Dioxin induces a novel nuclear factor, DIF-3, that is implicated in spermatogenesis

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Abstract 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin), a member of a class of environmental pollutants represented by polychlorinated dibenzo-*p*-dioxins and dibenzofurans, is one of the most toxic artificial compounds ever developed. In this study, we identified a novel TCDD target gene, DIF-3 (dioxin inducible factor-3), by cDNA representational difference analysis. DIF-3 protein is a nuclear factor and possesses a zinc-finger motif at its N-terminus. High DIF-3 mRNA expression in the testes was demonstrated by Northern blot analysis and abundant DIF-3 protein was detected during spermatogenesis. Thus, these results suggest that DIF-3 may be a target gene mediating the reproductive toxicity induced by TCDD.

Key words: Dioxin inducible factor-3; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Aryl hydrocarbon receptor; Zinc-finger protein

1. Introduction

In recent years, several reports have focused on certain man-made toxins known as endocrine disrupting chemicals (EDCs) that persist in the environment and are capable of altering the endocrine homeostasis of an animal, thereby causing serious reproductive and developmental defects [1,2]. One such compound is the xenobiotic agent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin). It is one of the most potent members of a family of EDCs, the polyhalogenated aromatic hydrocarbons. Exposure to TCDD induces transcriptional activation of genes encoding xenobiotic metabolizing enzymes including cytochromes P450 (CYP1A1, CYP1A2, CYP1B1) and phase II enzymes such as UDP-glucuronyl-*S*-transferases and sulfotransferases [3,4]. Changes in gene expression induced by TCDD and related chemicals are initiated upon their binding to the aryl hydrocarbon receptor (AhR), which then dimerizes with the AhR nuclear translocator, forming a complex which interacts with gene regulatory elements, known as xenobiotic response element motifs [3,4].

TCDD exposure can result in carcinogenesis, immunosuppression, and tissue and organ toxicity, as well as developmental toxicity and teratogenesis, when administered prenatally [5]. Alterations in gene expression that result from TCDD exposure are thought to contribute to the mechanisms underlying many of the effects induced by this compound. Studies with AhR-deficient mice revealed TCDD-induced tissue specific toxicity and transcriptional activation of genes encoding xenobiotic metabolizing enzymes to be mediated by AhR [6–8]. Although the AhR mediates many of the TCDD-induced changes in gene expression, the putative target genes responsible for toxicity have yet to be identified. Moreover, we have described the AhR-independent gene suppression by TCDD [9], and it is suggested that AhR-independent mechanisms may contribute to TCDD-induced developmental toxicity [10]. Clearly, the precise molecular mechanisms underlying TCDD toxicity in humans are still essentially unknown.

Based on these previous findings, we attempted to identify TCDD target genes using mouse embryonic stem (ES) cells. The identification of such downstream genes should shed light on the molecular mechanisms underlying EDC toxicity.

2. Materials and methods

2.1. Cell culture

TT2 ES cells were provided by Dr. Tetsu Akiyama (The University of Tokyo, Tokyo, Japan). TT2 ES cells were maintained as previously described [11].

$AhR^{-/-}$ embryos were generated from intercrossed $AhR^{+/-}$ mice [6]. Mouse embryonic fibroblasts (MEFs) with wild-type or mutant genotypes were generated from day 14.5 mouse embryos [11]. HeLa cells and MEFs were grown as previously described [9].

2.2. Chemicals

TCDD (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was prepared in dimethylsulfoxide (DMSO).

2.3. cDNA synthesis and representational difference analysis (RDA)

All procedures were performed as described previously [12,13]. ES cells at ~80% confluence were treated with 0.1% DMSO alone or with 1 μM TCDD by the exchange of the conditioned maintenance

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Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DIF-3, dioxin inducible factor-3; RDA, cDNA representational difference analysis

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media for fresh media containing these chemicals. After 4 h treatment, the cells were harvested. Poly(A)+RNA was prepared from the ES cells and double-stranded cDNA synthesis was performed using a TaKaRa cDNA synthesis kit (TaKaRa, Tokyo, Japan). In the first and second rounds of subtractive hybridization, tester:driver ratios of 1:100 and 1:800 respectively, were used. After two cycles of subtraction and amplification, the differential products were subcloned into a pGEM-3Z vector (Promega, Madison, WI, USA). The cloned cDNA fragments were then sequenced and used as probes for Northern blot analysis.

2.4. RNA isolation, Northern blot and RT-PCR analysis

For cDNA synthesis and Northern blot analysis, total RNA from ES cells and MEFs was prepared as previously described [9]. The dioxin inducible factor-3 (DIF-3) cDNA fragments (nucleotides 1152–2416) were used as probes. Northern blot analysis was performed as previously described [9].

To obtain the murine DIF-3 cDNA fragment, we performed RT-PCR using total RNA from ES cells. The nucleotide sequences of the primers used were as follows: DIF-3 primer 1S, CCCACACTCCCCGTGGCGCGAGCGGCTG; and DIF-3 primer 2AS, GCCACTAAGTTGCTTTAGTAGTCTC. The amplified sequences were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA).

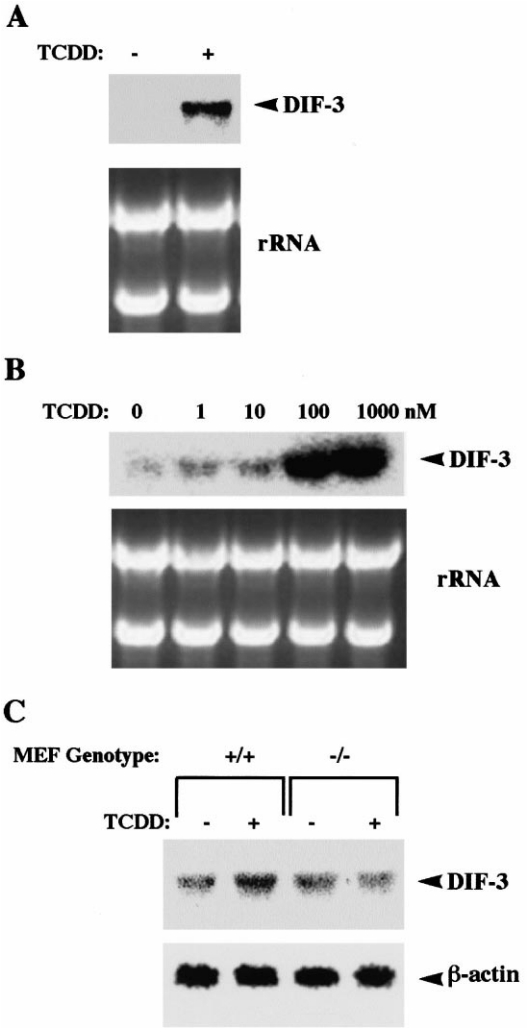

Fig. 1. TCDD treatment induced DIF-3 expression in ES cells and MEFs. A: Northern blot analysis of total RNA from ES cells. ES cells were treated with 0.1% DMSO alone or with 1 μM TCDD for 2 h. An arrow indicates the DIF-3 signal. An ethidium bromide-stained gel is shown as control. B: Northern blot analysis of the dose-dependent induction of DIF-3 by TCDD. ES cells were treated with 0.1% DMSO and TCDD (0, 1, 10, 100, 1000 nM each) for 2 h. An arrow indicates the DIF-3 signal. An ethidium bromide-stained gel is shown as control. C: DIF-3 induction by TCDD mediated by an AhR-dependent pathway. Northern blot analysis demonstrates that DIF-3 mRNA is not induced in $AhR^{-/-}$ MEFs treated with 0.1% DMSO alone or with 100 nM TCDD for 6 h. On the other hand, DIF-3 is activated by TCDD in $AhR^{+/+}$ MEFs treated with 0.1% DMSO alone or with 100 nM TCDD for 6 h. An arrow indicates the DIF-3 signal. The β-actin signals were also shown to confirm the induction of DIF-3 mRNAs.

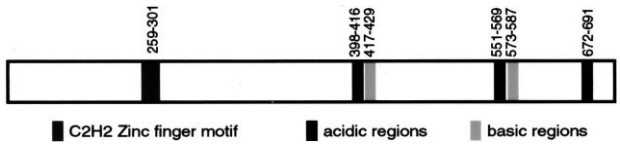

Fig. 2. The predicted domain structure of mouse DIF-3 is depicted in cartoon form. The nucleotide sequence of the cDNA has been deposited in DDBJ/EMBL/GenBank database under accession number AB064543.

2.5. Preparation of antiserum, Western blot analysis, indirect immunofluorescence staining and immunohistochemical analysis

To generate antiserum against DIF-3, cDNA containing the entire DIF-3 open reading frame was inserted into the *Escherichia coli* expression vector pET3a (Stratagene, La Jolla, CA, USA) at the *Eco*RI site and transformed into the BL21(DE3)pLysS strain of *E. coli*. The expression of recombinant DIF-3 linked to a 6× histidine tag at the N-terminus was induced by treatment of the bacterial culture with IPTG. Soluble recombinant protein was purified with a HisTrap kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. Rabbits were immunized with purified DIF-3 and antiserum was developed.

Immunoblotting was performed as previously described [9]. The nuclear and cytoplasmic fractions of HeLa cells were prepared as previously described [14]. Membranes were probed with the DIF-3 antiserum at a dilution of 1:1000. Pre-immune serum was used for control experiments. The signals were detected with an ECL plus Western blotting detection system (Amersham Pharmacia Biotech, UK).

The details of the cell staining procedure were as previously described [15]. The cells were stained with the DIF-3 antiserum at a dilution of 1:100.

To obtain frozen sections of the testis, excised mouse testes were

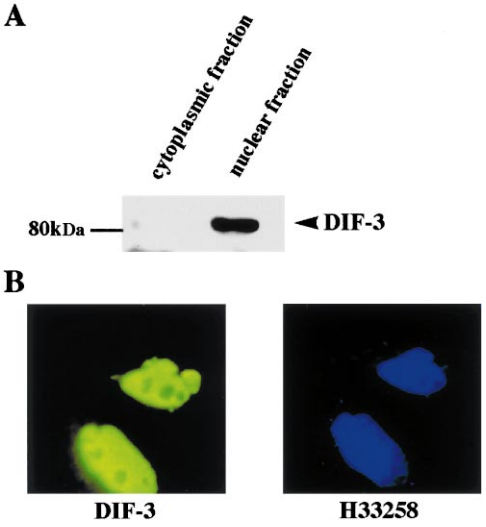

Fig. 3. DIF-3 expressed in the nucleus. A: Western blot analysis of the cytoplasm and nuclear fractions from HeLa cells. An arrow indicates the DIF-3 signal. No signal was detected using pre-immune serum (data not shown). B: DIF-3 immunostaining of fixed HeLa cells. DIF-3 protein localized in the nucleus. Nuclear morphology of the same cells stained with the DNA-binding dye H33258. No signal was detected using pre-immune serum (data not shown).

embedded with an optical cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen rapidly with ethanol/dry ice. Testicular slices were prepared and fixed with 4% paraformaldehyde phosphate-buffered saline for 10 min. Immunostaining was performed with the DIF-3 antiserum at a dilution of 1:100. Control staining was performed using an equivalent concentration of pre-immune rabbit serum.

To make paraffin sections, excised mouse testes were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 5-μm sections. The sections were incubated with DIF-3 antiserum at a dilution of 1:100 for 1 h. An LSABC kit (Dako Japan, Kyoto, Japan) was used as the detection system and 3,3′-diaminobenzidine as a chromogen. Hematoxyline was used for counterstaining.

3. Results and discussion

To isolate genes induced by TCDD, we performed RDA of cDNA derived from ES cells treated with TCDD and compared the results with those from untreated ES cells. Two cycles of RDA yielded several distinct bands on a 2% agarose gel. DNA fragments corresponding to these bands were subcloned and used as probes for Northern blots prepared from TCDD-treated or untreated ES cells. This procedure led to the identification of several unique genes that were overexpressed in TCDD-treated cells. One cDNA clone exhibiting particularly strong differential expression corresponded to a novel gene termed DIF-3 (Fig. 1A). Next, we examined the TCDD dose-dependency of DIF-3 expression. ES cells were treated for 2 h with several concentrations of TCDD. Fig. 1B shows that DIF-3 was indeed dose-dependently induced by TCDD.

The effects of TCDD are reportedly mediated by the AhR [4,6–8]. To determine the involvement of the AhR-mediated pathway in the TCDD-mediated induction of DIF-3, we examined DIF-3 expression in AhR-deficient ($AhR^{-/-}$) MEFs treated with 100 nM TCDD for 6 h by Northern blot analysis. DIF-3 induction was not detected in $AhR^{-/-}$ MEFs (Fig. 1C), indicating that DIF-3 expression following TCDD treatment is mediated by an AhR-dependent pathway.

To obtain the full-length murine DIF-3 cDNA, we searched the expressed sequence tag (EST) database. The 2711-nucleotide-long mouse DIF-3 cDNA was then amplified by RT-PCR using primers based upon EST clones AI 788249 and BE 985276 from mouse testicular cDNA. Examination of the

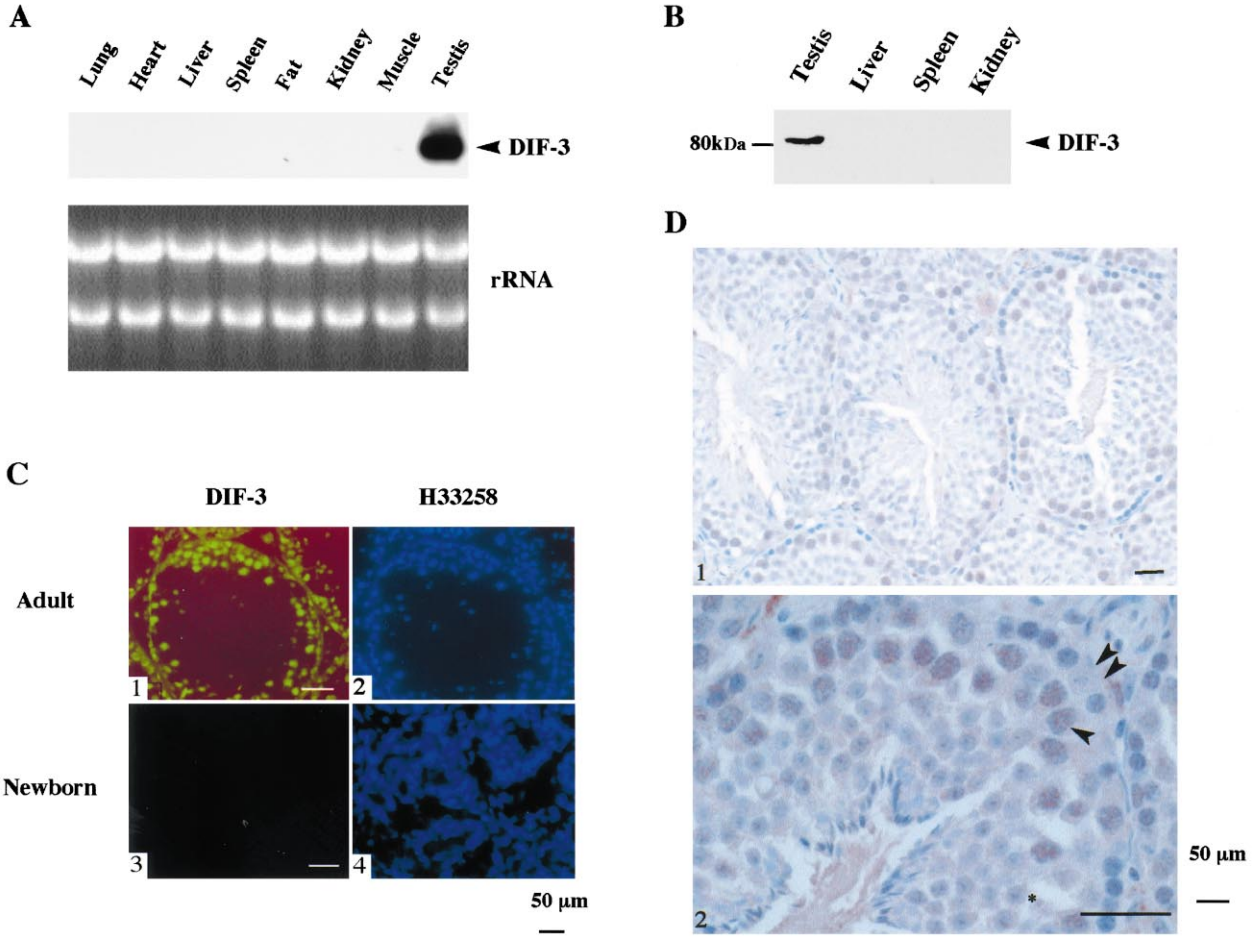

Fig. 4. DIF-3 expressed in mouse testis. A: Total RNA from the indicated organs of adult mice were hybridized with DIF-3 cDNA. An arrow indicates the DIF-3 signal. An ethidium bromide-stained gel is shown as control. B: Proteins from the indicated organs of adult mice were incubated with anti-DIF-3 antibody. An arrow indicates the DIF-3 signal. C: DIF-3 indirect immunofluorescence staining in testes. Frozen sections of adult mice (panels 1 and 2) and newborn mice (panels 3 and 4) were reacted with anti-DIF-3 antibody, and visualized with Alexa 488-labeled rabbit IgG antibody. Tissues were stained with DNA-binding dye H33258 to reveal the presence of cells with condensed chromatin. D: Formalin-fixed and paraffin-embedded sections of adult testes were reacted with anti-DIF-3 antibody. The signals were visualized with diaminobenzidine and counterstained with hematoxylin (panels 1 and 2). Specific cell types in the seminiferous epithelium are identified: arrowhead, pachytene spermatocyte; double arrowhead, Sertoli's cell; asterisk, round spermatid (panel 2). No signal was detected when pre-immune serum was used instead of anti DIF-3 antibody as control (data not shown).

cDNA sequence revealed that it encodes a protein of 741 amino acids with a C2H2 zinc-finger domain (residues 259–301), two basic regions (residues 417–429, 573–587) and three repeated acidic regions (residues 398–416, 551–569, 672–691) (Fig. 2). The complete sequence data have been deposited into the DDBJ/EMBL/GenBank database under accession number AB064543. From these results, we predicted DIF-3 to be a nuclear factor. To confirm the localization of DIF-3, we made a polyclonal antibody against recombinant DIF-3 protein. DIF-3 protein was detected in the nuclear fraction from HeLa cells as an ~80 kDa protein. The molecular weight is coincided with the calculated molecular weight, 84 kDa, of mouse DIF-3 protein. On the other hand, the cytoplasmic fraction was negative for DIF-3 protein by Western blot analysis (Fig. 3A). Staining of fixed HeLa cells with the DIF-3 antibody revealed the presence of reactive antigen in the nucleus (Fig. 3B).

Northern and Western blot analysis of normal mouse tissues revealed the DIF-3 mRNA and protein were detected only in the testis (Fig. 4A,B). Indirect immunofluorescence staining revealed DIF-3 to be abundantly expressed during spermatogenesis (Fig. 4C). In newborn animals, in which spermatogenesis has not yet progressed beyond the earliest stages, DIF-3 was not detectable (Fig. 4C). These results indicate that DIF-3 may not be involved in initial testicular development.

Finally, to examine DIF-3 expression in more detail, immunohistochemical analyses were performed using paraffin sections. DIF-3 is expressed most strongly in the large pachytene spermatocytes, which are distinguished by their size and loose chromatin organization (Fig. 4D, arrowhead). DIF-3 immunoreactivity was also present in the round spermatids (Fig. 4D, asterisk) but was undetectable in condensing forms of sperm. Spermatogonia and Sertoli cells exhibited only background levels of staining.

What is the physiological function of DIF-3? Sequence analysis revealed that DIF-3 has a C2H2 zinc-finger motif, two basic regions and three repeated acidic regions, suggesting that DIF-3 may bind to DNA and act as a transcriptional modulator. Immunohistochemical analysis showed DIF-3 to be localized in the nuclei of mouse testicular cells. Northern blot analysis of normal tissues showed that DIF-3 mRNA is abundantly expressed in the testis. Furthermore, DIF-3 protein was detected during spermatogenesis in adult, but not neonatal, testes. In addition, DIF-3 was immunohistochemically shown to be strongly expressed in pachytene spermatocytes. Although further study is required to clarify these issues, DIF-3 is anticipated to play an important role in transcriptional regulation of meiotic events during spermatogenesis.

In recent years, several reports have focused on certain man-made toxins known as EDCs that persist in the environment and are capable of altering the endocrine homeostasis of an animal, thereby causing serious reproductive and developmental defects, as well as testicular oncogenesis [1,2,16]. Several reports have also provided evidence of a decline in semen quality and/or sperm counts over the same period ([16] and references therein). Among various EDCs, TCDD and estrogenic chemicals such as diethylstilbestrol have been demonstrated to possess the greatest reproductive toxicity [17–19]. Fetal and/or neonatal exposure to these chemicals is especially problematic [16,20–22]. However, which types of molecules mediate the toxicity induced by TCDD during spermatogenesis remains unclear. Although more experiments are needed to provide direct evidence linking DIF-3 with TCDD-induced reproductive toxicity, DIF-3 might be involved with TCDD-induced reproductive toxicity by interfering with spermatogenesis.

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