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Identification of a putative calcium-binding protein as a dioxin-responsive gene in zebrafish and rainbow trout

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is a widespread environmental contaminant that causes multiple effects in vertebrates. TCDD elicits its toxicity through aryl hydrocarbon receptor (AhR)-mediated modulation of gene regulation, increasing intracellular free calcium, and inducing calcium-mediated apoptosis in cell culture. Two TCDD-responsive cDNAs, which encode putative calcium-binding proteins, have been isolated from zebrafish and rainbow trout. The zebrafish and rainbow trout sequences are 88% similar to each other at the amino acid level and are orthologs of the human S100A4 calcium-binding protein. In zebrafish liver cell culture, treatment with TCDD increases S100A4a mRNA abundance. In juvenile rainbow trout, S100A4 mRNA was constitutively expressed in the heart, kidney, intestine, and spleen, but not in the liver. Exposure to TCDD significantly increased rainbow trout S100A4 mRNA abundance in the rainbow trout kidney. Taken together, these findings demonstrate in zebrafish and rainbow trout that dioxin increases expression of this EF-hand calcium-binding protein gene in a tissue-dependent fashion. However, demonstration that the encoded S100A4 proteins actually bind calcium and play a role in dioxin toxicity will require further study.
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1. Introduction

The polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls are global contaminants. They are resistant to chemical and biological degradation, persist in the environment, and bioaccumulate in animals posing a potential risk to fish, wildlife, and human. 2,3,7,8-Tetrachloro-

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dibenzo-*p*-dioxin (TCDD, dioxin) is the most potent congener and the prototype for investigating effects of exposure to the halogenated aromatic hydrocarbons. TCDD evokes a number of endpoints of toxicity, including adverse developmental, endocrine and reproductive effects, immune suppression, carcinogenesis, and induction of xenobiotic metabolizing enzymes, in a variety of vertebrates including fish (Pohjanvirta and Tuomisto, 1994; Walker and Peterson, 1994; Tanguay et al., in press). The mechanism of TCDD toxicity has been extensively studied in mammals and to a lesser extent in fish. It is generally believed in both classes of vertebrates that TCDD elicits its toxicity through aryl hydrocarbon receptor/Ah receptor nuclear translocator (AhR/ARNT)-mediated modulation of transcription (Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997; Hahn, 2002a; Hahn, in press, Tanguay et al., in press). Both AhR and ARNT are transcription factors belonging to the PAS (Per, ARNT, AhR, Sim) protein family. After TCDD binds to cytosolic AhR, liganded AhR translocates to the nucleus and dimerizes with ARNT. The heterodimer then binds to dioxin-responsive elements upstream of target genes, increasing or decreasing their expression (Fujisawa-Sehara et al., 1987; Whitlaw et al., 1993; Whitlock, 1991).

Most known dioxin-responsive genes have been isolated from mammals. The most well-characterized, dioxin-responsive genes encode xenobiotic metabolizing enzymes such as P4501A1, -1A2, -1B1, glutathione S-transferase Ya subunit, and NADH: quinone reductase (Schmidt and Bradfield, 1996). TCDD also regulates other dioxin-responsive genes that may be involved in the regulation of cell proliferation and differentiation including IL-1β, PAI-2, TGF-α, ecto-ATPase, c-fos, and c-jun (Sutter and Greenlee, 1992; Gao et al., 1998; Puga et al., 1992; Choi et al., 1991). Exposure to TCDD also substantially increases the intracellular free Ca^{2+} level in several cell types including B-lymphocytes, hepatocytes, and hepatoma cells (Puga et al., 1997; Canga et al., 1988, 1993; Hanneman et al., 1996; Al-Bayati et al., 1988).

Fish are among the most sensitive vertebrates to TCDD. Exposure to TCDD during fish embryonic and larval development leads to yolk sac edema, craniofacial malformation, cardiovascular dysfunction, growth retardation, and mortality (Walker and Peterson, 1991, 1994; Henry et al., 1997; Hornung et al., 1999; Tanguay et al., in press). The AhR signaling pathway is functional in fish (Hahn et al., 1997; Hahn, 2002a; Hahn, in press, Tanguay et al., in press). Both AhR and ARNT cDNAs have been isolated from rainbow trout and zebrafish (Pollenz et al., 1996; Abnet et al., 1999; Tanguay et al., 1999, 2000; Andreasen et al., 2002), and it is reasonable to believe that TCDD elicits toxicity in fish species through AhR-mediated modulation of gene expression (Hahn, 2002a; Hahn, in press, Tanguay et al., in press). In contrast to the extensive studies of TCDD-responsive genes in mammalian species, few have been identified in fish. In this study, we used subtractive hybridization and PCR-based techniques to identify TCDD-responsive genes in two fish species. We isolated cDNA clones designated as S100A4 that encode putative calcium-binding proteins from zebrafish liver cell cultures and juvenile rainbow trout tissues, respectively. TCDD exposure was discovered to increase the abundance of S100A4a mRNA in zebrafish liver cell culture and S100A4 mRNA in the kidney of juvenile rainbow trout.

2. Materials and methods

2.1. Cell culture and TCDD exposure

All chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise. TCDD ($>$ 98% purity) was purchased from Chemsyn (Lenexa, KS). Zebrafish liver cells were obtained from David Barnes (Oregon State University, Corvallis, OR) and were cultured in modified Eagle's medium supplemented with 5% fetal bovine serum and 0.5% rainbow trout serum at 27 °C in an atmosphere of normal air. As previously described (Henry et al., 2001), confluent cells were treated with vehicle (0.1% DMSO) or TCDD (50 nM final concentration) 24 h after a final media change and harvested at specific times (0–48 h) for RNA isolation.

2.2. Experimental animals

Three juvenile rainbow trout (approximately 250 g, provided by the Aquaculture Program, University of Wisconsin, Madison, WI) were injected with 10 μg TCDD/kg body weight, i.p., in a vehicle of 5% acetone and 95% corn oil. Control fish ($n = 3$) were injected i.p. with vehicle alone. Treated fish were held 3 days without feeding in a tank provided with continuous flowing water at 12 ± 2 °C. They were then euthanized and the liver, kidney, heart, spleen, and intestine removed, snap-frozen in liquid nitrogen, and stored at −70 °C.

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated from TCDD- and vehicle-exposed cells and tissues using TRI reagent (Molecular Research Laboratories, Inc., Cincinnati, OH). Total RNA was directly used for Northern blot analysis or treated with RNase-free DNase (Promega, Madison, WI) to remove contaminating DNA, and used for cDNA synthesis or mRNA differential display. For Northern blot analysis, 10 μg of total RNA was size-fractionated through 1.2% agarose formaldehyde gels, blotted onto nylon membranes (Boehringer, Mannheim, Germany), and hybridized with [^{32}P]-labeled probes (1×10^5 cpm/ml probe was used for hybridization). Prehybridization and hybridization conditions were described previously (Cao et al., 2000). Quantitation of the RNA was performed using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). β-Actin and rainbow trout GAPDH-specific probes were used to normalize the amount of RNA loaded in zebrafish and rainbow trout samples, respectively.

2.4. PCR subtractive hybridization

Total RNA (5 μg) isolated from either vehicle- or TCDD-exposed zebrafish liver cell cultures was reverse-transcribed into first-strand cDNA using oligo-dT$_{15}$ primer (Promega, Madison, WI). Double-stranded cDNA was synthesized by the Gubler-Hoffman method and ligated to a linker (5′-AACTGGTCAGAATTCGATGGAGC-3′). The cDNA was PCR-amplified for 30 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 45 s) in a Perkin–Elmer 9600 thermalcycler using the linker sequence as primer. Biotin-dATP was incorporated into the PCR products from the DMSO-exposed sample. The unlabeled PCR products from the TCDD-treated sample were mixed in a 1:20 ratio with biotin-labeled PCR products. Subtractive hybridization was performed as described previously (Wang and Brown, 1991).

2.5. Differential screening of the subtracted cDNA

After three rounds of subtractive hybridization, the remaining cDNA products were PCR-amplified with the linker primer, digested with *Eco*RI, ligated into pGEM3Z vector (Promega, Madison, WI), and transfected into JM109 competent cells (Promega, Madison, WI). Individual transformants were transferred to a nylon membrane and hybridized with [^{32}P]-labeled first-strand cDNA probe synthesized from 10 μg DMSO-treated total RNA. Following autoradiography, the probes were stripped from the membranes by boiling in 0.1% SDS and re-hybridized with the cDNA probe prepared from the TCDD-exposed sample. Candidate clones were further analyzed by Northern blot and sequenced using a Sequenase kit (USB, Cleveland, OH). A 320 bp clone was identified as being most closely related to the mammalian S100A4 calcium-binding protein, a member of the S100 superfamily of calcium-binding proteins (Kretsinger, 1980). Following the recommended nomenclature guidelines for duplicated genes in which the zebrafish gene should be given the name of the human ortholog followed by an “a” or “b”, we have tentatively designated this cDNA as zebrafish S100A4a.

2.6. Zebrafish cDNA library screening

A cDNA library constructed from zebrafish liver cells (Tanguay et al., 1999) was screened for a full-length S100A4a cDNA. Library screening was performed according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Briefly, the library was plated onto ten 15-cm plates (about 50,000 pfu/plate), transferred to nylon membrane,

and hybridized with a [^{32}P]-labeled riboprobe derived from the 320 bp S100A4a cDNA fragment. Phage from positive plaques was plated at 200–300 pfu/plate for secondary screen and final purification. The positive clones were converted to plasmids using ex-Assist-helper phage (Stratagene, La Jolla, CA) and nonsuppression XLOLR host bacteria. Positive clones were sequenced using fluorescent dye-labeling cycle sequencing (Biotechnology Center, University of Wisconsin, Madison, WI).

2.7. Cloning of rainbow trout S100A4 cDNA by degenerate PCR

The 3′-sequence of the trout S100A4 ortholog was PCR-amplified with a degenerate primer (GGNAARGARGGNGAYAA, N = G/A/T/C, R = A/G, Y = C/T designated "Cal 3") and a specific "T-primer" (5′-CGGGCAGTGAGCG-CAACGT$_{(12)}$-3, kindly provided by Dr. Carl Lowenberger, University of Wisconsin, Madison, WI). Total RNA (10 μg) from rainbow trout kidney, liver, and spleen was combined and reverse-transcribed using the specific T-primer. The cDNA was PCR-amplified for 30 cycles (94 °C, 30 s; 50 °C, 90 s; 72 °C, 30 s) with Cal 3 primer (based on the first calcium-binding motif of zebrafish S100A4a) and T-primer. The resulting 480 bp PCR product was inserted into the Sma I site of pGEM3Z vector and sequenced.

The 5′-end of the rainbow trout S100A4 cDNA was obtained from a rainbow trout cDNA library using a PCR-based approach. A primer (5′-ACTGCCATCTCACTTAGCGTCCAG-3′, designated "Cal 5") was designed based on the partial rainbow trout S100A4 3′-nucleotide sequences. Using 0.1 μg of a rainbow trout λ phage cDNA library derived from a rainbow trout gonadal (RTG-2) cell line (Abnet et al., 1999) as template in a PCR reaction for 30 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) with the Cal 5 primer and the T_3 promoter vector primer (Promega, Madison, WI), the 5′-end of rainbow trout S100A4 cDNA was amplified. The 165 bp PCR product was inserted into the Sma I site of the pGEM3Z vector and sequenced.

3. Results

3.1. Isolation of zebrafish S100A4a, a novel TCDD-regulated gene

PCR subtractive hybridization techniques were employed to identify TCDD-responsive genes in zebrafish liver cell culture. A novel TCDD-inducible cDNA clone was identified. Sequence analysis revealed that the gene encodes a putative calcium-binding protein, closely related to mammalian S100A4 and catfish ictacalcin calcium-binding proteins (Porta et al., 1996). The full-length cDNA clone, designated S100A4a, is 360 nucleotides long with an open reading frame encoding a 95 amino acid protein with a theoretical molecular weight of 10.4 kDa. Notably, the predicted amino acid sequence contains two EF-hand calcium-binding motifs common in the S100 superfamily of proteins (underlined in Fig. 1).

3.2. Constitutive expression of zebrafish S100A4a mRNA in zebrafish liver and effect of TCDD treatment

To determine the tissue distribution of the S100A4a mRNA in tissues of adult zebrafish, total RNA was isolated from certain organs. Northern blot analysis revealed that S100A4a mRNA is constitutively expressed in the liver of

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CCAGAACCACC 11
ATGGCTACGTCAGATACCCAGAAAGCAATGGCTATGCTCATTGCA 56
M  A  T  S  D  T  Q  K  A  M  A  M  L  I  A   15
ACCTTCCACAAATACTCTGGAAAGGAGGGCGACAAATTGACCCTG 101
T  F  H  K  Y  S  G  K  E  G  D  K  L  T  L   30
TCCAAAGGCGAGCTGAAGGAACTGCTCTCTGCAGAGTTGGGCGAC 146
S  K  G  E  L  K  E  L  L  S  A  E  L  G  D   45
ATCTTTGGGAAAACTACAGACAAGGCAGCTTTGGACATGATATTC 191
I  F  G  K  T  T  D  K  A  A  L  D  M  I  F   60
AAGGATCTGGATGCAAATGCTGATGGTTCTGTGGACTTTCAGGAG 236
K  D  L  D  A  N  A  D  G  S  V  D  F  Q  E   75
TACATCACATTGATTGCCTGTATCACAATGCTTTGCAATGAGTTT 281
Y  I  T  L  I  A  C  I  T  M  L  C  N  E  F   90
TTCACAGGAAAAAAATAAGTGTCCTTACAGAATAGATTTGTCTGT 326
F  T  G  K  K  *                              95
AAGAAAAAAAATGAATAAAGGTTTTTAATGTTTTG           360
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Fig. 1. Nucleotide and predicted amino acid sequence of zebrafish S100A4a cDNA. The initiation codons (ATG) and the polyadenylation signal sequence (AATAAA) are indicated in boldface. The stop codon is in boldface and indicated by an asterisk. The conserved EF-hand calcium-binding domains are underlined.

adult zebrafish (Cao, unpublished results) and is highly abundant in zebrafish liver cell cultures exposed to DMSO for 24 h (Fig. 2A). Treatment of zebrafish liver cell culture with TCDD (50 nM) for 24 h was found to increase S100A4a mRNA abundance (Fig. 2A). 50 nM TCDD was selected as the exposure concentration since in other studies using these cells 50 nM TCDD led to the greatest overall induction of *CYP*1A and AhR2 mRNA (Henry et al., 2001; Tanguay et al., 1999). Furthermore, in a preliminary TCDD dose finding experiment, 50 nM TCDD produced the greatest increase in S100A4a mRNA abundance in zebrafish liver cell culture at 24 h. Therefore, this TCDD concentration was used to determine the time course for TCDD induction of S100A4a mRNA expression (Fig. 2B). S100A4a mRNA abundance in the TCDD group did not begin to increase, above that in the DMSO group, until 20 h. The TCDD-induced increase in S100A4a mRNA level was maximal at 24 h, and was maintained at this level, approximately 2.5 times greater than the DMSO group, at 42 and 48 h (Fig. 2B).

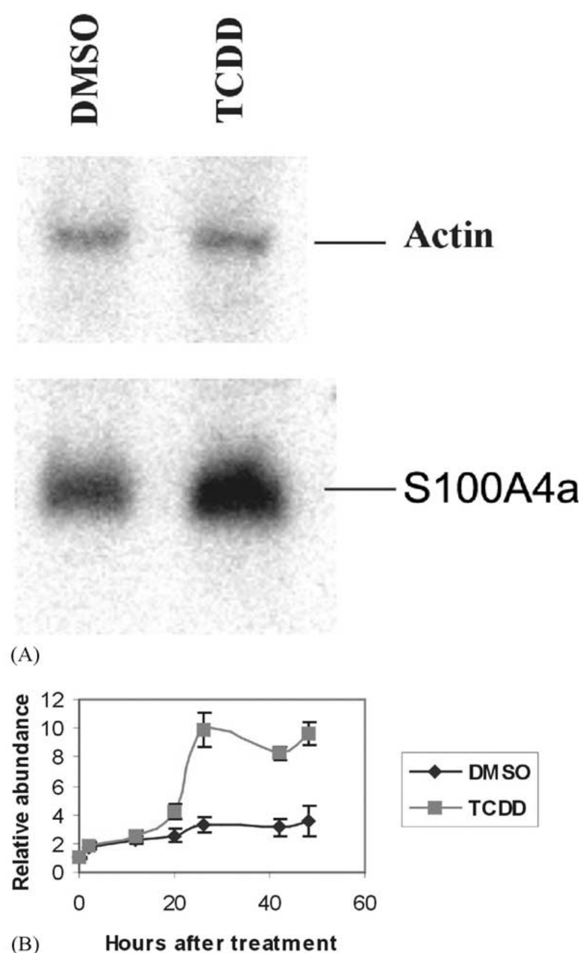

Fig. 2. TCDD exposure increases zebrafish S100A4a mRNA expression in zebrafish liver cell culture. (A) Representative Northern blot of S100A4a mRNA levels in zebrafish liver cell cultures exposed to either 0.1% DMSO (vehicle) or 50 nM TCDD for 24 h. The β-actin probe was used as a normalization control. (B) Time course for induction of zebrafish S100A4a mRNA by 50 nM TCDD relative to vehicle-exposed cultures. Each point and associated vertical line represent mean ± SE ($n = 3$).

3.3. *Cloning of the rainbow trout S100A4 ortholog*

To determine if TCDD regulation of S100A4 gene expression is conserved in other fish species, a PCR-based approach was used to isolate the rainbow trout (*Oncorhynchus mykiss*) S100A4 cDNA. The amplified rainbow trout S100A4 cDNA contains a single open reading frame similar to the zebrafish S100A4 and catfish ictacalcin amino acid sequences. The rainbow trout S100A4 clone also contains two EF-hand calcium-binding motifs. The full-length cDNA contains 606 bp and encodes a 92 amino acid polypeptide with a theoretical molecular weight of 10.4 kDa (Fig. 3).

3.4. *Constitutive expression of the rainbow trout S100A4 mRNA in different organs of rainbow trout and effect of TCDD treatment*

In order to determine the abundance of rainbow trout S100A4 mRNA in different organs of juvenile rainbow trout, total RNA was isolated from the heart, intestine, kidney, liver, and spleen for Northern blot analysis. The trout S100A4 mRNA is constitutively expressed in the heart, intestine, kidney, and spleen of juvenile rainbow trout, but not in the liver (Fig. 4A). Three days after treatment of juvenile rainbow trout with TCDD (10 μg/kg, i.p.), there was a significant 2.5-fold increase in S100A4 mRNA abundance in the kidney, but not in the other organs (Fig. 4A

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         TCTGACTGTCCAACTCCTCGCACAACTTCGTCACC 36
ATGGCACAGGTCCAGCAGGGTATGGCATTGCTCATCTCAGCCTTC 81
M  A  Q  V  Q  Q  G  M  A  L  L  I  S  A  F   15
CACAAGTACTCTGGCAAGGAGGGCGACAAGACGACCCTGAGCAAG 126
H  K  Y  S  G  K  E  G  D  K  T  T  L  S  K   30
GGAGAACTCAAAGATCTGCTCAACGCTGAGCTTGGAGAGATGATG 171
G  E  L  K  D  L  L  N  A  E  L  G  E  M  M   45
GGGAAAAACACTGACCAGGCAAAGGTTGACAAGATCTTCAAAGAT 216
G  K  N  T  D  Q  A  K  V  D  K  I  F  K  D   60
CTGGACGCTAACTCAGATGGCAGTGTGGACTTCCAGGAGTATGTC 261
L  D  A  N  S  D  G  S  V  D  F  Q  E  Y  V   75
ACACTGGTGGCCTGCCTGACCATGATGTGCAATGAGTTCTTCACC 306
T  L  V  A  C  L  T  M  M  C  N  E  F  F  T   90
AAGAAGTGAAACAGCTCCCCCTGCCCACTCTCCACAGAACAGCAG 351
K  K  *                                       92
CTCTTGCTCCCATGAGTGAATTCATGATAAGATTAGCAAGATCCT 396
CAATGCAGGTTCAAAGGGACAGAGATGCAGGTTTACACACATACA 441
CAG AAAAAGGGTTGGGTTGATTATACACACCACACATAGACAGA 486
CAGGTGAAGGACACAGACACTGCATGTACACATACAGACATATAG 531
GCTACTATCTCATGAGATGGAGAGGAAGCTCTTTGGTTTGTTTAA 576
AGTGAAAAAATGAAATAAACTATTTAATATG               606
```

Fig. 3. Nucleotide and predicted amino acid sequence of the rainbow trout S100A4 cDNA. The initiation codon (ATG) and the polyadenylation signal sequence (AATAAA) are indicated in boldface. The stop codon is in boldface and is indicated by an asterisk. The conserved EF-hand calcium-binding domains are underlined.

and B). The increased abundance of S100A4 mRNA in the kidney is representative of that observed in other trout exposed to TCDD. The slight tendency for S100A4 mRNA abundance to be increased in the spleen of the TCDD-exposed rainbow trout shown (Fig. 4A), however, was not observed in other TCDD-exposed rainbow trout.

3.5. Cross-species comparisons of calcium-binding proteins

Comparisons of the deduced amino acid sequences of the zebrafish and rainbow trout S100A4 cDNAs with the Genbank database reveal that both proteins are closely related to the mammalian S100 calcium-binding protein superfamily (Fig. 5A). The genes in this family all encode small proteins that contain two highly conserved EF-hand calcium-binding domains. Although these genes have been identified in several mammalian species and in catfish, their precise function is largely unknown. The zebrafish and rainbow trout S100A4 proteins are 72% identical and 88% similar to each other at the amino acid level and are most closely related to the catfish ictacalcin ortholog sharing 69% identity and 81% similarity overall (Fig. 5A and B). Of all

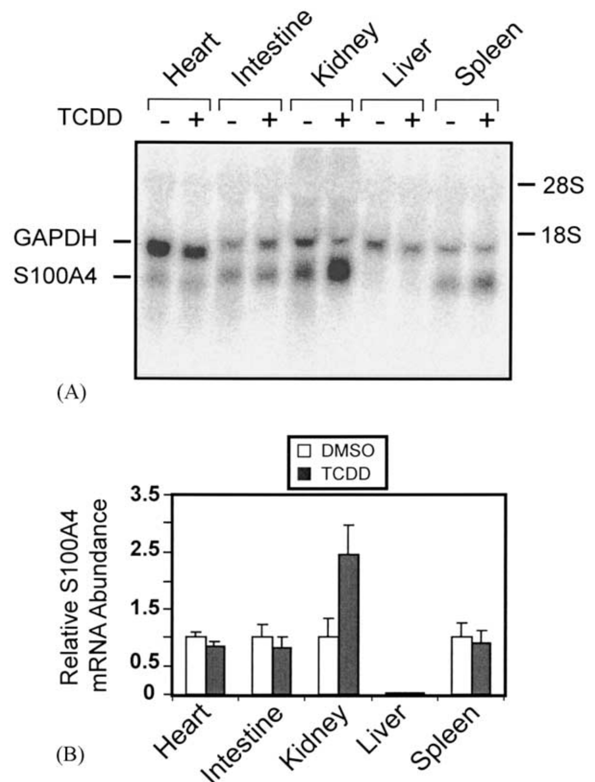
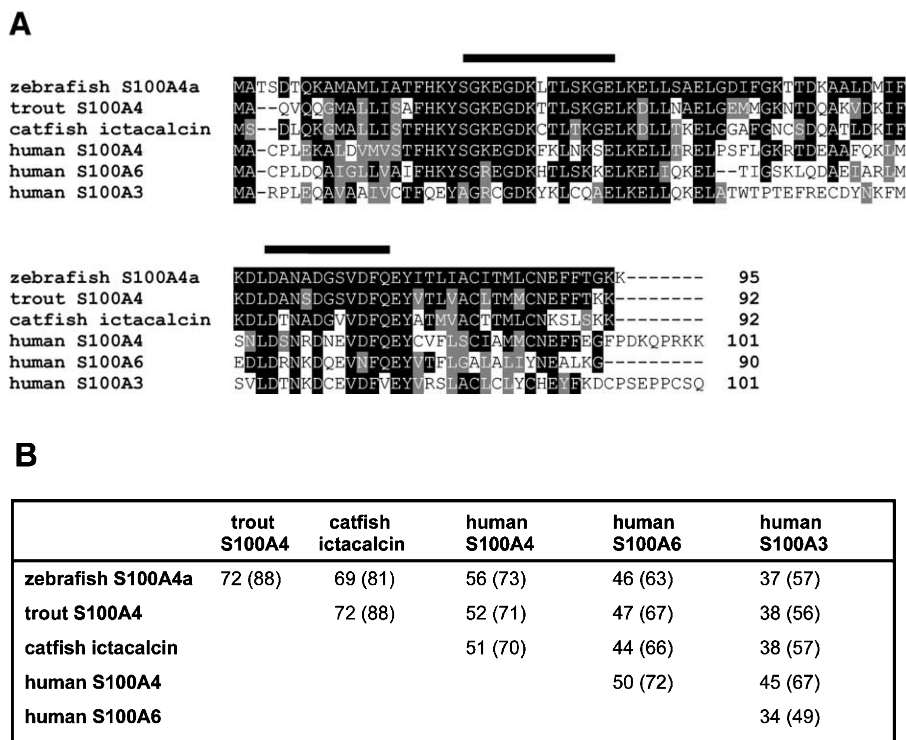


Fig. 4. Constitutive and TCDD-induced expression of rainbow trout S100A4 mRNA in organs of juvenile rainbow trout. Total RNA obtained from heart, intestine, kidney, liver, and spleen was analyzed by Northern blot analysis using [^{32}P]-labeled rainbow trout S100A4 and GAPDH (used as an RNA loading control) riboprobes. (A) Representative Northern blot analysis of S100A4 and GAPDH mRNA abundance in tissues of a TCDD- and vehicle-exposed juvenile rainbow trout. (B) Expression levels of S100A4 mRNA in organs obtained from TCDD- and vehicle-exposed trout. The height of each bar and associated vertical line for the heart, intestine, kidney, and spleen are fold induction in TCDD-treated relative to vehicle-treated fish (mean ± SE, $n = 3$).

the previously characterized human S100 class calcium-binding proteins, the zebrafish and rainbow trout cDNAs are most closely related to the S100A4 gene (also known as calvasculin or placental calcium-binding protein) with 56 and 52% overall identity, respectively. For comparisons, the next closest related human S100 protein to these fish proteins is the S100A6 (also known as prolactin receptor-associated protein) and S100A3 protein (Fig. 5A and B). Closer examination of the



	trout S100A4	catfish ictacalcin	human S100A4	human S100A6	human S100A3
zebrafish S100A4a	72 (88)	69 (81)	56 (73)	46 (63)	37 (57)
trout S100A4		72 (88)	52 (71)	47 (67)	38 (56)
catfish ictacalcin			51 (70)	44 (66)	38 (57)
human S100A4				50 (72)	45 (67)
human S100A6					34 (49)

Fig. 5. Comparison of the aligned amino acid sequences of the zebrafish, rainbow trout, and catfish putative calcium-binding proteins with the human S100 class proteins. (A) ClustalW sequence alignment of the zebrafish S100A4a, rainbow trout S100A4, catfish ictacalcin, and the human S100A4, S100A6, and S100A3 deduced amino acid sequences. The putative EF-hand domains are indicated by the bar. Black shading designates sequence identity with the zebrafish S100A4a residues and the gray shading indicates functionally conserved residues. (B) Amino acid conservation between aligned putative calcium-binding proteins. Percent identities were calculated from full-length proteins. Gaps were excluded from the alignment calculations. Numbers indicate percent identity and numbers in parentheses indicate percent similarity between compared sequences. Swissprot protein accession numbers used: catfish ictacalcin, Q91061; human S100A4, P26447; human S100A6, P06703; human S100A3, P33764.

EF-hand domains of the aligned sequence reveals remarkable sequence conservation. In the N-terminal EF-hand domain, there is only a single amino acid difference between the trout and zebrafish proteins. This residue appears not to be essential for structure since it is different in all of the human S100 proteins. There are three additional differences between the fish and human S100A4 sequences within this domain. The C-terminal EF-hand domain is also well conserved between the zebrafish and rainbow trout, with only a single difference, alanine versus serine. There are a total of four different residues between the fish and mammalian S100A4 sequences. Importantly, these residues are also not conserved between the S100A class human proteins. Taken together, the zebrafish and rainbow trout proteins are members of the S100A superfamily; however, modest divergences within the EF-hand domains make it possible that structurally and functionally the fish S100A4 genes may be distinct.

4. Discussion

4.1. Dioxin-responsive genes

Almost all of the characterized dioxin-responsive genes are from mammalian species. The exception are the *CYP*1A genes that have been characterized in many fish species and are induced at the transcriptional level by exposure to TCDD

or TCDD-like congeners (Nelson et al., 1996; Cao et al., 2000). The hypersensitivity of fish embryos and larvae to TCDD suggests that TCDD may modulate the expression of genes involved in signal transduction and cell proliferation or differentiation. Using subtractive hybridization and PCR-based approaches, we have identified two TCDD-responsive cDNA clones from zebrafish liver cell cultures and rainbow trout tissues. These two clones are closely related to the mammalian S100 superfamily and catfish ictacalcin proteins. The mRNA levels of these genes were significantly increased in zebrafish liver cell culture and in the kidney of juvenile rainbow trout exposed to TCDD. The mechanism for the TCDD-induced increase in zebrafish S100A4a and rainbow trout S100A4 mRNA levels is not clear. Increased mRNA abundance could result directly from AhR-mediated transcription by a secondary process downstream from AhR-mediated gene regulation or by altered mRNA stability.

4.2. AhR and Ca^{2+} signaling pathways

The importance of AhR in mediating TCDD toxicity has been confirmed by AhR knockout studies in mice (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). TCDD activation of the AhR signaling pathway could up-regulate gene expression through AhR/ARNT-mediated transactivation, as occurs for the induction of drug metabolizing enzymes. ARNT is also required for the activity of the hypoxia-inducible factor (Hif-1α), another PAS family transcription factor (Wang et al., 1995), and ARNT also associates with other PAS proteins (reviewed in Schmidt and Bradfield, 1996). It has been suggested that competition between PAS family members for the ARNT protein could contribute to the mechanism of TCDD toxicity. Specific TCDD toxicity endpoints could result from AhR-mediated regulation of dioxin-responsive genes, or from the down-regulation of genes under control of other PAS proteins.

The involvement of Ca^{2+} in the AhR pathway adds another opportunity for regulation. Ca^{2+} is the most common intracellular messenger in the signal transduction pathway and is required for cell growth and survival. $Ca_3(PO_4)_2$ is insoluble and the normal intracellular Ca^{2+} level is usually maintained at a very low concentration. To achieve this, Ca^{2+} is pumped out of the cell into the extracellular space or into local cellular compartments such as the endoplasmic reticulum. This allows cells to use phosphate (ATP) as their energy source and Ca^{2+} as an intracellular messenger (Kretsinger, 1980). Increases in cytosolic Ca^{2+} concentration initiates gene expression and cell cycle progression, and prolonged elevated cytosolic Ca^{2+} levels activate nuclease activity resulting in programmed cell death (Clapham, 1995; Talor and Broad, 1998). Exposure to TCDD has been reported to significantly increase intracellular free Ca^{2+} in cell cultures (Puga et al., 1997; Canga et al., 1988, 1993; Hanneman et al., 1996; Al-Bayati et al., 1988). In rat thymocytes, TCDD exposure increased the intracellular calcium level, triggering Ca^{2+}-mediated endonuclease activity leading to apoptosis (McConkey et al., 1988). Apoptosis also has been observed in medaka, fundulus, and zebrafish embryos exposed to TCDD (Cantrell et al., 1996, 1998; Toomey et al., 2001; Dong et al., 2001, 2002). Extracellular Ca^{2+} has been reported to be an essential factor required for the induction of *CYP*1A1 in human keratinocytes and it affects AhR-mediated induction of plasminogen activator inhibitor-2 (PAI-2) in mouse hepatoma cells when exposed to TCDD (Sutter et al., 1991; Berghard et al., 1990). The induction of c-fos and c-jun by dioxin in human hepatocytes is dependent on the activity of protein kinase C (PKC), a calcium-dependent protein kinase (Puga et al., 1992). PKC activity is also involved in *CYP*1A1 induction through AhR phosphorylation (Chen and Tukey, 1996; Carrier et al., 1992; Berghard et al., 1993). Taken together, these studies suggest that Ca^{2+} is involved in TCDD action.

4.3. EF-hand calcium-binding proteins

As an intracellular second messenger, Ca^{2+} plays a number of roles that are critical for the regulation of many cellular functions. Calcium-binding proteins are believed to be the targets and mediators of many actions of calcium at the

cellular level. The general importance of calcium-binding proteins in the control of transcription is beginning to be understood (reviewed in Ikura et al., 2002). Calmodulin is a ubiquitous EF-hand calcium-binding protein that regulates several protein kinases involved in transcriptional regulation including calmodulin-dependent protein kinases, calcineurin, AP-1, and NF-AT (reviewed in Ikura et al., 2002). The most recently described DREAM (also named calsenilin or KChlP3) is a Ca^{2+}-regulated, direct DNA-binding transcriptional repressor (Carrion et al., 1999).

The S100 family of calcium-binding proteins is a group of small proteins that contain two characteristic EF-hands forming the calcium-binding structure (Kretsinger et al., 1991). The expression of these genes is under growth control as mRNA levels increase significantly after cells are stimulated by serum and growth factors such as EGF and PDGF (Hirschhorn et al., 1984; Calabretta et al., 1986). No clear function for S100 proteins has been established; however, S100 proteins may participate in the activity of PKC and inhibit PKC-modulated protein phosphorylation on brain membranes (Patel and Marangos, 1982; Patel et al., 1983; Kligman and Patel, 1986). The human S100B has been shown to regulate the activity of the p53 tumor suppression gene and consequently the expression levels of a number of cancer-related genes (Delphin et al., 1999).

The catfish ictacalcin is abundant in epithelial cells of the olfactory barbels, skin, and gills suggesting a role in calcium homeostasis (Bettini et al., 1994; Porta et al., 1996). Although the functions of zebrafish S100A4a and rainbow trout S100A4 proteins are not clear, the modulation of the mRNA encoding these EF-hand calcium-binding proteins by TCDD in zebrafish liver cell cultures and in rainbow trout kidney raises the possibility of interactions between the calcium and AhR signaling pathways occurring in TCDD-exposed fish. In global mammalian microarray and EST frequency studies conducted in tissues and cells treated with TCDD, S100 Ca^{2+}-binding proteins have not been identified as TCDD responsive (Bradfield et al., Web site: http://edge.oncology.wisc.edu/EDGEWebSite/people_behind_bradfield.htm; Frueh et al., 2001). It is important to note that these studies have focused on the adult liver and liver-derived HEPG2 cell responses to TCDD. In the rainbow trout studies reported here, S100A4 was not detected or induced in the adult liver, but instead TCDD-mediated induction was restricted to the kidney. S100A4a mRNA levels were elevated in cultured zebrafish liver cells, and it remains to be determined if the zebrafish S100A4a mRNA levels are modulated by TCDD in vivo.

4.4. Dioxin toxicity

The endpoints of TCDD developmental toxicity in rainbow trout, and zebrafish embryos and larvae include reduced cardiac output, reduced blood flow to various vascular beds, stunted body growth, craniofacial malformations, pericardial and yolk sac edema, apoptosis and necrosis of various tissues secondary to ischemia, and impaired swim bladder inflation culminating in mortality (Walker and Peterson, 1994; Henry et al., 1997; Teraoka et al., 2002; Dong et al., 2001, 2002; Tillitt and Papoulias, 2002; Tanguay et al., in press). The TCDD-responsive genes involved in producing these endpoints of toxicity in fish early life stage development, including the prominent cardiovascular toxicity endpoints, are unknown.

Under normal conditions, the heart, acting as a pump, provides the pressure to keep blood flowing. The barrier function of the vascular endothelium and the osmotic pressure provided by plasma proteins keep the extracellular fluid component of blood inside the vasculature so that edema does not occur. TCDD exposure in the mouse increases intracellular Ca^{2+} and decreases the β-adrenergic response of heart muscle fibers; in rainbow trout larvae it inhibits heart development; and in the chick embryo it causes cardiac malformations, inhibits coronary artery development, and decreases cardiac myocyte proliferation (Cheung et al., 1981; Canga et al., 1988; Hornung et al., 1999; Ivnitski et al., 2001). Taken together, these findings indicate that the heart is a target organ of TCDD toxicity in vertebrates.

The kidney is the most important organ for the homeostasis of water and electrolytes. Most of the salts and proteins filtered in kidney glomeruli are

reabsorbed at later sites in the nephrons. Kidney dysfunction can lead to a loss of plasma proteins, such as albumin, in the urine. The hypo-osmotic effect in blood would drive extracellular fluid out of the vasculature leading to edema. TCDD exposure also significantly increases AhR2 mRNA levels in the kidney of juvenile rainbow trout (Abnet et al., 1999) suggesting that the kidney may be sensitive to TCDD toxicity. The finding that the rainbow trout S100A4 is induced by TCDD exposure in the kidney of juvenile rainbow trout coupled with the finding of edema in trout exposed to TCDD raises the possibility that renal dysfunction may play a role in the TCDD-induced edema in fish. However, further research would need to determine what role, if any, increased expression of a putative calcium-binding protein in rainbow trout kidney plays in the edema response.

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