15 s at 94 °C, 45 s at 50 °C, and 1 min at 72 °C). A final 72 °C, 7 min extension followed the last cycle. The PCR products were cloned into pGEM-T Easy (Promega) vector and sequenced as described earlier (Karchner et al., 1999).

For 5′- and 3′-rapid amplification of cDNA ends (RACE), adaptor-ligated, oligo(dT)-primed, double-stranded liver cDNA was synthesized using a Marathon cDNA Amplification kit (Clontech). For 3′-RACE, gene specific primers (HSL-3′4) were coupled with adaptor primers in the PCR reactions and the products were cloned and sequenced. For 5′-RACE, primer Bel, which was designed based on the beluga AHR mRNA sequence (Jensen and Hahn, 2001), and seal-specific primer HSL-5′7 were used. For determination of the full length seal AHR sequence, at least three clones were sequenced completely (forward and reverse).

2.4. Sequence analysis

AHR amino acid sequences were aligned using ClustalX (Thompson et al., 1994, 1997) and these aligned sequences were used to construct phylogenetic trees using distance and maximum parsimony criteria within PAUP*4.0b8 (Swofford, 1998). Positions with gaps were omitted.

2.5. Expression construct

The Bel/HSL-3′15 primer pair was used to amplify a 2650 bp (full-length) harbor seal cDNA from liver. The PCR product was ligated into pGEM-T Easy to make plasmid pGEM_PvAHR encoding the full-length harbor seal AHR protein. Advantage cDNA polymerase mix (Clontech) was used in the PCR reactions to maximize fidelity of amplification. All cDNA constructs were verified by complete sequencing.

2.6. In vitro protein synthesis

TNT Quick coupled Reticulocyte Lysate Systems (Promega) were used to synthesize unlabeled or [^{35}S]methionine-labeled proteins following manufacturer's directions. One-half μg of pGEM_PvAHR or pSPORTmAHR was used in each 25 μml reaction. Five μl of the TNT reactions was separated on 10% SDS-polyacrylamide gel electrophoresis. Gels were fixed, dried onto filter paper, and exposed to film overnight. The [^{35}S]methionine-labeled proteins were quantified by scintillation counting of excised gel fragments.

2.7. Velocity sedimentation analysis

AHR proteins were expressed by in vitro transcription and translation and analyzed by velocity sedimentation on sucrose gradients in a vertical tube rotor (Tsui and Okey, 1981). For each receptor or the unprogrammed lysate (UPL) control, two identical TNT reactions (100 μl total) were combined, diluted 1:1 with MEEDMG buffer (25 mM MOPS, pH 7.5 at the rate of 20 °C, containing 1 mM dithiothreitol, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 20 mM Na_2MoO_4, 10% (v:v) glycerol), split into two 100 μl aliquots and incubated with [^{3}H]TCDD (2 nM) ± TCDF (400 nM) for 18 h at 4 °C. Velocity sedimentation analysis was performed as described earlier (Karchner et al., 1999).

2.8. Saturation binding analysis

The specific binding of [^{3}H]TCDD to in vitro expressed harbor seal and mouse AHRs was measured using a hydroxylapatite binding assay (Gasiewicz and Neal, 1982), with modifications. The seal and mouse AHR proteins were synthesized by in vitro transcription and translation as described above. For each AHR, four 50 μl reactions were combined and then diluted four-fold in MEEDG buffer (25 mM MOPS, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 10% (v:v) glycerol, 1

mM DTT, pH 7.5). The diluted TNT-expressed AHRs were then split into 50 µl aliquots that were incubated in 10 × 75 mm glass tubes for 22 h at 4 °C with each of eight concentrations of [^{3}H]TCDD in DMSO (0.1, 0.2, 0.5, 1, 2, 4, 8, 10 nM, each in duplicate). (Initial experiments showed that equilibrium was attained after overnight incubation at 4 °C). A 5 µl aliquot was taken from each tube to determine the total concentration of [^{3}H]TCDD. After the incubation, 200 µl of resuspended HAP (50%, v:v in MEEDG) were added to each tube and the tubes were incubated on ice for 30 min. A 0.5 ml aliquot of MEEDGT buffer (MEEDG buffer plus 0.1% Tween 20) was then added and the contents were transferred to a 1.5 ml Eppendorf tube, which was then centrifuged at 700 × g. After two additional washes with MEEDGT, the HAP pellet was transferred to a scintillation vial and the radioactivity (total bound [^{3}H]TCDD) was measured on a Beckman 5000 scintillation counter. For the measurement of nonspecific binding, 'unprogrammed' TNT lysate (UPL; TNT lysate plus an empty expression vector) was incubated with the same concentrations of [^{3}H]TCDD and analyzed by the HAP assay; UPL lacks specific [^{3}H]TCDD binding as assessed by velocity sedimentation and other methods (Karchner et al., 1999; Jensen and Hahn, 2001). The binding of [^{3}H]TCDD to UPL was plotted as a function of the free [^{3}H]TCDD concentration and fit to a linear model; this relationship was used to calculate the non-specific binding at each concentration of free [^{3}H]TCDD in the incubations with in vitro synthesized AHRs. Specific binding for each AHR was calculated as the difference between the actual total binding and the calculated non-specific binding.

The equilibrium dissociation constants (K_d) for harbor seal and mouse AHRs were obtained from the respective specific binding curves using the equation for the Langmuir binding isotherm: $B = B_{max}[L]/[L] + K_d$, where B is specifically bound [^{3}H]TCDD, B_{max} is maximum bound receptor, L is the concentration of free ligand, and K_d is the equilibrium dissociation constant. Non-linear regression analysis was performed using Prism version 3 software for the Macintosh (GraphPad) as described earlier (Jensen and Hahn, 2001).

3. Results

3.1. Cloning of a harbor seal AHR cDNA

Using the RT-PCR approach described in Section 2, we obtained a full-length AHR cDNA sequence from harbor seal liver. Initially, degenerate primers Qf and B2 produced a 849-bp partial AHR cDNA. The remainder of the 5′ coding sequence was obtained using the Bel/HSL-5′7 primer pair; 3′ coding and UTR sequences were obtained by 3′-RACE with the HSL-3′4 primer (Fig. 1). The harbor seal AHR cDNA has an open reading frame of 843 amino acid residues that encodes a protein with a predicted molecular mass of 94.6 kDa (Fig. 2). The C-terminal sequence includes 105 bp of 3′-UTR with a poly $(A)^+$ tail preceded by a polyadenylation signal sequence (AATAAA).

3.2. Sequence comparisons

Alignment of the amino acid sequences of mammalian AHRs revealed high overall amino acid identities among harbor seal, beluga, human, and mouse AHRs (Fig. 3). The harbor seal AHR is most closely related to the beluga AHR (82% overall amino acid identity) and shares 79 and 66% identity with the human and mouse AHRs, respectively. The N-terminal half of harbor seal

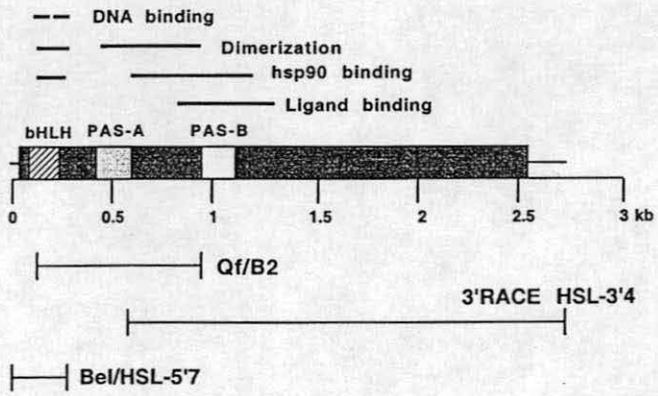

Fig. 1. Strategy for cloning and sequencing of the harbor seal AHR cDNA. A schematic diagram of the seal AHR cDNA is shown. For orientation, the functional domains of human and murine AHRs are indicated above the cDNA. Below the cDNA, the initial RT-PCR product (Qf/B2) and RACE products are indicated. See text for details.

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1    CCA AGC TTG GGC ACC ATG AAC AGC AGC AGC GCC CAC ATC ACC TAC GCC AGT CGC AAG CGG   60
                     M   N   S   S   S   A   H   I   T   Y   A   S   R   K   R
61   CGG AAG CCG GTG CAG AAA ACT GTC AAG CCA ATC CCA GCT GAA GGA ATC AAG TCA AAT CCT  120
      R   K   P   V   Q   K   T   V   K   P   I   P   A   E   G   I   K   S   N   P
121  TCC AAG CGA CAT AGA GAC CGA CTT AAT ACA GAG TTG GAC CGT TTG GCT AGT CTG CTG CCT  180
      S   K   R   H   R   D   R   L   N   T   E   L   D   R   L   A   S   L   L   P
181  TTT CCA CAA GAT GTT ATT AAT AAG CTG GAT AAA CTT TCA GTT CTT AGG CTC AGT GTC AGT  240
      F   P   Q   D   V   I   N   K   L   D   K   L   S   V   L   R   L   S   V   S
241  TAC CTA AGA GCC AAG AGC TTC TTT GAT GTT TCA TTA CAG TCC TCC CCA GCT GAC AGA AAT  300
      Y   L   R   A   K   S   F   F   D   V   S   L   Q   S   S   P   A   D   R   N
301  GAA GTC CAG GAA AAC TGT AGA ACA AAA TTC AGA GAA GAC CTG CAT CTG CAA GAA GGA GAA  360
      E   V   Q   E   N   C   R   T   K   F   R   E   D   L   H   L   Q   E   G   E
361  TTC TTA CTA CAG GCT CTC AAT GGC TTT GTG CTG GTT GTC ACC ACA GAT GCT TTG GTC TTT  420
      F   L   L   Q   A   L   N   G   F   V   L   V   V   T   T   D   A   L   V   F
421  TAT GCT TCT TCT ACC ATA CAA GAT TAC CTA GGG TTT CAG CAG TCT GAC GTC ATA CAT CAG  480
      Y   A   S   S   T   I   Q   D   Y   L   G   F   Q   Q   S   D   V   I   H   Q
481  AGC GTA TAT GAA CTT ATT CAT ACT GAA GAC CGA GGT GAA TTT CAG CGT CAG CTA CAC TGG  540
      S   V   Y   E   L   I   H   T   E   D   R   G   E   F   Q   R   Q   L   H   W
541  ACA TTA AAC CCT TCA CAG TGT ACA GAC TCT GGA CAA AGA ATT GAT GCA GCT AAT GGG CTG  600
      T   L   N   P   S   Q   C   T   D   S   G   Q   R   I   D   A   A   N   G   L
601  CCA CAG GCA GTA GGC TGT CAC ACC CCA GAC CAG CTT CCT CCA GAA AAC TCT TCC TGT ATG  660
      P   Q   A   V   G   C   H   T   P   D   Q   L   P   P   E   N   S   S   C   M
661  GAA AGG AGC TTT GTG TGC CGA CTC AGA TGT CTC CTG GAT AAT TCA TCT GGT TTT CTG GCA  720
      E   R   S   F   V   C   R   L   R   C   L   L   D   N   S   S   G   F   L   A
721  ATG AAT TTC CAA GGC AGG TTA AAG TAT CTT CAT GGA CAG AAC AAG AAA GGG AAA GAT GGA  780
      M   N   F   Q   G   R   L   K   Y   L   H   G   Q   N   K   K   G   K   D   G
781  TCA GTA CTA CCA CCT CAG TTG GCT TTG TTT GCC ATA GCG ACT CCC CTT CAG CCA CCA TCC  840
      S   V   L   P   P   Q   L   A   L   F   A   I   A   T   P   L   Q   P   P   S
841  ATC CTT GAG ATC CGA ACC AAA AAT TTC ATC TTC AGA ACC AAA CAC AAA CTA GAC TTT ACA  900
      I   L   E   I   R   T   K   N   F   I   F   R   T   K   H   K   L   D   F   T
901  CCC ACT GCT TGT GAT GCC AAA GGA AAA CTT GTT TTA GGC TAT ACT GAA GCA GAG TTG TGC  960
      P   T   A   C   D   A   K   G   K   L   V   L   G   Y   T   E   A   E   L   C
961  ATG AGA GGA TCA GGA TAT CAA TTT ATT CAT GCT GCT GAT ATG CTT TAT TGT GCT GAG TAC 1020
      M   R   G   S   G   Y   Q   F   I   H   A   A   D   M   L   Y   C   A   E   Y
1021 CAT ATC CGG ATG ATT AAG ACT GGA GAG AGT GGC CTG ATA GTA TTC AGG CTT CTT ACC AAA 1080
      H   I   R   M   I   K   T   G   E   S   G   L   I   V   F   R   L   L   T   K
1081 GAA AAT CGA TGG ACC TGG GTT CAG TCT AAT GCA CGC TTA GTG TAT AAA AAT GGA AGA CCA 1140
      E   N   R   W   T   W   V   Q   S   N   A   R   L   V   Y   K   N   G   R   P
1141 GAT TAT ATC ATT GCA ACT CAG AGA CCT CTA ACA GAT GAA GAA GGA ACA GAA CAT TTA CGA 1200
      D   Y   I   I   A   T   Q   R   P   L   T   D   E   E   G   T   E   H   L   R
1201 AAG CGA AAT ATG AAG ATG CCA TTT ATG TTT ACT ACT GGA GAA GCT GTG TTA TAT GAG ATA 1260
      K   R   N   M   K   M   P   F   M   F   T   T   G   E   A   V   L   Y   E   I
1261 ACC AGT CCT TTT CCT CCC GTG ATG GAT CCC TTA CCA ATA AGG ACT AAA TAT GGT ACA AGT 1320
      T   S   P   F   P   P   V   M   D   P   L   P   I   R   T   K   Y   G   T   S
1321 GGG ATA GAT TCT GCT ACC AAA TCA GCT CTC AAT AAG GAT TCT CTC AAT CCC AGT TCC CTC 1380
      G   I   D   S   A   T   K   S   A   L   N   K   D   S   L   N   P   S   S   L
1381 CTG GCT GCC ATC ATG CAA CAG GAT GAG TCT ATT TAT CTC TAT CCC GCC TCA GGT AGC ACC 1440
      L   A   A   I   M   Q   Q   D   E   S   I   Y   L   Y   P   A   S   G   S   T
1441 CCG TTT GGA AGA AAT CTT TTT AGT GAA CCT GTG AAT GAA TGC AGT AAT TGG CAA GAC AAT 1500
      P   F   G   R   N   L   F   S   E   P   V   N   E   C   S   N   W   Q   D   N
1501 ATC GCA CCA ATG GGA AGT GAC GGT ATC CTG AAA CAT GAG GAA ATA GAT CAT TCT CAG GAA 1560
      I   A   P   M   G   S   D   G   I   L   K   H   E   E   I   D   H   S   Q   E
1561 ATG AAC CCA ACA CTC TCT GGA GGT CCG ACA GGG CTC TTT CCA GAC AAC AGA AAT AGT GAC 1620
      M   N   P   T   L   S   G   G   P   T   G   L   F   P   D   N   R   N   S   D
1621 TTG TAT GGT ATT ATG AAA CAG CTA GGC ATT GAT TTT GAA GAT ATC AAA CAC ATG CAA CAG 1680
      L   Y   G   I   M   K   Q   L   G   I   D   F   E   D   I   K   H   M   Q   Q
1681 AAT GAG GAA TTT TTC AGA ACT GAC TTT TCG GTT GAG GAT GAC TTC CGA GAT ATT GAC CTA 1740
      N   E   E   F   F   R   T   D   F   S   V   E   D   D   F   R   D   I   D   L
1741 ACA GAT GAA ATC CTG ACC TAC GTC CAA GAT TCT TTA AGC AAG CCC ACC TTC GGG TGT TCA 1800
      T   D   E   I   L   T   Y   V   Q   D   S   L   S   K   P   T   F   G   C   S
1801 GAC TAC CCG CAG CCA CAG TCC CTG GCT CTG AGC TCG AGC TGT GCG GTA CAG GAG CCC CTG 1860
      D   Y   P   Q   P   Q   S   L   A   L   S   S   S   C   A   V   Q   E   P   L
1861 CAG TTC GCA CAG CTG CAG CAC CGC CCC GAG CAC GGG GCC GTG GAG CGG GCA CAG CTG TGT 1920
      Q   F   A   Q   L   Q   H   R   P   E   H   G   A   V   E   R   A   Q   L   C
1921 CAG AAA ATG CAG CAT ATG CAA GTT AAC AGC ATG TTT GCC GAC TGG AAC CCT CAC CCG TCC 1980
      Q   K   M   Q   H   M   Q   V   N   S   M   F   A   D   W   N   P   H   P   S
1981 GTA CCT CGG AGT TGT CCT CAG CAA GAT CTG CAG CAG TAT AGT GTC TTC TCA GAC GTC CCT 2040
      V   P   R   S   C   P   Q   Q   D   L   Q   Q   Y   S   V   F   S   D   V   P
2041 GGG ACC AGT GAA GCG TTT CCC TAC AAA CCT GAG CTC AGT CCT GTA CCG TAC ACA CAG AAC 2100
      G   T   S   E   A   F   P   Y   K   P   E   L   S   P   V   P   Y   T   Q   N
2101 TTT ATT CCC TGT AAT CAG CCT GTG TTA CCA CAG CAT TCC AAC GGG ACA CAG TTA GGC TTT 2160
      F   I   P   C   N   Q   P   V   L   P   Q   H   S   N   G   T   Q   L   G   F
2161 CCC ATA GGG AAT TTT GAA CCA TCC CCA TAC CCT ACT AAT AAT TTA GAA GAT TTT GTC ACA 2220
      P   I   G   N   F   E   P   S   P   Y   P   T   N   N   L   E   D   F   V   T
2221 TGT TTA CAA GTT CCT GAA AAC CAA ACC CAT GGA CTG AAT CCA GAG TCA ACC ACA GTA AGT 2280
      C   L   Q   V   P   E   N   Q   T   H   G   L   N   P   E   S   T   T   V   S
2281 CCT CAG GCC TGT TAC GCC GGG GCC GTG TCC CTG TAT CAG TGC CAG CCG GAG CCA CCA CCC 2340
      P   Q   A   C   Y   A   G   A   V   S   L   Y   Q   C   Q   P   E   P   P   P
2341 AGC AGT GTG GCC CGC ATG CCC TAC GAT CCA GCG GCA GCG CCA CAG GCA TTT TTA AAC AAG 2400
      S   S   V   A   R   M   P   Y   D   P   A   A   A   P   Q   A   F   L   N   K
2401 TTT CAG AAT GGA GGA GTT TTA AAT GAA ACC TAT CCT GCT GAA TTA AAT AAT ATA GGT AAC 2460
      F   Q   N   G   G   V   L   N   E   T   Y   P   A   E   L   N   N   I   G   N
2461 ACT CAG ACT ACC ACA CAT CTT CAG GCC CTT CAT CAC CCA TCA GAA GCC AGA CCT TTC CCT 2520
      T   Q   T   T   T   H   L   Q   A   L   H   H   P   S   E   A   R   P   F   P
2521 GAT TTG ACA TCC AGT GGA TTC CTA TAA TTC CAA CCC CAT TTT ATG CAC CTT GGT TTT CGG 2580
      D   L   T   S   S   G   F   L   *
2581 ATG AGT TTG CAG AAG ATT ACA GAA TAA TAA AAC TGT CAG TGT TGG TCA TCG AAA AAA AAA 2640
2641 AAA AAA AAA                                                                   2649
```

Fig. 2. The nucleotide and deduced amino acid sequence for a harbor seal AHR cDNA. This sequence has been deposited into the Genbank and DDBJ databases with accession number AB056700.

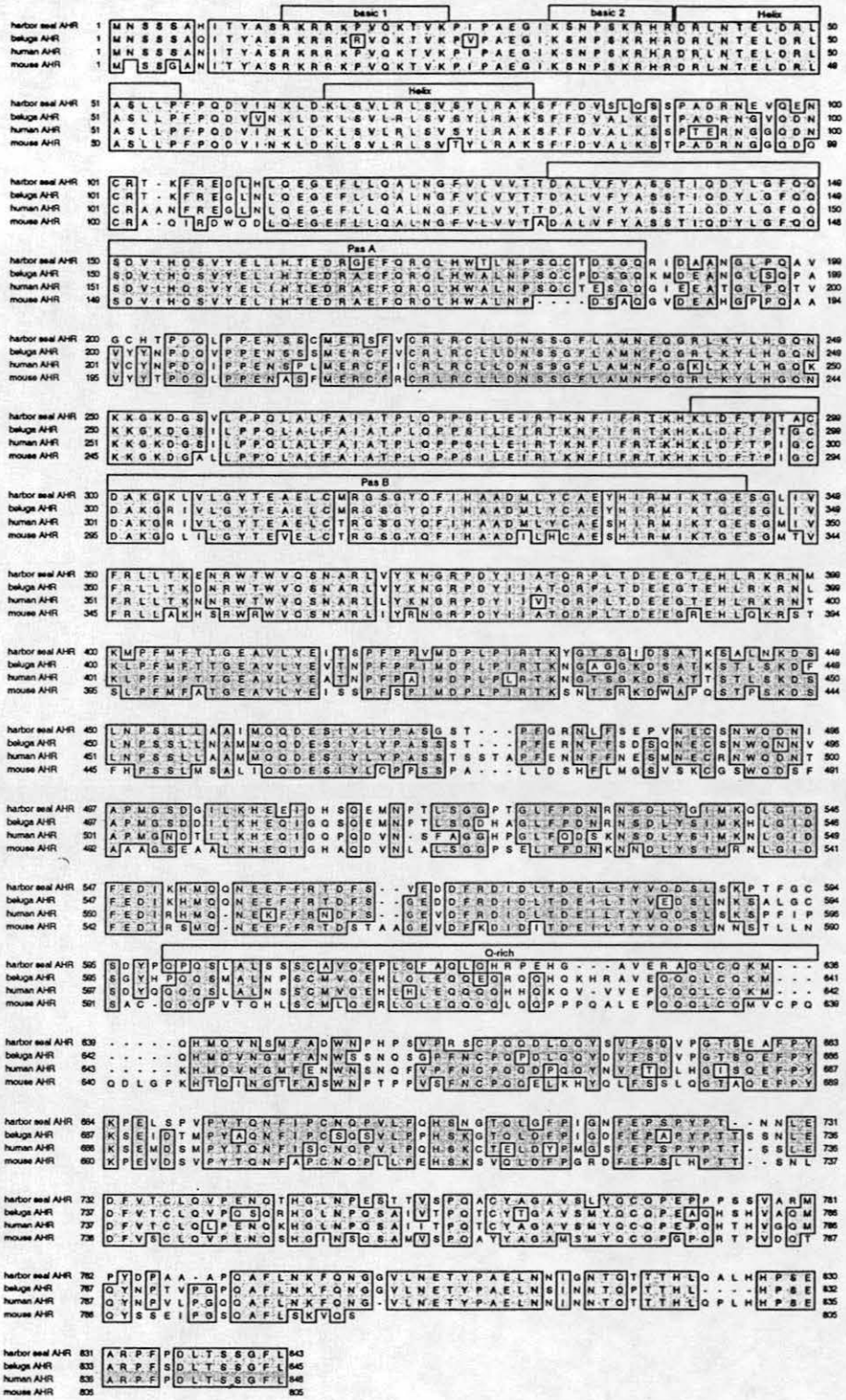

Fig. 3. Alignment of harbor seal, beluga, human, and mouse AHR amino acid sequences. Sequences were aligned with Clustal. Accession numbers and references for sequences used are: beluga AHR (Jensen and Hahn, 2001) (accession number AF332999), human AHR (Dolwick et al., 1993a) (accession number L19872), and mouse AHR ($Ah^{b\text{-}1}$ allele, Burbach et al., 1992) (accession number M94623). The functional domains labeled were identified by homology to other mammalian AHRs (see text). Identical amino acids are shaded and boxed.

Table 1
Amino acid identity (%) in full-length and, N- and C-terminal regions of mammalian AHRs

	Harbor seal	Beluga	Human	Mouse
Harbor seal		82	79	66
Beluga	91 (N)		83	51
	74 (C)			
Human	88 (N)	91(N)		53
	70 (C)	76 (C)		
Mouse	83 (N)	85 (N)	85 (N)	
	49 (C)	51 (C)	53 (C)	

*AHR amino acid sequences were aligned using Clustal. The boundaries between the N- and C-terminal regions for this table are residues 423, 423, 424, and 418 for harbor seal AHR, beluga AHR, human AHR and mouse AHR, respectively. Full-length comparisons at upper right; N- (N) and C-terminal (C) comparisons at lower left.

AHR showed 95% amino acid identity with beluga AHR in the basic regions, 100% in the HLH motif, and 95% in the PAS domain. The percent identity of the harbor seal AHR in comparison with the human AHR is 100% in the bHLH region and 94% in the PAS domain. There is also high identity with beluga (74%), and human (70%) AHRs in the C-terminal half, which is usually more divergent than the N-terminal half among AHRs (Table 1).

Phylogenetic analysis of published mammalian AHR sequences showed that the seal AHR grouped with AHRs from beluga, human, and rabbit; a separate clade of mammalian AHRs included those from mouse, rat, and hamster (Fig. 4). Phylogenetic analyses using all vertebrate AHR sequences (not shown) indicated that the seal AHR amino acid sequence groups with the 'AHR1 clade' rather than the 'AHR2 clade' recently identified in some vertebrates (Hahn et al., 1997; Karchner et al., 1999).

Differential dioxin sensitivity between C57BL/6 and DBA/2J mice is due to the properties of their AHRs ($Ah^{b\text{-}1}$ and Ah^{d} alleles, respectively). A single amino acid change (Ala^{375} to Val^{375}) appears to be responsible for the altered ligand binding affinity (Poland et al., 1994). The corresponding amino acid residue in the harbor seal AHR is alanine (Ala^{380}), as seen in the high affinity mouse $Ah^{b\text{-}1}$ allele. Cys^{216}, which is a critical residue for AHR function (Sun et al., 1997), is also conserved in the harbor seal AHR.

3.3. In vitro translation and functional characterization

An in vitro transcription/translation system was used to demonstrate the ability of the harbor seal AHR cDNA to encode protein. Fig. 5 shows that the [^{35}S]methionine-labeled translation product of the harbor seal AHR cDNA was a single band of approximately 110 kDa. This is slightly higher than its predicted size (94.6 kDa); similar differences in predicted size and mobility on SDS-PAGE gels has been noted also for other AHRs, and may be due to anomalous migration on denaturing gels or to post-translational processing (Burbach et al., 1992; Dolwick et al., 1993a; Carver et al., 1994; Jensen and Hahn, 2001). The seal AHR band was slightly larger than that of the mouse AHR, consistent with their relative

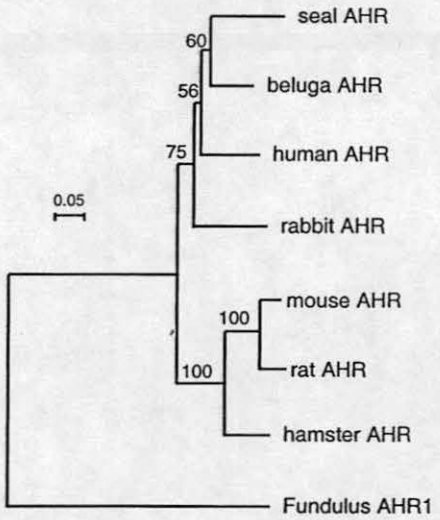

Fig. 4. Phylogenetic analysis of mammalian AHR amino acid sequences.Full-length AHR sequences were aligned using ClustalX and a neighbor-joining tree was constructed using PAUP*4.0b8. Positions with gaps were omitted and distances were corrected for multiple substitutions. Numbers represent bootstrap values based on 100 replicates. An identical topology was obtained in one of the two most parsimonious trees. Sequences and accession numbers are as listed in the legend to Fig. 3, plus the following: rabbit (Takahashi et al., 1996) (D38226), rat (Carver et al., 1994) (U09000), hamster (Korkalainen et al., 2000) (AF275721). *Fundulus* AHR1 (Karchner et al., 1999) (AF024591) was used as an outgroup.

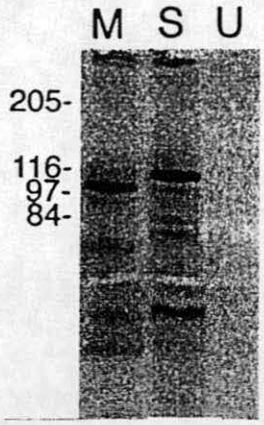

Fig. 5. In vitro expression of harbor seal and mouse AHRs.Harbor seal and mouse AHRs were synthesized by in vitro transcription and translation in the presence of [^{35}S]-labeled methionine. One-half μg of pGEM_PvAHR or pSPORTmAHR was used in each 25 μl reaction. Five μl of the TNT reactions was separated on 10% SDS-polyacrylamide gel electrophoresis and analyzed by fluorography. Positions of molecular weight standards (in kDa) are shown. Abbreviations: Mouse AHR (M), seal AHR (S), unprogrammed lysate (U).

predicted sizes (94.6 kDa for seal vs. 90.3 kDa for mouse).

The ability of the harbor seal AHR to bind ligand was tested with in vitro expressed AHR protein (Fig. 6). Lysates containing unlabeled in vitro translated harbor seal AHR were incubated with [^{3}H]TCDD and analyzed by velocity sedimentation on sucrose density gradients. The harbor seal AHR exhibited a peak of [^{3}H]TCDD specific binding that was displaced by a 200-fold excess of 2,3,7,8-TCDF (Fig. 6A). The sedimentation coefficient of harbor seal AHR was 10.7 S, similar to that of the mouse AHR (Fig. 6B). No specific binding was detected using in vitro translation products of the empty vector (UPL; Fig. 6C). This result shows that harbor seal AHR encodes protein capable of specific binding of [^{3}H]TCDD.

To determine the [^{3}H]TCDD-binding affinity of the seal AHR, saturation binding analysis was performed using an hydroxylapatite adsorption assay (Gasiewicz and Neal, 1982) with varying concentrations of [^{3}H]TCDD; the results were compared with those obtained with the Ah^{b-1} allele from a dioxin sensitive mouse strain

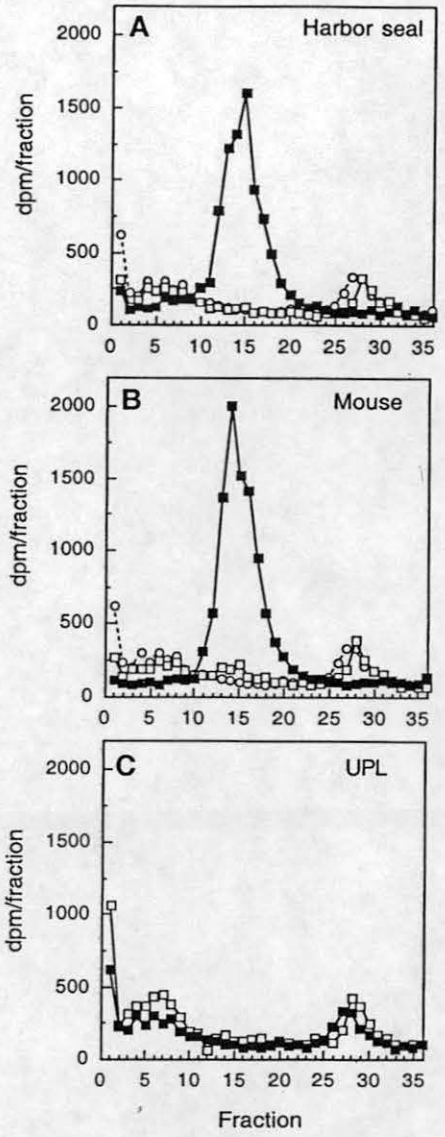

Fig. 6. Specific binding of [^{3}H]TCDD to in vitro-expressed harbor seal and mouse AHRs as assessed by velocity sedimentation on sucrose gradients.AHR proteins were expressed by in vitro transcription and translation and incubated with [^{3}H]TCDD (2 nM) ±400 nM TCDF for 18 h at 4 °C. Reactions were applied to 10–30% sucrose gradients and centrifuged for 140 min at 60 000 rpm in a VTi 65.2 rotor. Total bound [^{3}H]TCDD (dpm) is indicated by the filled squares, and non-specific binding (bound [^{3}H]TCDD that is not displaced by 200-fold excess cold TCDF) is indicated by the open squares. (A) Harbor seal AHR expressed from pGEM_PvAHR; (B) Mouse AHR expressed from pSportmAHR; (C) UPL (TNT lysate programmed with empty vector). The UPL total bound [^{3}H]TCDD shown in (C) has also been plotted in (A) and (B) as open circles. The sedimentation markers [^{14}C]ovalbumin (3.6 S) and [^{14}C]catalase (11.3 S) eluted at fractions 3–4 and 14–15, respectively.

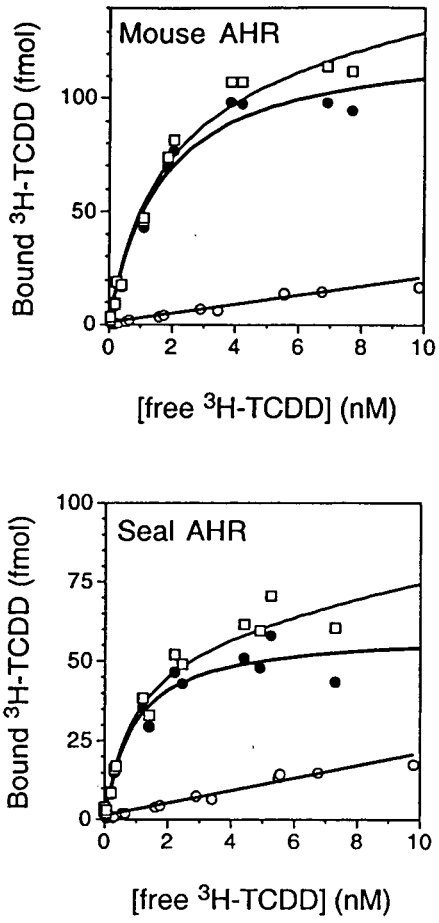

Fig. 7. Saturation binding of [^{3}H]TCDD to in vitro-expressed harbor seal and mouse AHRs as assessed by a hydroxylapatite adsorption assay.AHR proteins were expressed by in vitro transcription and translation and incubated with various concentrations of [^{3}H]TCDD for 22 h at 4 °C, followed by separation of bound and free [^{3}H]TCDD by adsorption to hydroxylapatite as described in Section 2. Open squares represent total binding and open circles represent non-specific binding as determined using unprogrammed lysate. Filled circles represent specific binding determined by subtracting the calculated non-specific binding values from the total binding in each tube. The curve through the specific binding points was derived by a non-linear curve fit to the Langmuir binding isotherm as described in Section 2. The curves shown are from one of two independent experiments, which produced similar results.

(C57BL/6) (Fig. 7). The harbor seal and mouse AHR cDNAs were expressed and assayed under identical conditions. Nonspecific binding was measured by saturation binding analysis of UPL, as done earlier (Jensen and Hahn, 2001). Specific binding was calculated as the difference between total and nonspecific binding, as described in Section 2, and equilibrium dissociation constants (K_d) were determined by non-linear regression analysis of the specific binding curves. The K_d for the harbor seal AHR (0.93 nM; 95% confidence interval 0.51–1.35 nM)) was not significantly different from that of the mouse AHR (1.70 nM; 95% confidence interval 1.11–2.23 nM).

4. Discussion

4.1. The harbor seal AHR sequence is highly similar to those of beluga and human

The cloning and sequencing of AHRs from humans and experimental animals have led to major advances in our understanding of the mechanisms underlying sensitivity to dioxin toxicity (Burbach et al., 1992; Ema et al., 1992, 1994; Poland et al., 1994). However, the structural and functional characteristics of AHRs in marine mammals are poorly understood (Hahn, 1998a). In marine mammals, a full-length AHR cDNA sequence has been reported only from beluga (Jensen et al., 1999; Jensen and Hahn, 2001). Here we describe the first full-length AHR cDNA sequence from a pinniped, the harbor seal-a species that is known to accumulate high concentrations of PHAHs in its tissues. Given the differences in sensitivity to TCDD toxicity among mammalian species (Poland and Knutson, 1982; Pohjanvirta and Tuomisto, 1994), it was of interest to compare the primary structures of several mammalian AHRs. The harbor seal AHR protein is most similar to the beluga AHR in amino acid sequence. This was not unexpected because although these two species represent distinct evolutionary lineages (pinnipeds and cetaceans) within class mammalia, they both are members of the Laurasiatheria and thus more closely related to each other than either is to rodents or primates (Madsen et al., 2001; Murphy et al., 2001). Harbor seal and beluga AHR cDNAs encode proteins of almost identical size (843 and 845 amino-acid, respectively) that are 82% identical in overall amino acid sequence. These results indicate that harbor seal and beluga express AHR proteins that are closely related structurally.

The harbor seal AHR cDNA demonstrated strong N-terminal sequence conservation with the

beluga and human AHRs. The greatest similarity between the sequences is found within the conserved bHLH domain, which has been shown to mediate DNA binding and play a secondary role in AHR/ARNT dimerization in mammalian AHRs (Dolwick et al., 1993b; Fukunaga et al., 1995; Fukunaga and Hankinson, 1996; Swanson and Yang, 1996). The PAS domain, which is important in ligand binding and AHR/ARNT dimerization (Dolwick et al., 1993b; Whitelaw et al., 1993), is also highly conserved. Furthermore, the seal and other mammalian AHRs show high similarities even in the carboxyl-terminal half (74 and 70% with beluga and human, respectively). As seen in beluga, human, and mouse AHRs, the harbor seal AHR contains a Gln-rich region, which is thought to play an important role in mediating transactivation by the AHR (Jain et al., 1994; Whitelaw et al., 1994). Overall, the sequence comparisons demonstrate that there is high degree of conservation of AHR structure between terrestrial and some marine mammals, and suggest that the basic mechanism of TCDD toxicity may be similarly conserved in these two groups.

4.2. Harbor seal may be sensitive to PHAH effects

The harbor seal AHR protein synthesized by in vitro transcription and translation demonstrated specific and high affinity binding of [^{3}H]TCDD. The TCDD-binding affinity of the harbor seal AHR was at least as high as that of the AHR from a dioxin-sensitive strain of mice (K_d values of 0.93 and 1.70 nM, respectively; lower K_d indicates higher affinity),[1] suggesting that this seal species also may be sensitive to PHAH effects. Consistent with the notion that harbor seals are capable of responding to PHAHs at environmentally relevant levels of exposure, Boon et al. (1997) and van den Brink et al. (2000) presented evidence for induction of CYP1A in harbor seal, as inferred from PCB concentration-dependent patterns of PCB congener accumulation. Our results also indicate that the TCDD-binding affinity of the seal AHR is likely to be greater than that of the human AHR, as determined in a earlier study comparing the TCDD-binding affinities of human, beluga, and mouse AHRs (Jensen et al., 1999; Jensen and Hahn, 2001). The high binding affinity of harbor seal AHR is also supported by the presence of an alanine at position 380. A comparison of the amino acid sequence and binding affinities of the mouse $Ah^{b\text{-}1}$ and Ah^{d} alleles shows that the Alanine^{375} → Valine^{375} polymorphism is responsible for the difference in binding affinities, and comparison of the human and mouse AHR sequences suggests that a similar difference (Valine^{381}) could contribute to the relatively low ligand binding affinity of human AHR as well (Ema et al., 1994; Poland et al., 1994). Harbor seal and beluga AHRs contain Alanine at the corresponding residue (Alanine^{380}) and show TCDD-binding affinities much higher than that of the human AHR (this study, Jensen and Hahn, 2001).

Marine mammals such as harbor seal and beluga are known to accumulate high levels of PHAHs in blubber and other tissues (Bergek et al., 1992; Hutchinson and Simmonds, 1994; Norstrom and Muir, 1994; Colborn and Smolen, 1996). A variety of factors, including differences in pharmacokinetics and in the capacity to metabolize TCDD and PHAHs (Tanabe et al., 1988; Boon et al., 1992; van den Berg et al., 1994), may contribute to interspecies variability in susceptibility to chemical effects in marine mammals. In addition, differences in other components of the AHR pathway could contribute to species differences in sensitivity to PHAHs (reviewed in Hahn, 1998b). Nevertheless, the high TCDD-binding affinity of marine mammal AHRs and the accumulation of high concentrations of PHAHs predicts that certain marine mammals, such as

[1] The absolute K_d values obtained using [^{3}H]TCDD in this and other experimental systems are overestimates of the true K_ds, because the concentration of free [^{3}H]TCDD is overestimated in the presence of large amounts of nonspecific binding proteins (Bradfield et al., 1988). The rabbit reticulocyte lysate used for the in vitro transcription and translation reactions unavoidably contributes a sizeable amount of protein to the binding assay. Thus, as is the case for experiments using tissue cytosol, the K_d values obtained in this system reflect relative binding affinities that are useful only in comparing samples evaluated under identical conditions, and cannot be compared directly with K_d values measured in other systems.

harbor seal and beluga, may experience a greater threat from TCDD and PHAHs than terrestrial mammals.

4.3. AHR polymorphisms

Our conclusions regarding the sensitivity of harbor seals to PHAHs assumes that the AHR cDNA that we isolated from a single animal is representative of harbor seal AHRs generally. However, polymorphisms at the AHR locus have been demonstrated in several species, including humans (Kawajiri et al., 1995; Micka et al., 1997; Smart and Daly, 2000; Wong et al., 2001), mice (Poland et al., 1986, 1994; Chang et al., 1993; Ema et al., 1994), rats (Poland and Glover, 1987; Pohjanvirta et al., 1998), and fish (Roy and Wirgin, 1997; S. Karchner and M. Hahn, unpublished). In mice, the allelic variants differ in ligand-binding affinities (Ema et al., 1994 Poland et al., 1994), while no functional differences have been seen among human variants (Wong et al., 2001). Whether AHR polymorphisms exist in harbor seal is not yet known; such polymorphisms could result in AHR proteins with greater or lesser dioxin-binding affinity than the one described here. Nevertheless, variation in AHR structure and function among individuals within species is almost certain to be much lower than variation among species. Our results therefore suggest that at least some individual seals may be sensitive to PHAH effects.

4.4. The AHR may be a useful biomarker for dioxin susceptibility in marine mammals

Biomarkers are generally defined as biochemical, physiological, or other types of biological changes that indicate the presence (exposure) or effects of xenobiotic compounds (Committee on Biological Markers of the National Research Council, 1987). In addition, some biological characteristics can be used as biomarkers of susceptibility (Eubanks, 1994; Perera, 1997; Puga et al., 1997; Nebert et al., 1999). Most, if not all, effects of TCDD are thought to result from interactions with the AHR (Schmidt and Bradfield, 1996; Gonzalez and Fernandez-Salguero, 1998). Thus, the expression and characteristics of AHRs may control the sensitivity of animal species to the effects of PHAHs. The use of AHR knock-out mice, in which the AHR gene is inactivated, has confirmed that the AHR is necessary for the biochemical, lethal, and teratogenic effects of TCDD (Fernandez-Salguero et al., 1995, 1996; Schmidt et al., 1996; Mimura et al., 1997; Peters et al., 1999). In addition, several studies have shown that differences in AHR characteristics, including ligand-binding affinity, appear to be critical determinants of differential dioxin sensitivity (Okey et al., 1989; Poland et al., 1994; Sanderson and Bellward, 1995; Pohjanvirta et al., 1998). Although the AHR is not the only factor that can influence the susceptibility to PHAH effects, it is clear that the AHR plays an important and perhaps primary role in determining susceptibility to PHAH toxicity.

The essential role of the AHR in PHAH toxicity suggests that this protein might be useful as a biomarker of susceptibility to dioxin toxicity in aquatic mammals or other species of wildlife. For example, cloning of AHR cDNAs and characterization of AHR ligand-binding affinities in distinct lineages of aquatic mammals (pinnipeds, cetaceans, mustelids, ursids, and sirenians) could indicate whether any of these are particularly sensitive to dioxin-like chemicals. In addition, in vitro competitive binding studies or transactivation assays using cloned AHRs could reveal species-specific structure-activity relationships for AHR binding and activation, which are important in the TCDD toxic-equivalency approach to risk assessment.

4.5. Conclusion

Assessing the impact of PHAHs and other contaminants on marine mammal health is a formidable challenge (Marine Mammal Commission, 1999). Because direct toxicity testing of chemicals is difficult or impossible in most marine mammals, indirect approaches must be used to infer marine mammal sensitivity to, and effects of, chemical contaminants. In this regard, the challenges associated with marine mammal toxicology are similar to those encountered in human toxi-

cology, where direct approaches are also precluded. In the latter situation, in vitro approaches are being widely used to provide data that can be used to infer human sensitivity to chemicals (MacGregor et al., 2001). We suggest that similar in vitro studies can also be valuable in marine mammal toxicology. For example, characterization of cytochrome P450s and other xenobiotic-metabolizing enzymes can contribute to an understanding of differences in the metabolism and persistence of PHAHs in marine mammals (White et al., 1994; Bandiera et al., 1995; Godard et al., 2000; Teramitsu et al., 2000; White et al., 2000; Boon et al., 2001). Similarly, and as shown for other groups of wildlife (Karchner et al., 1999; Hawkins et al., 2000; Matthews and Zacharewski, 2000), the study of receptors involved in the mechanisms of xenobiotic toxicity in marine mammals may provide important information about receptor diversity and species-specific properties, such as ligand-binding affinity and structure-binding relationships, that may underlie species differences in susceptibility (Jensen, 2000; Jensen and Hahn, 2001). The 'weight of evidence' approach for assessing the effect of chemicals in marine mammals (Marine Mammal Commission, 1999; Ross, 2000) provides a useful framework within which to interpret such data. In vitro studies such as those described above can make an important contribution to this approach. The results presented here provide a foundation for understanding the mechanistic basis of PHAH toxicity in harbor seals and show how molecular and biochemical studies may contribute to assessing the health risks of environmental contaminants in protected marine species.

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