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Role of Dioxin Response Element and Nuclear Factor-κB Motifs in 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Mediated Regulation of Fas and Fas Ligand Expression

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ABSTRACT

We have demonstrated previously that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) up-regulates Fas and FasL in immune cells, although the molecular mechanisms remain unknown. We investigated the regulation of Fas or FasL promoter by TCDD in EL4 T cells using luciferase reporter constructs. We observed 20 ± 5- and 14 ± 4-fold induction of promoter activity for Fas and FasL, respectively, after TCDD exposure. The induction of luciferase was significantly reduced (2 ± 1-fold) in the presence of α-naphthoflavone, an aryl hydrocarbon receptor (AhR) antagonist. We noted the presence of a dioxin response element (DRE) and five nuclear factor-κB (NF-κB) motifs on Fas promoter, and no DRE but two NF-κB motifs on FasL promoter. When we investigated the role of DRE and NF-κB, we observed varying levels of luciferase induction (9 ± 2-fold for DRE and 8 ± 2-fold for NF-κBs of Fas promoter and 6 ± 3-fold for NF-κBs of FasL promoter). Mutations in DRE of Fas promoter or NF-κBs of FasL promoter led to decreased luciferase induction, further supporting our results. Probes for DRE or NF-κB motifs of Fas and/or FasL promoters demonstrated mobility shift in the presence of nuclear extract from TCDD-treated EL4 cells. Furthermore, we observed supershift in mobility when DRE and NF-κB probes were incubated in the presence of anti-mouse AhR, and anti-NF-κB (RelA/p65 and p50) antibodies, respectively. Administration of TCDD into mice caused significant increase in Fas and FasL transcripts in thymus and liver. These data demonstrate that TCDD regulates Fas and FasL promoters through DRE and/or NF-κB motifs via AhR.

Aryl hydrocarbon receptor (AhR), a member of the basis-helix-loop-helix–PER/AhR nuclear translocator (ARNT)/SIM family (Poland and Knutson, 1982; Denison and Heath-Pagliuso, 1998), has been shown to play a central role in mediating detoxification induced by toxic compounds, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hankinson, 1995; Fernandez-Salguero et al., 1996; Schmidt and Bradfield, 1996; Gonzalez and Fernandez-Salguero, 1998; Gu et al., 2000; Safe, 2001; Denison et al., 2002; Mimura and Fujii-Kuriyama, 2003; Camacho et al., 2005). In the absence of its ligand, AhR is inactive and present in the cytosol associated with the 90-kDa heat shock protein (Denis, 1998), an immunophilin (Ma and Whitlock, 1997), and p23 proteins (Kazlauskas et al., 1999). Once a ligand, such as TCDD, binds to AhR, the complex becomes active and translocates into the nucleus where it binds with ARNT and forms AhR/ARNT complex. AhR/ARNT complex binds to specific DNA sequences known as xenobiotic response elements or dioxin response elements (DREs) and regulates the expression of various genes (Schmidt and Bradfield, 1996; Gonzalez and Fernandez-Salguero, 1998; Tian et al., 1999; Whitlock, 1999; Sulentic et al., 2000; Dertinger et al., 2001; Matikainen et al., 2001; Nazarenko et al., 2001; Mimura and Fujii-Kuriyama, 2003).

TCDD is a ubiquitous environmental pollutant that elicits a broad spectrum of toxic and biochemical responses leading to deleterious effects on humans and wildlife. Studies from our laboratory and elsewhere have shown that apoptosis may play a crucial role in regulating the toxic effects of TCDD (Holsapple et al., 1991). In particular, we have shown that TCDD-induced up-regulation of Fas and FasL in the thymus may play an important role in thymic atrophy caused by TCDD (Rhile et al., 1996; Kamath et al., 1997, 1999; Cama-

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, AhR nuclear translocator; DRE, dioxin response element; NF-κB, nuclear factor-κB; ANF, α-naphthoflavone; RLU, relative light unit; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; NE, nuclear extract; bp, base pair(s); Ab, antibody.

cho et al., 2001, 2002, 2004a, 2005). However, the precise molecular mechanisms of TCDD-induced up-regulation of Fas and FasL are unclear.

Regulation of Fas and FasL expression is controlled by distinct protein–DNA interactions at their respective promoters. Previous studies have identified c-myc and Nur77 as transcription factors involved in regulation of Fas and FasL expression (Davis and Lau, 1994). In recent years, several other transcription factors that include nuclear factor of activated T cells, p53, ceramide, members of c-Jun NH_2-terminal kinase/stress-activated protein stress-activated pathways, and NF-κB, have been identified to regulate the expression of Fas and FasL genes (Sharma et al., 2000). Recent studies from our laboratory have demonstrated the presence of a functional DRE motif on Fas promoter (Fisher et al., 2004). Thus, TCDD-induced up-regulation of Fas may be due to TCDD-mediated activation of AhR and binding of AhR to DRE found in Fas promoter. In contrast, FasL promoter did not exhibit any DRE motif, but it had NF-κB motifs (Camacho et al., 2005), suggesting that its regulation may result from activation of NF-κB interactions. There are reports demonstrating that ligand-activated AhR interacts with NF-κB (Tian et al., 1999). Several studies have also shown functional interactions between AhR and NF-κB (Puga et al., 2000; Schlezinger et al., 2000; Sulentic et al., 2000; Baba et al., 2001). We have recently shown that TCDD/AhR interaction in thymic stromal cells led to activation of NF-κB and its migration from the cytosol to the nucleus, where it participated in up-regulation of FasL expression (Camacho et al., 2005). In other previous studies, it has been shown that AhR and NF-κB interaction may be responsible for dioxin toxicity (Tian et al., 1999, 2002). Furthermore, NF-κB has also been shown to up-regulate the expression of Fas (Puga et al., 2000; Schlezinger et al., 2000; Sulentic et al., 2000; Baba et al., 2001; Zheng et al., 2001).

The precise mechanism of TCDD-mediated regulation of Fas and FasL genes with respect to the specific transcription factors has not been well characterized and understood. Therefore, the aim of the present study was to elucidate the molecular mechanisms of TCDD-mediated regulation of Fas and FasL gene expression and examine the role of DRE and NF-κB motifs in regulation of these genes.

Materials and Methods

Mice. C57BL/6(H-2^b) mice were purchased from the National Institutes of Health (Bethesda, MD). All animals were housed in the University of South Carolina Animal facility. Care and maintenance of animals were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted by Institutional and National Institutes of Health guidelines.

Cell Line. The mouse T lymphoma cell line (EL4) was used in this study. EL4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM L-glutamine, 10 mM HEPES, and 100 μg/ml penicillin/streptomycin at 37°C and 5% CO_2.

Reagents and Antibodies. We purchased L-glutamine, HEPES, gentamicin, RPMI 1640, Dulbecco's modified Eagle's medium, phosphate-buffered saline, and fetal bovine serum from Invitrogen (Carlsbad, CA). The following mouse monoclonal antibodies were purchased from BD Biosciences PharMingen (San Diego, CA): anti-mouse IgG, FcBlock, anti-FasL-PE (Kay-10), and anti-Fas-PE (Jo2). Goat anti-mouse AhR polyclonal antibody, goat anti-mouse NF-κBp65 (c-20), and rabbit anti-mouse NF-κB p50 (c-19) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). pGL3-basic vector was from Promega (Madison, WI), pCMV-β-galactosidase vector was from Clontech (Mountain View, CA), QuikChange II Motifs-directed mutagenesis kit was from Stratagene (La Jolla, CA), Effectene transfection kit was from QIAGEN (Valencia, CA), and the Dual-Light system was purchased from Applied Biosystems (Foster City, CA). RNeasy Mini kit and iScript cDNA synthesis kit were purchased from QIAGEN. Epicenter's PCR premix F and Platinum *Taq* Polymerase kits were purchased from Invitrogen.

Generation of the Mouse Fas Promoter Reporter Constructs and Motif-Directed Mutagenesis. The mouse Fas promoter cloned in pGL3 vector was a gift from Dr. Amar A. Beg (Columbia University, New York, NY). The details of cloning mouse Fas promoter/luciferase reporter construct in pGL3 vector have been described previously (Zheng et al., 2001).

To elucidate the role of DRE and NF-κB in regulation of Fas promoter upon TCDD exposure, we generated several Fas promoter constructs. The various Fas promoter constructs were assigned names based on the size of their fragments. These subclones were designated as Fas973 (contains all NF-κB motifs), Fas266 (contains one NF-κB1motif located between −1169 and −903), Fas317 [contains two NF-κB (NF-κB2 and NF-κB2) motifs located between −792 and −475], Fas270 [contains two NF-κB (NF-κB4 and NF-κB5) motifs located between −447 and −197], and Fas244 (contains only DRE motifs located between −244 and +1) (Fig. 2B). The primers used to generate various fragments of mouse Fas promoter are shown in Table 1. For directional cloning, all the upstream primers included SacI restriction sites and downstream primers included XhoI restriction sites.

The fragments generated from Fas promoter were first subcloned into the polylinker sites of Topo PCR2.1 T/A vector and sequenced to identify the orientation and fidelity. The right clones for each construct were chosen, digested with SacI and XhoI, gel-purified, and then subcloned into luciferase reporter pGL3 vector. All the generated pGL3 clones were sequenced and the correct clones with right orientation were chosen for use in this study.

To confirm the role of DRE motif in TCDD-mediated regulation of mouse Fas promoter, we generated mutation (GCGTG->AAATA) in DRE motif using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. pGL3-

TABLE 1
Fas and FasL primers

Fas	
Fas244	
Upstream	5′-GGGGAGCTCGACCAGCAGAACTTTCTCG-3′
Downstream	5′-GGGGAGCTCGTCTGCAGCAAGGGAAAACAG-3′
Fas973	
Upstream	5′-GGGGAGCTCCTATAGTCTTGGCAACAG-3′
Downstream	5′-GGGGAGCTCCACAATCTTCGTAAAGCCC-3′
Fas266	
Upstream	5′-GGGGAGCTCCTATAGTCTTGGCAACAG-3′
Downstream	5′-GGGGAGCTCGACGATGAATGGGAATAC-3′
Fas317	
Upstream	5′-GGGGAGCTCCGTGGTTCTAAAGATTCTG-3′
Downstream	5′-GGGGAGCTCGAGACAAAGACCATTTTGC-3′
Fas270	
Upstream	5′-GGGGAGCTCCAGAGTTCAGAATTCCTTC-3′
Downstream	5′-GGGGAGCTCCACAATCTTCGTAAAGCCC-3′
FasL	
FasL231	
Upstream	5′-GGGGAGCTCCTCAGTTTTCATCTGGTG-3′
Downstream	5′-GGGGAGCTCGATATCTCCACATCTGAG-3′.
FasL191	
Upstream	5′-GGGGAGCTCCAGGGAAGGGACTTC-3′
Downstream	5′-GGGGAGCTCGTCAGCGCTGGATACC-3′
FasL324	
Upstream	5′-GGGGAGCTCAGCCTGGTTTACCAGCC-3′
Downstream	5′-GGGGAGCTCCAGCGTGACCAGAAAG-3′

Faspro1.4 was used as the template for mutagenesis. The primers used for mutation in DRE motif are shown in Table 2 Several mutant clones were sequenced and analyzed, and the correct mutant clones were selected and used in this study.

Generation of the Mouse FasL Promoter Reporter Constructs and Motifs-Directed Mutagenesis. Mouse FasL promoter cloned in pGL3 vector was a gift from Dr. Timothy L. Ratiff (University of Iowa, Iowa City, IA). The cloning of FasL promoter and generation of luciferase reporter construct in pGL3 vector have been described previously (Crist et al., 2003).

To determine the role of NF-κB in regulation of FasL promoter in the presence of TCDD, we generated three FasL promoter constructs with or without NF-κB motifs. We designated these constructs FasL231, FasL191, and FasL324 based on the size of the respective PCR-amplified fragments. The primers used to generate various fragments of mouse FasL promoter are shown in Table 1. For directional cloning, all upstream primers included SacI restriction site and downstream primers included XhoI restriction site.

The amplified PCR fragments FasL231, FasL191, and FasL324 were first cloned into the polylinker sites of Topo PCR2.1 T/A vector and then sequenced to identify the orientation and fidelity of the clones. The right clones for each construct were chosen, digested with SacI and XhoI, gel-purified, and then subcloned into pGL3 vector. These pGL3 subclones were sequenced, and the subclones with correct sequence and right orientation were chosen and used in this study.

To further examine the role of NF-κB motifs in regulation of FasL promoter, mutations in NF-κB1 and NF-κB2 motifs of FasL promoter were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The template used for mutagenesis was pGL3-FasL754. Primers used to generate mutations in NF-κB1 and NF-κB2 motifs are shown in Table 2. All the mutant subclones were sequenced, analyzed, and the correct clones were selected and used in this study.

Transfection and Luciferase Assays. EL4 cells (1×10^7) were transfected using Effectene tranfection reagent kit (QIAGEN) with 5 to 10 μg of the indicated reporter plasmid and 1 μg of the pCMV-βGal control vector. Two days after transfection, the cells were replated in triplicate in 24-well plate and the cells were treated with 10 to 100 nM TCDD and incubated for 24 h at 37°C, 5% CO_2. After incubation, cells were harvested, washed with phosphate-buffered saline, and centrifuged, and nuclear extracts (NEs) were prepared by lysing cells in 70 μl of reporter lysis buffer (Promega). Luciferase activity was determined by mixing 20 μl of extract with 100 μl of luciferase substrate (Promega) and immediately reading the sample in a Monolight 2010 luminometer (BD Biosciences, San Jose, CA). Likewise, β-galactosidase activity was determined by mixing 20 μl of extract with Galacto-light β-galactosidase substrate (Tropix, Bedford, MA), incubating for 1 h at 25°C, adding 300 μl of Galacto-light accelerator (Tropix), and reading the sample with a luminometer. Under similar conditions, 100 ng/ml α-naphthoflavone (ANF), an antagonist for TCDD, was used in the culture at least 1 h before TCDD treatment, and luciferase and β-galactosidase assays were performed. Luciferase activity was normalized by dividing the mean luciferase RLUs by the mean β-galactosidase RLUs. The normalized luciferase RLUs from the stimulated samples were divided by the normalized RLUs of the untreated sample, and values ware expressed as "normalized-fold induction."

TABLE 2

Primers used to generate mutations in DRE (Fas promoter) and NFκB1 and -2 (Fas L promoter) motifs

Underlined characters indicate changes in bases for mutation.

DRE	
Mut1	5′-GCGGTTTG TGTAAATACCAGGGGGCG-3′
Mut2	5′-CGCCC CCTGGTATTTACACAAACCGC-3′
NF-κB1	
Mut1	5′-CCTTGGTCTTTTAAACATGCCTCAGC-3′
Mut2	5′-GCTGAGGCATGTTTAAAAGACCAAGG-3′
NF-κB2	
Mut1	5′-GAGAAAGGTGTTTAAATTGACTGC-3′
Mut2	5′-GCAGTCAATTTAAACACCTTTCTC-3′

Preparation of Nuclear Extracts. To prepare nuclear extract for EMSA, EL4 cells were first activated with 2.5 μg/ml concavalin A for 24 h and then treated with 100 nM TCDD and incubated for 24 h at 37°C, 5% CO_2. After TCDD treatment, the cells were harvested by centrifuging at 1100 rpm for 5 min at 4°C. All subsequent steps were done on ice. The cell pellet was resuspended in 200 μl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol) and lysed by passing four times through a 28-gauge needle. The nuclei were then pelleted by centrifugation for 10 s, and the supernatant was aspirated. The crude nuclei preparation was then extracted by adding 120 μl of buffer C [20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride] and incubating for 15 min on ice. Then, 120 μl of buffer D (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) was added, and the preparation was centrifuged for 10 min at 15,000 rpm. The supernatant was harvested, snap frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined using the BCA protein determination kit from Pierce Chemical (Rockford, IL), using albumin as a protein standard.

EMSA and Supershift Analysis. The hairpin oligonucleotide probes corresponding to DRE and NF-κB motifs of mouse Fas promoter and NF-κB motifs of mouse FasL promoter were synthesized at Virginia Commonwealth University core facility (Richmond, VA). The sequences of hairpin oligonucleotide probes are shown in Table 3. The hairpin oligonucleotide probes were 5-end-labeled by mixing 1 pmol of hairpin oligonucleotide with 10 μCi of [γ-^{32}P]ATP (MP Biomedicals, Aurora, OH), and 8 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in 1× polynucleotide kinase buffer and incubating for 1 h at 37°C. After incubation, the end-labeled hairpin oligonucleotides were purified from free ATP by passing over a NICK column (GE Healthcare, Little Chalfont, Buckinghamshire, UK). One to 5 μg of nuclear protein was mixed with 1 μl of radiolabeled oligonucleotide (50,000 cpm) in a reaction mix containing 1 μl of binding buffer [10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, and 10% (v/v) glycerol] and 1 μg of poly(dI-dC) (GE Healthcare) as a nonspecific inhibitor in a final volume of 25 μl and incubated for 30 min at 25°C. The samples were resolved on a 6% polyacrylamide gel in Tris borate-EDTA that has been pre run for 30 min. The gels were dried and exposed to X-ray film. For the specific and nonspecific competition analyses, equimolar amounts of the unlabeled hairpin oligonucleotide competitors were added to the binding reaction before the addition of the labeled oligonucleotide probes. For the supershift analyses, various amounts of the appropriate polyclonal anti-DRE antibody and monoclonal antibody against NF-κB p65 or p50 (Santa Cruz Biotechnology, Inc.) were added to the reaction mixtures during the 30-min incubation before electrophoresis.

Real-Time Reverse Transcriptase Polymerase Chain Reaction for Quantitative Analysis of mRNA Expression of Fas and FasL in Vivo after TCDD Exposure. Total RNA was isolated from thymus or liver of C57BL6 mice that were treated either with various doses of TCDD (1–10 μg/kg body weight) or the vehicle control (DMSO) for 1 to 3 days using RNeasy Mini kit and following the protocol of the company (QIAGEN, Valencia, CA). First-strand cDNA synthesis was performed in a 20-μl reaction mix containing 2 μg of total RNA using iScript kit and following the protocol of the manufacturer (Bio-Rad, Hercules, CA). The primers used are shown in Table 4. After first strand synthesis, 2 μl (10% of the reaction volume) was used as a template for quantitative PCR in 50 μl of PCR master mix consisting of 1× SYBR Green Supermix [stock solution (2 times) of SYBR Green Supermix contains 100 mM KCl, 40 mM Tris-HCl, pH 8.4, a 0.4 mM concentration of each dNTP (dATP,

dGTP, dCTP, and dTTP), 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers; Bio-Rad, Hercules, CA] and 100 nM concentrations of each forward and reverse primer. The amplification conditions were as follows: 5 min at 95°C, 30 cycles at 95°C for 30 s, annealing temperature 58°C for 40 s, and 72°C for 60 s, and 1 final cycle at 72°C for 10 min. In 96-well plates, each sample was monitored in triplicate for each mRNA. Wells containing same amount of SYBR Green were used as background control, and wells containing all reagents except cDNA were used as negative control. CYP1A1 was used as a positive control for TCDD-mediated up-regulated gene expression and 18S was used as an internal standard control. The relative standard curve method was used, and the levels of Fas, FasL, and CYP1A1 mRNA were normalized to that of 18S.

Statistical Analysis. Data presented for luciferase expression were derived from at least three independent experiments and depicted as mean ± S.E.M. Likewise, results for real-time PCR represent at least three animals for each treatment and expressed as the mean ± S.E.M. Statistical analyses were performed using Student's *t* test or two-factor analysis of variance as appropriate, with a P value of ≤ 0.05 considered to be statistically significant.

Results

TCDD Regulates Mouse Fas Promoter through AhR. To determine the role of TCDD in regulation of Fas promoter, EL4 cells were transfected with constructs containing full-length upstream region of Fas (pGL3-Fas1272) promoter. Two days after transfection, the transiently expressing EL4 cells were treated with various concentrations of TCDD (1–1000 nM) or vehicle (DMSO). Luciferase assays were performed 24 h after treatment with TCDD or vehicle. We observed TCDD-mediated increase in expression of luciferase when Fas promoter was used for transfection, and the cells were treated with TCDD for at least 24 h (Fig. 1A). The effect of TCDD on luciferase expression was very minimal at lower doses (1–10 nM; Fig. 1A). At 50 nM, the effect of TCDD was moderately significant. However, we observed highly significant increase (20 ± 5-fold) in luciferase expression when 100 nM or higher doses (500–1000 nM) of TCDD were used (Fig. 1, A and B). We observed minimal level (2 ± 1) of luciferase expression when the cells were transfected with Fas promoter and treated with vehicle as well as in pGL-3 controls (Fig. 1, A and B). The observed up-regulation of luciferase expression was Fas promoter-specific and TCDD-induced, because the luciferase expression was significantly higher ($p < 0.05$) in cells transfected with Fas promoter compared with untreated (none) or vehicle-treated cells (Fig. 1, A and B). The data obtained clearly demonstrated that TCDD-induced up-regulation of luciferase expression was through Fas promoter.

To further understand TCDD-induced regulation of mouse Fas promoter and participation of AhR, we performed luciferase assays as described above but in the presence of ANF, an antagonist of AhR, added to cultures at least 2 h before TCDD treatment of the transfected cells. We observed significant decrease in luciferase expression in cultures treated with TCDD + ANF compared with TCDD alone (Fig. 1C). These data suggested a role for AhR in TCDD-induced regulation of Fas promoter.

Role of DRE in TCDD-Induced Regulation of Fas Promoter. We have previously reported the presence of at least one DRE motif (Fig. 2A) in Fas promoter (Fisher et al., 2004). In this context, we sought to determine the precise role of DRE as a regulatory element for TCDD-mediated induction of Fas promoter. To this end, we generated a subclone (Fas244) pGL3 reporter vector containing DRE motif on the Fas promoter (Fig. 2B). Luciferase activity was measured in EL4 cells transfected with Fas244 subclone after treatment with TCDD for 24 h. We observed a 9 ± 2-fold increase in luciferase expression compared with vehicle control that showed minimal expression (Fig. 2C). To further elucidate the role of DRE as a regulatory element for Fas promoter, we generated a DRE mutant subclone (pGL3-Fas DRE mutant) containing mutation (GCGTG→AAATA) in DRE. Luciferase assays performed on EL4 cells transfected with DRE mutant subclone and treated with TCDD demonstrated approximately 5 ± 2-fold luciferase expression (Fig. 2D) compared with 20 ± 5-fold (Fig. 1A) induction seen with pGL3-Fas. Together, these data demonstrated that DRE acts as a *cis*-regulatory element involved in TCDD-mediated regulation of Fas promoter. It should be noted that luciferase induction using pGL3 vector containing DRE motif alone was 9 ± 2,

TABLE 4
PCR primers

Mouse FasL	
Forward	5′-CGG TGG TAT TTT TCA TGG TTC TGG-3′
Reverse	5′-CTT GTG GTT TAG GGG CTG GTT GTT-3′
Mouse Fas (486 bp)	
Forward	5′-TCT GGT GCT TGC TGG CTC AC-3′
Reverse	5′ CCA TAG GCG ATT TCT GGG AC-3′
Mouse CYP1A1 (499 bp)	
Forward	5′-CCC ACA GCA CCA CAA GAG ATA-3′
Reverse	5′-AAG TAG GAG GCA GGC ACA ATG TC-3′
Mouse 18S	
Forward	5′-GCC CGA GCC GCC TGG ATA C-3′
Reverse	5′-CCG GCG GGT CAT GGG AAT AAC-3′

TABLE 3
Hairpin loop probes

Mouse Fas promoter	
Fas DRE motif	GAGAGGTTGTGCGTGCCTTAGCTTGGCACGCACAACCTCT
Fas DRE mutant motif	GAGAGGTTGTAAATACCTTAGCTTGGTATTTACAACCTCT
CYP1A1 DRE motif	GAGAGGTTGTACGTGGCGTGTCTTGTCGCGGGCACGCACAACCTCT
FAS NF-κB1 motif	GAGAAAAGGGGACTCCCTTTAGCTAAAAGGGAGTCCCCTTTTCT
FAS NF-κB2 motif	GAGAAAAGGGACATTCCCCTTTAGCTAAGGGGAATGTCCCTTTTCT
FAS NF-κB3 motif	GAGAAAAGGGACCTCCCTTTAGCTAAGGGAGGTCCCTTTTCT
FAS NF-κB4 motif	GAGAAAAGGGGAATGCCCATTTAGCTAATGGGCATTCCCCTTTTCT
FAS NF-κB5 motif	GAGAAAAGGGTTTCCCCTTTAGCATAAGGGGAAACCCTTTTCT
Mouse FasL promoter	
NF-κB1 motif	GAGATGGTCTTTTCCCCAATTAGCTTTTGGGGAAAAGACCATCT
NF-κB1 mutant motif	GAGATGGTCTTTTAAACAATTAGCTTTTGTTTAAAAGACCATCT
NF-κB2 motif	GAGAAAAGGTGTTTCCCTTTTAGCTTAAGGGAAACACCTTTTCT
NF-κB2 mutant motif	GAGAAAAGGTGTTTAAATTTTAGCTTAATTTAAACACCTTTTCT

compared with full-length Fas promoter that showed 20 ± 5-fold increase (Fig. 2C). This suggested that other *cis*-regulatory elements, in addition to DRE, might also be involved in TCDD-mediated regulation of Fas gene.

To further confirm the participation of DRE motif as a *cis*-regulatory element, we performed EMSA using radiolabeled single-strand loop Fas DRE or CYP1A1DRE probe incubated with nuclear extracts from TCDD-treated EL4 cells. We observed shift in mobility of DRE probe when incubated with NEs from EL4 cells treated with 100 nM TCDD for 24 h (Fig. 3A). We also observed shift in CYP1A1DRE probe in the presence of NEs from TCDD-treated EL4 cells (Fig. 3A). No mobility shift of Fas DRE probe was observed when incubated without nuclear extracts or when mutant DRE probe was used after incubation with nuclear extracts (Fig. 3A). Furthermore, mobility shift of Fas DRE probe in the presence of nuclear extract was reversed when cold (unlabeled DNA; Comp) DRE probe was added in the reaction mixture (Fig. 3A).

NF-κB Motifs Found on Fas Promoter Also Act as *cis*-Regulatory Elements and Participate in TCDD-Mediated Regulation of Fas Gene. Although we observed a definite role of DRE motif as a *cis*-regulatory element for TCDD-mediated regulation of Fas gene using luciferase assays, the reporter gene expression was lower using promoter with DRE motif alone compared with full-length Fas promoter (9 ± 2- versus 20 ± 5-fold; Figs. 1, A and B, and 2C). These observations indicated that DRE motif of Fas might not be the only *cis*-regulatory element that participates in TCDD-mediated regulation of Fas gene. In this context, we examined transcription factor NF-κB and its role in TCDD-mediated regulation of Fas gene, because there are reports indicating the role of NF-κB transcription factor in regulation of Fas gene (Zheng et al., 2001). We noted at least five NF-κB motifs present in Fas promoter (Fig. 2A). To determine the role of NF-κB motifs in TCDD-mediated regulation of Fas gene, we generated several subclones of Fas promoter that contain either one, two, or all NF-κB motifs (described under *Materials and Methods* and shown in Fig. 2B). Luciferase assays were performed using EL4 cells that were transfected with various Fas promoter subclones and treated with TCDD for at least 24 h. We observed 8 ± 2-fold increase in luciferase expression in EL4 cells transfected with Fas973, a subclone containing all the NF-κB motifs of Fas promoter (Fig. 2C). We observed a 2 ± 1-fold increase in luciferase expression in cells transfected with Fas266, a 5 ± 1-fold increase with Fas317, and a 3 ± 1-fold increase with Fas270 subclones (Fig. 2C). Fas266 contained one NF-κB motif, whereas Fas317 and Fas270 contained at least two NF-κB motifs of the Fas promoter (Fig. 2B). These data demonstrated that NF-κB motifs of Fas promoter might also act as *cis*-regulatory elements and participate in TCDD-mediated

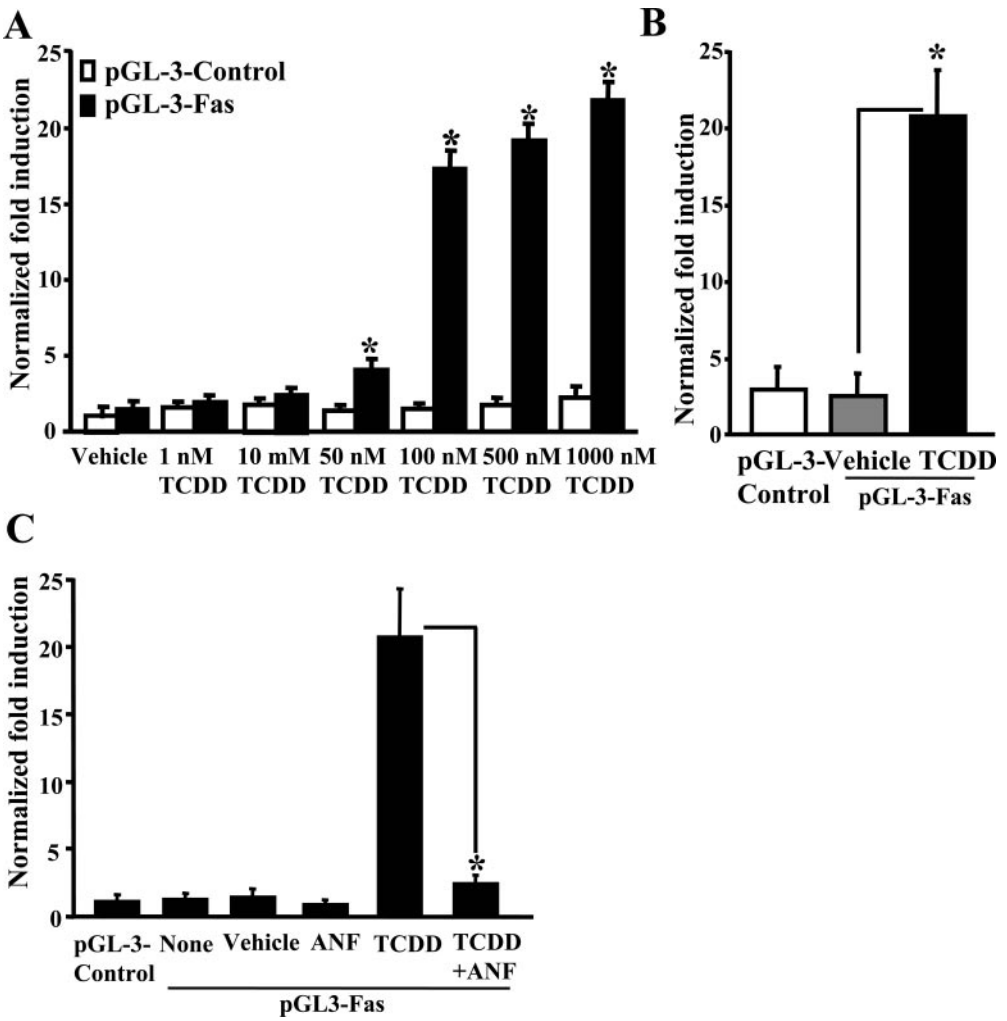

Fig. 1. TCDD regulates Fas promoter activity. A and B, EL4 cells were transfected with luciferase reporter constructs containing full-length Fas promoter (pGL-3-Fas) or pGL-3 control and pcDNA3.1 containing pCMV-β-galactosidase gene. Two days after transfection, the transiently expressing EL4 cells were treated with various concentrations of TCDD (1–1000 nM/ml) or DMSO (vehicle). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bar represents mean ± S.E.M. from three independent experiments. C, EL4 cells were transfected with various luciferase reporter constructs; pGL-3-control, pGL-3-Fas, and pcDNA3.1 containing pCMV-β-galactosidase gene. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated with DMSO (vehicle), 100 ng/ml ANF alone (ANF), 100 nM TCDD (TCDD), 100 nM TCDD + 100 ng/ml ANF (TCDD+ANF). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bars represent mean ± S.E.M. from three independent experiments. *, $p < 0.05$.

regulation of Fas. However, the response of these NF-κB motifs to TCDD varied (weak, 2 ± 1-fold versus moderate, 5 ± 1), and this variation may be due to differences in response to TCDD-mediated regulatory proteins.

To further confirm the regulatory role of Fas NF-κB motifs in TCDD-mediated regulation of Fas gene, we performed EMSA assays using various radiolabeled probes containing different NF-κB motifs of Fas promoter. We observed mobility shift of all NF-κB probes incubated with nuclear extracts from EL4 cells treated with 100 nM TCDD for 24 h (Fig. 3B). No mobility shift was observed in the absence of nuclear extract (Fig. 3B). We further examined the role of NF-κB subunits in TCDD-mediated regulation of Fas promoter and performed supershift assays using anti-mouse monoclonal antibodies against NF-κB/p50 and NF-κB/p65 subunits. As shown in Fig. 3B, we observed supershift in NF-κB probes in the presence of subunit NF-κB/p50 (Fig. 3B) but not in the presence of NF-κB/p65 (Fig. 3B). In the presence of cold probe (Comp), no shift in NF-κB probes was observed with nuclear extract (Fig. 3). Mutant NF-κB probes also yielded no mobility shift (Fig. 3B) compared with normal NF-κB probes (Fig. 3B). The data obtained from EMSA assays confirmed that NF-κB motifs present in Fas promoter also participate in TCDD-mediated regulation of Fas gene.

TCDD-Induced Regulation of Mouse FasL Promoter via AhR. Regulation of FasL promoter by TCDD was determined by transfecting EL4 cells with constructs containing full-length upstream region of FasL (pGL3-FasL720) promoter. The transiently expressing EL4 cells were treated with various concentrations of TCDD (1–1000) or vehicle (DMSO) 48 h after transfection. Luciferase assays were performed on these cells 24 h after TCDD treatment. We observed TCDD-mediated increase in expression of luciferase when FasL promoter was used for transfection, and the cells were treated with 100 nM or higher doses of TCDD for at least 24 h (Fig. 4, A and B). The effect of TCDD on luciferase expression was very minimal at lower doses (1–50 nM; Fig. 4A). We observed minimal level (2 ± 1) of luciferase expression when the cells were transfected with FasL promoter and not treated or treated with vehicle (DMSO) (Fig. 4, A and B). The data demonstrated that TCDD induces up-regulation of luciferase expression via FasL promoter.

To further understand TCDD-induced regulation of mouse FasL promoter, we performed luciferase assays as described above, but ANF, an antagonist of AhR, was added in the culture at least 2 h before TCDD treatment of the transfected cells. There was significant inhibition of luciferase expression in cultures treated with TCDD + ANF compared with those treated with TCDD alone (Fig. 4C). These data suggested a role for AhR in TCDD-induced regulation of FasL promoter.

NF-κB Motifs of FasL Promoter Act as *cis*-Regulatory Elements in TCDD-Mediated Regulation of FasL Gene. Because mouse FasL promoter does not contain a DRE motif, we speculated that TCDD-mediated regulation of

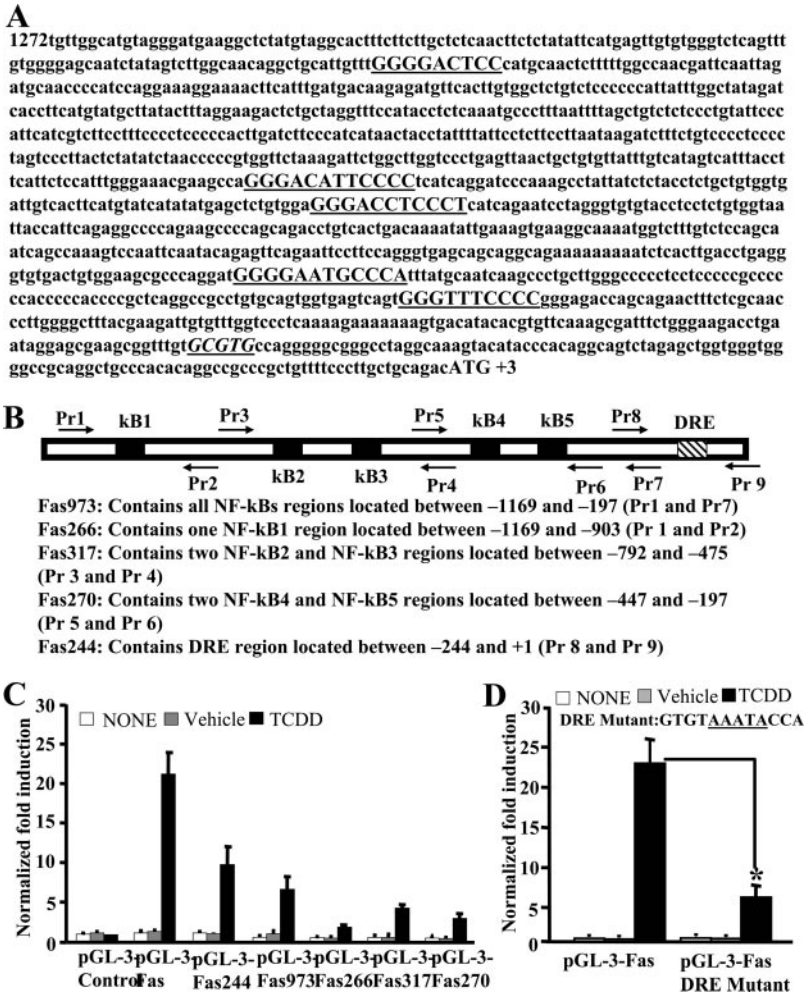

Fig. 2. TCDD regulates Fas promoter activity through DRE and NF-κB. A, sequence of a 1272-bp mouse Fas promoter upstream of the Fas translational start site is depicted. DRE binding sites are shown in italics, bold, and underline, whereas NF-κB binding sites (five sites) are shown in bold and underlined. B, sites and sets of primers used to amplify different regions of Fas promoter containing either DRE or various NF-κB sites. C, EL4 cells were transfected with various luciferase reporter constructs containing pGL-3 control or full-length Fas promoter (pGL-3-Fas) and pcDNA3.1 containing pCMV-β-galactosidase gene. Additional constructs included those containing DRE motif (NpGL-3-Fas244) or various NF-κB motifs; pGL-3-Fas973, pGL-3-Fas266, pGL-3-Fas317, and pGL-3-Fas270. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated either with 100 nM TCDD or vehicle (DMSO). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bars represent mean ± S.E.M. from three independent experiments. D, EL4 cells were transfected with luciferase reporter constructs pGL-3-Fas, pGL-3-Fas DRE mutant, and pcDNA3.1 with pCMV-β-galactosidase gene. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated with vehicle (DMSO) or 100 nM TCDD (TCDD). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bars represent mean ± S.E.M. from three independent experiments. *, $p < 0.05$.

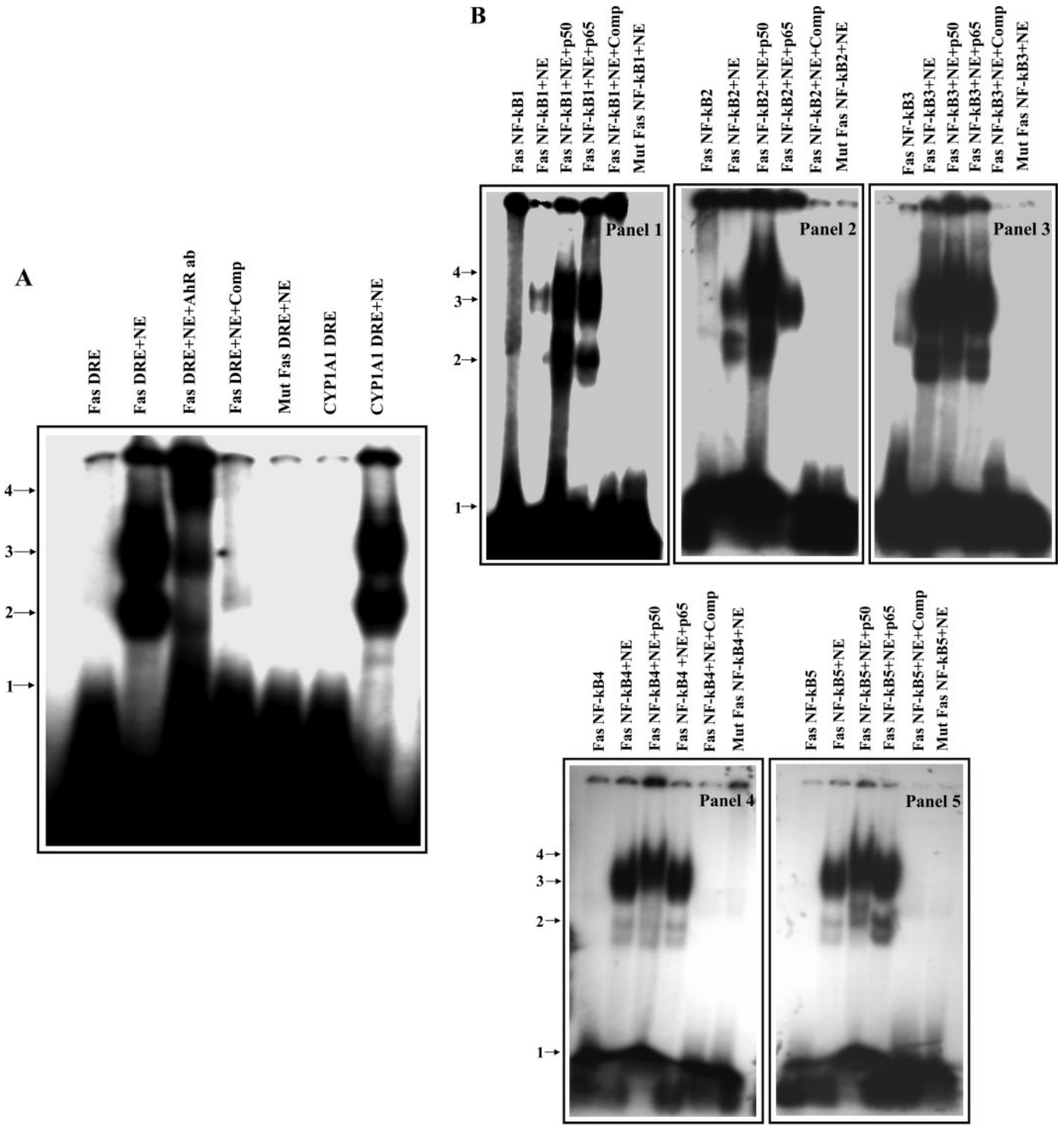

Fig. 3. EMSA analysis of DRE and NF-κB motifs on Fas promoter. A, single-strand hairpin loop probes containing DRE site of mouse Fas promoter or mouse CYP1A1 promoter were generated. Nuclear extract was generated from TCDD-treated EL4 cells, and 4 to 5 μg of NE was used in each reaction. Radiolabeled ^{32}P-DRE probes were either directly used or used after incubation with nuclear extract, nuclear extract + goat anti-mouse AhR polyclonal antibody, or nuclear extract containing cold DNA (unlabeled). Arrow 1, Fas DRE probe DNA band; arrows 2 and 3, Fas DRE/nuclear extract complexes; and arrow 4, supershifted complex in the presence of Abs against mouse AhR. The lanes are labeled as Fas DRE (Fas DRE probe without nuclear extract), Fas DRE+NE (Fas DRE probe + NE), Fas DRE+NE+AhR Ab (Fas DRE probe + NE + AhR Ab), Fas DRE+NE+Com [Fas DRE probe + NE + cold (unlabeled) Fas DRE probe for competition, Mut DRE+NE (mutant DRE probe + NE) CYP1A1 DRE (DRE probe from mouse CYP1A1 without nuclear extract), and CYP1A1DRE+NE (CYP1A1 DRE probe + nuclear extract) are shown. B, single-strand hairpin loop probes containing various NF-κB motifs of Fas promoter were generated. Nuclear extract (4–5 μg) generated from TCDD-treated EL4 cells and goat anti-mouse monoclonal antibody against NF-κB (p65 or p50) was used in various binding reactions. Radiolabeled ^{32}P-NF-κB probes were either directly used or used after incubation with nuclear extract or with p65 or p50 subunits of NF-κB. Panel 1 shows Fas NF-κB1 probe without nuclear extract (lane 1), Fas NF-κB1 probe + NE (lane 2), Fas NF-κB1 probe + NE + p50 antibody (lane 3), Fas NF-κB1 probe + NE + p65 antibody (lane 4), Fas NF-κB1 probe + NE + unlabeled DNA (comp; lane 5), and mutant Fas NF-κB1 probe + NE (lane 6). Panels 2 to 5 show Fas NF-κB2 to Fas NF-κB5 probes and lanes 1 to 6 represent the same orientation as described for panel 1 above. Arrow 1 shows NF-κB probes' DNA band, arrows 2 and 3 demonstrate various NF-κB-nuclear extract complexes, and arrow 4 shows supershift of NF-κB probes.

FasL promoter is DRE-independent. We noted at least two NF-κB motifs present in mouse FasL promoter (Fig. 5A). In an effort to examine the role of NF-κB motifs in TCDD-mediated regulation of FasL promoter, we generated several reporter NF-κB subclones as described under *Materials and Methods*. These subclones were designated as FasL191 (contains NF-κB1 motif), FasL324 (contains NF-κB2 motif), and FasL231 (without NF-κB motifs). Luciferase assays, performed using TCDD-treated EL4 cells transfected with these subclones, yielded up-regulated luciferase expression in the presence of NF-κB motifs and minimal luciferase expression in the absence of NF-κB motifs (Fig. 5C). Luciferase induction was 5 ± 2-fold when subclone FasL191 containing NF-κB1 was used for transfection compared with full-length FasL promoter (pGL3-FasL), which showed 14 ± 4-fold induction (Fig. 5C). Likewise, we observed 6 ± 2-fold increase in luciferase expression when subclone FasL324 containing NF-κB2 was used for transfection (Fig. 5C). However, we observed minimal levels (~1-fold) of luciferase expression when subclone FasL231, without any NF-κB motif, was used for transfection (Fig. 5C). To further examine the regulatory role of NF-κB in TCDD-mediated regulation of FasL promoter, we generated several FasL promoter mutant constructs designated as FasL NF-κB1 mutant, FasL NF-κB2 mutant, and FasL NF-κB1+NF-κB2 mutant based on the NF-κB motif mutated (as described under *Materials and Methods*). Luciferase assays performed using TCDD-treated EL4 cells transfected with these FasL promoter mutant subclones demonstrated significant reduction in luciferase expression compared with full-length FasL promoter (Fig. 5D). We observed 4 ± 1- and 3 ± 1-fold increase in luciferase expression in FasL NF-κB1 and FasL NF-κB2 mutant-transfected cells, respectively (Fig. 5D). In the same experimental settings, we observed minimal luciferase expression when subclone containing both FasL NF-κB1 and -κB2 mutant was used for transfection (Fig. 5D). These data suggested that NF-κB motifs present in mouse FasL promoter participate in TCDD-induced regulation of FasL promoter.

EMSA assays were also performed to further confirm the regulatory role of NF-κB motifs in TCDD-mediated FasL regulation. Hairpin loop probes specific to NF-κB1 and NF-κB2 motifs were generated as described under *Materials and Methods*. Radiolabeled NF-κB probes were incubated in the presence or absence of nuclear extracts from EL4 cells treated with 100 nM TCDD for 24 h. The data obtained from EMSA assays demonstrated that both NF-κB1 and NF-κB2 probes shifted their mobility in the presence of nuclear extract (Fig. 6). However, the probes that were not incubated with nuclear extracts migrated in normal manner (Fig. 6). We also observed no shift in migration when NF-κB1 or NF-κB2 mutant probes were used in EMSA assays (Fig. 6). Furthermore, we observed that both NF-κB1 and NF-κB2 probes supershifted when incubated with nuclear extracts and monoclonal antibodies against p50 subunit of mouse NF-κB but not very clearly in the presence of p65 subunit of mouse NF-κB (Fig. 6). These probes when incubated in the presence of cold probes (unlabeled probes) showed no shift or supershift of bands (Fig. 6). These data further confirmed that NF-κB motifs of FasL promoter act as *cis*-regulatory elements in TCDD-mediated regulation of FasL gene.

TCDD Up-Regulates the Expression of Fas and FasL in Vivo. After confirming that TCDD mediates regulation of Fas and FasL promoters via DRE and/or NF-κB motifs, we further tested TCDD-induced up-regulation of Fas and FasL

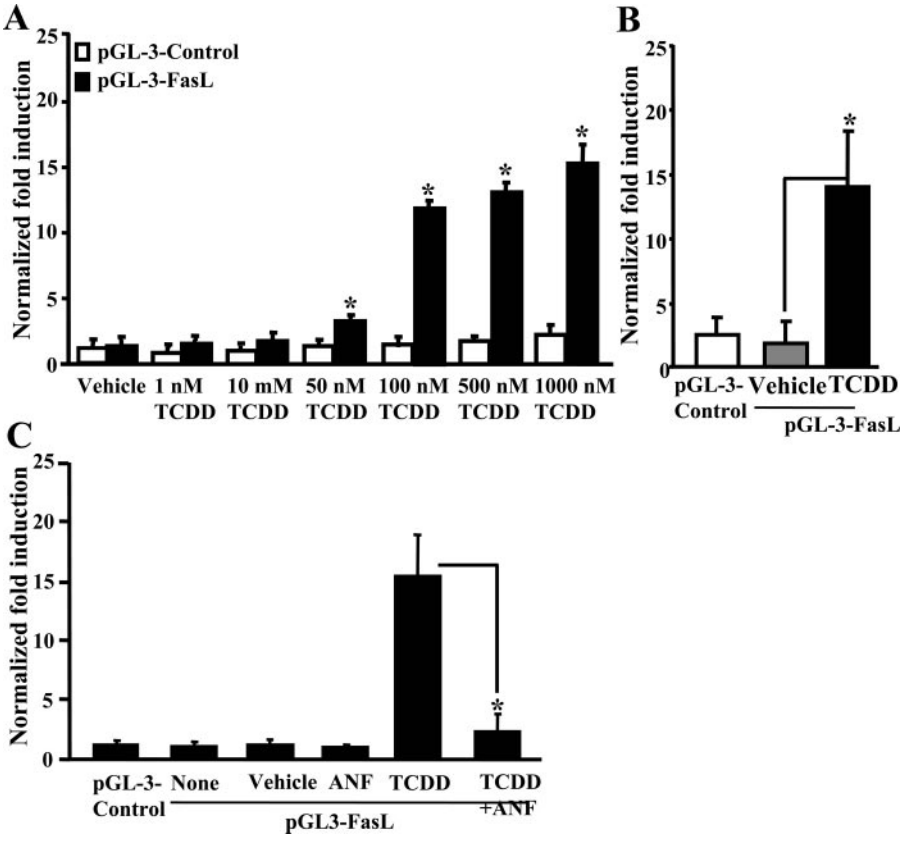

Fig. 4. TCDD regulates FasL promoter activity. A and B, EL4 cells were transfected with luciferase reporter construct containing full-length FasL promoter (pGL-3-FasL) or pGL-3 control and pcDNA3.1 containing pCMV-β-galactosidase gene. Two days after transfection, the transiently expressing EL4 cells were treated with various concentrations of TCDD (1–1000 nM/ml) or DMSO (vehicle). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bar represents mean ± S.E.M. from three independent experiments. *, $p < 0.05$. C, EL4 cells were transfected with luciferase reporter constructs containing pGL-3 control or pGL-3-FasL and pcDNA3.1 with pCMV-β-galactosidase. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated either with DMSO (vehicle), 100 ng/ml ANF alone (ANF), 100 nM TCDD (TCDD), 100 nM TCDD + 100 ng/ml ANF (TCDD+ANF). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bars represent mean ± S.E.M. from three independent experiments. *, $p < 0.05$.

in vivo. To this end, C57BL6 mice were given either TCDD (1, 5, or 10 μg/kg) or vehicle (DMSO), and thymus and liver, heart, and lungs were harvested on day 1 and 3 after treatment with TCDD or vehicle. Next, we analyzed mRNA levels of Fas and FasL in thymus and liver by performing real-time PCR using iCycler (Bio-Rad). The mRNA levels for Fas and FasL were increased both in thymus and liver by TCDD in a dose-dependent and time-dependent manner (Fig. 7, A–C) compared with vehicle controls. The effect of TCDD on CYP1A1 expression was also obtained by a parallel analysis of CYP1A1 mRNA levels in thymus and liver (Fig. 7, A–C). As expected, exposure to TCDD triggered marked increase in CYP1A1 in a dose- and time-dependent manner, compared with the controls. The -fold increase in CYP1A1 after treatment with TCDD was significantly greater compared with the increases seen in expression of Fas and FasL at the respective doses and time points.

Discussion

Our laboratory has previously shown that Fas/FasL-mediated apoptosis constitutes one of the key mechanisms responsible for TCDD-induced killing of T cells and immunotoxicity (Rhile et al., 1996; Kamath et al., 1997, 1998, 1999; Camacho et al., 2001, 2002, 2004a,b, 2005; Fisher et al., 2004). We have also demonstrated that C57BL/6 mice carrying mutations in Fas and FasL were more resistant to TCDD-mediated thymic atrophy at concentrations of <50 μg/kg body weight (Rhile et al., 1996; Kamath et al., 1999; Camacho et al., 2002, 2005). In addition, TCDD-induced up-regulation of Fas in the thymus

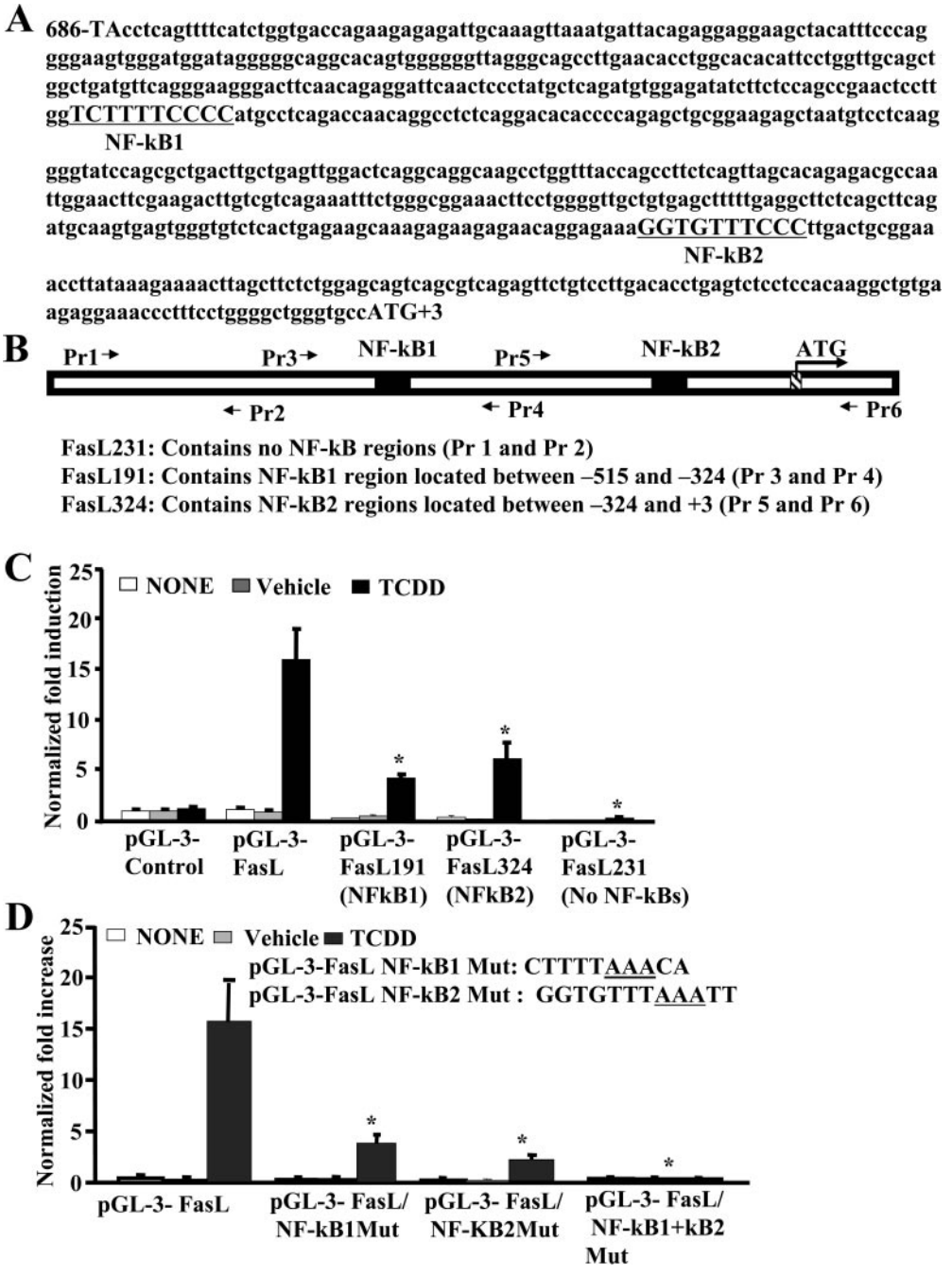

Fig. 5. TCDD regulates FasL promoter activity through NF-κB. A, sequence of a 689-bp mouse Fas promoter upstream of the FasL translational start site has been depicted. NF-κB binding sites (two sites) have been shown in bold and underlined. B, sites and sets of primers used to amplify different regions of FasL promoter with or without NF-κB. C, EL4 cells were transfected with various luciferase reporter constructs containing pGL-3 control or full-length FasL promoter (pGL-3-FasL) or containing various NF-κB motifs; pGL-3-FasL231, pGL-3-FasL191, pGL-3-FasL324, and pcDNA3.1 with pCMV-β-galactosidase. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated either with DMSO (vehicle) or 100 nM TCDD (TCDD). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bars represent mean ± S.E.M. from three independent experiments. *, $p < 0.05$ compared with pGL-3-FasL group. D, EL4 cells were transfected with luciferase reporter constructs pGL-3-FasL, pGL-3-FasL/NF-κB1 mutant, pGL-3-FasL/NF-kB2 mutant, pGL-3-FasL/NF-κB1 and kB2 mutant, and pcDNA3.1 with pCMV-β-galactosidase. Two days after transfection, the transiently expressing EL4 cells were left untreated (none) or treated either with DMSO (vehicle) or 100 nM TCDD (TCDD). The luciferase activity was normalized to β-galactosidase activity and expressed as normalized -fold induction. The vertical bar represents mean ± S.E.M. from three independent experiments. *, $p < 0.05$ compared with pGL-3-FasL group.

and FasL expression on thymic stromal cells in vivo (Fisher et al., 2004; Camacho et al., 2005) and that interaction between stromal cells and T cells was critical for TCDD-induced apoptosis in T cells (Camacho et al., 2005). Despite such studies, the precise mechanism of transcriptional regulation of Fas and FasL genes induced by TCDD has not been previously investigated. In the present study, therefore, we examined *cis*-regulatory elements of Fas and FasL promoters and their role in TCDD-mediated regulation of Fas and FasL gene expression.

The data from current study demonstrated that TCDD regulates of Fas and FasL promoters inasmuch as we observed increased luciferase expression in TCDD-treated EL4 cells transfected with constructs containing full-length Fas or FasL promoter. In addition, the up-regulation of luciferase expression was TCDD-specific, because we observed minimal (1- to 2-fold) luciferase expression when vehicle (DMSO) was used in the culture under the same settings (Figs. 1A and 3A). Furthermore, luciferase expression was completely blocked when EL4 cells transfected with Fas or FasL promoter were treated with α-naphthoflavone, an AhR antagonist, before TCDD treatment. This demonstrated the role of AhR in TCDD-mediated regulation of Fas and FasL promoters. Furthermore, we also observed the potential role of multiple sets of regulatory elements in TCDD-mediated Fas/FasL regulation. In the case of Fas promoter, we observed the participation of DRE motif as *cis*-regulatory element, as we observed after TCDD treatment, 6 ± 2-fold increase in luciferase expression in the presence of DRE motif, compared with the control (Fig. 2C). However, such reduced levels of luciferase expression seen with DRE motif alone compared with full-length Fas promoter (6 ± 2- versus 20 ± 5-fold) indicated that DRE might not be the only TCDD-induced regulatory motif in Fas promoter. Upon further examination, we noted five NF-κB motifs present in Fas promoter, and when we examined luciferase expression in the presence of various NF-κB regulatory motifs of Fas promoter, we observed varying degrees of luciferase expression (5 ± 2-fold) after TCDD treatment. Furthermore, more definite proof for the participation of DRE and NF-κB motifs came from EMSA assays when we observed shifts in Fas promoter probes containing DRE or different NF-κB motifs incubated with nuclear extracts from TCDD-treated EL4 cells. In FasL promoter, we observed no DRE motif but two NF-κB motifs. Upon examining the role of NF-κBs in TCDD-induced regulation of FasL promoter, we observed 2 ± 4-fold increase in luciferase expression in EL4 cells transfected with constructs containing either NF-κB motif of FasL promoter. Moreover, we observed shift in probes containing different NF-κB motifs of FasL that were incubated with nuclear extracts from TCDD-treated EL4 cells, thereby corroborating the role of NF-κB in the regulation of FasL promoter.

TCDD-induced regulation of Fas and FasL expression seems to require signaling through AhR because both Fas

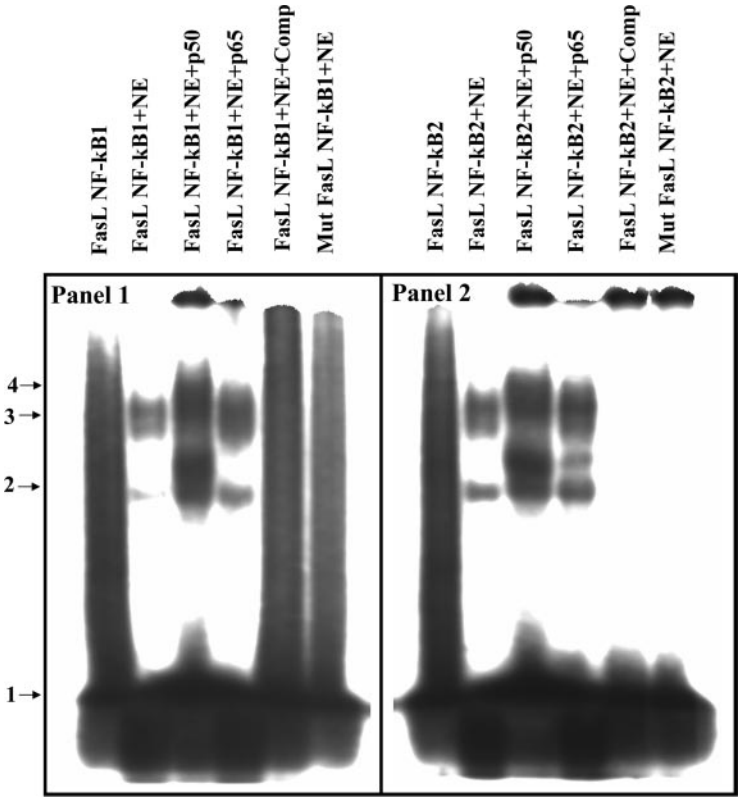

Fig. 6. EMSA analysis of NF-κB motifs on FasL promoter. 1, single-strand hairpin loop probe containing FasL promoter NF-κB1 motifs was generated. Nuclear extract (4–5 μg) generated from TCDD-treated EL4 cells was used in each reaction. Radiolabeled ^{32}P-FasL NF-κB1 probe was either directly used or used after incubation with nuclear extract or nuclear extract + goat anti-mouse NF-κB (p65 and p50) monoclonal antibody. Lane 1, FasL NF-κB1 probe without nuclear extract; lane 2, FasL NF-κB1 probe + nuclear extract; lane 3, FasL NF-κB1 probe + nuclear extract + NF-κB (p50) mAb; lane 4, FasL NF-κB1 probe + nuclear extract + NF-κB (p65) mAb; lane 5, FasL NF-κB1 probe + nuclear extract + cold DNA (comp); and lane 6, mutant FasL NF-κB1 probe + nuclear extract. Panel 2, similarly demonstrates FasL NF-κB2 probes and six lanes represent the same orientation described for FasL NF-κB1 probe above. Arrow 1, NF-κB1 or NF-κB2 probe DNA bands; Arrows 2 and 3, NF-κB1 or NF-κB2 nuclear extract complexes; arrow 4, NF-κB1 or NF-κB2 supershifted complex.

and FasL promoters failed to induce luciferase expression in the presence of ANF. We have shown that TCDD failed to up-regulate the expression of FasL in AhR-deficient mice (Camacho et al., 2005). In addition, we have observed that TCDD failed to induce apoptosis in thymic T cells from AhR-deficient mice (Camacho et al., 2005). It is known that AhR has much wider role than just induction of detoxification enzymes (Matikainen et al., 2001). In this context, our studies demonstrate that AhR may also participate in the induction of apoptosis through regulation of Fas and FasL expression. This may represent a normal physiological response to a chemical insult, thereby enabling the host to get rid of damaged and potentially precancerous cells.

TCDD-mediated regulation of Fas involves DRE motif present in Fas promoter because we obtained binding of nuclear protein obtained from TCDD-exposed EL4 cells, shift of DRE bands, and supershift of DRE bands in the presence of AhR antibodies. In addition to DRE, AhR has also been

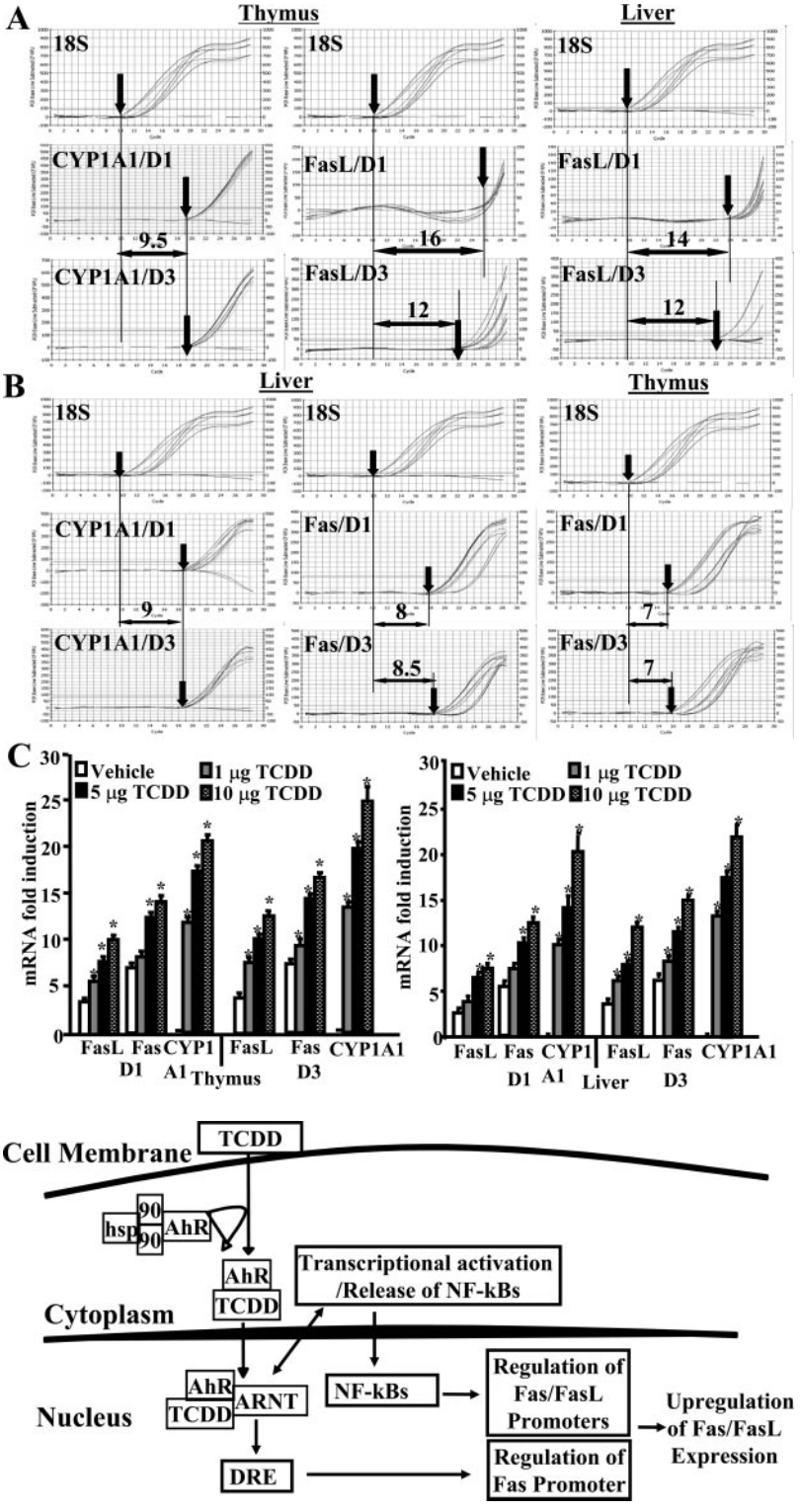

Fig. 7. TCDD up-regulates the expression of Fas and FasL in vivo. C57/BL6 mice were treated either with vehicle or 1, 5, or 10 μg/kg TCDD. On days 1 (D1) and 3 (D3) after treatment, thymus and liver were harvested, and total RNA from these organs was extracted. A and B, raw data obtained from real-time PCR on iCycler (Bio-Rad). ↔, represents difference in onset of amplification for FasL, Fas, and CYP1A1 compared with 18S. C, above-mentioned data expressed as -fold induction. The total RNA was probed for Fas, FasL, CYP1A1, and 18S genes in separate tubes using the relative standard method. The relative amounts of Fas, FasL, and CYP1A1 were calculated based on the corresponding standard curves, normalized to the 18S from the same treatment group, and then normalized to the vehicle-treated group to obtain -fold induction.

Fig. 8. Schematic representation of molecular mechanisms involved in TCDD-mediated regulation of mouse Fas and FasL genes leading to their up-regulated expression.

shown to interact with NF-κB, another transcription factor. Recent studies have shown that AhR and NF-κB physically interact and functionally modulate each other's activities (Kim et al., 2000). In this context, we have noted at least five NF-κB motifs in Fas promoter and two NF-μB motifs in FasL promoter. These NF-κB motifs may interact with AhR and participate in TCDD-mediated regulation of Fas and FasL expression inasmuch as we observed various levels of luciferase expression in the presence different NF-κB motifs of Fas and FasL.

Tian et al. (1999), in their review article, highlighted the interactions between AhR and NF-κB and the potential biological effects that result of these interactions (Tian et al., 1999). Both AhR and NF-κB are inducible transcription factors, and each governs the expression of distinct sets of genes that are important for normal physiological and pathophysiological responses. There are reports demonstrating the association of AhR with the p65 (RelA) subunit of NF-κB in mouse hepatoma cells (Tian et al., 1999). However, the impact of this association may differ depending on the cell type (Kim et al., 2000). For example, Tian and coworkers demonstrated in COS cells an AhR-mediated inhibition of NF-κB (RelA/p50) binding to a consensus κB element, which was augmented by TCDD treatment (Tian et al., 1999). In contrast, Kim and coworkers identified cooperation between AhR and RelA in transactivation of a c-myc promoter-reporter construct in mammary epithelial cells (Kim et al., 2000). A similar observation was also made in B cells in which AhR nuclear complex and NF-κB/Rel proteins were shown to converge at the DRE and κB motif to influence transcriptional activity of the hs4 enhancer fragment of immunoglobulin heavy chain gene (Sulentic et al., 2004). In addition, TCDD treatment of hepatoma cells was shown to activate NF-κB in an AhR-dependent manner (Puga et al., 2000). In this study, it was also shown that most of the increase in NF-κB binding activity could be accounted for by increases in p50/p50 complexes. Because these complexes are known to repress NF-κB-dependent gene transcription, it was suggested that this may constitute another mechanism through which TCDD may exert immunosuppression. Induction of NF-κB activity has also been shown in immature rat thymus after TCDD treatment (Olnes et al., 1994). Recent studies from our laboratory also demonstrated that in vivo exposure to TCDD led to nuclear translocation of NF-κB in thymic stromal cells leading to up-regulation of FasL (Camacho et al., 2005).

Regulation of Fas and FasL expression is a double-edged sword. Underexpression or mutation in Fas or FasL, on one hand, results in various diseases, including autoimmunity, severe lymphoproliferation, and susceptibility to lymphoid carcinoma, autoimmune lymphoproliferative syndrome, and systemic lupus erythematosus (Kovacs et al., 1997b; Gronbaek et al., 1998; Straus et al., 1999). In contrast, overexpression of Fas or FasL results in uncontrolled apoptosis that may contribute to neurodegenerative disorders, failure to clear pathogens, and cardioretinopathology (Kovacs et al., 1997a; Sharma et al., 2000). The immune privilege status of various organs, such as eyes and testes, is partially established by expression of high levels of FasL in these organs (Griffith et al., 1995). However, continuous FasL expression in testes has been shown to cause autoimmunity, leading to fertility problems (Hu et al., 2003). FasL constitutively expressed in the thymus plays an important role in the ontogenesis and negative selection of T cells (Kabelitz, 1993), but up-regulated expression of FasL in thymus also leads to development of autoimmunity (Kobata et al., 1997; Brochu et al., 1999). In addition, in the thyroid gland, inflammation leading to interaction between Fas and FasL can cause thyrocyte destruction and trigger Hashimotos thyroiditis (Stassi et al., 2000). In the current study, we noted that Fas and FasL were up-regulated in the thymus and liver after in vivo TCDD administration. We have also noted that Fas is up-regulated in the spleen, lungs, and heart and FasL in the spleen and lungs but not heart (N. Singh, M. Nagarkatti, and P. Nagarkatti, unpublished data). Together, our studies suggest a common pathway of up-regulation of these molecules in various tissues that could potentially trigger tissue injury as well as autoimmune disease.

In conclusion, the current study demonstrates that TCDD-induced transcriptional regulation of Fas and FasL genes involves AhR-mediated signaling and participation of DRE and/or NF-κB motifs present in their respective promoters (Fig. 8).

Acknowledgments

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References

Baba T, Mimura J, Gradin K, Kuroiwa A, Watanabe T, Matsuda Y, Inazawa J, Sogawa K, and Fujii-Kuriyama Y (2001) Structure and expression of the Ah receptor repressor gene. *J Biol Chem* **276:**33101–33110.

Brochu S, Rioux-Masse B, Roy J, Roy DC, and Perreault C (1999) Massive activation-induced cell death of alloreactive T cells with apoptosis of bystander postthymic T cells prevents immune reconstitution in mice with graft-versus-host disease. *Blood* **94:**390–400.

Camacho IA, Hassuneh MR, Nagarkatti M, and Nagarkatti PS (2001) Enhanced activation-induced cell death as a mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced immunotoxicity in peripheral T cells. *Toxicology* **165:**51–63.

Camacho IA, Nagarkatti M, and Nagarkatti PS (2002) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces Fas-dependent activation-induced cell death in superantigen-primed T cells. *Arch Toxicol* **76:**570–580.

Camacho IA, Nagarkatti M, and Nagarkatti PS (2004a) Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on maternal immune response during pregnancy. *Arch Toxicol* **78:**290–300.

Camacho IA, Nagarkatti M, and Nagarkatti PS (2004b) Evidence for induction of apoptosis in T cells from murine fetal thymus following perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Toxicol Sci* **78:**96–106.

Camacho IA, Singh N, Hegde VL, Nagarkatti M, and Nagarkatti PS (2005) Treatment of mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* **175:**90–103.

Crist SA, Griffith TS, and Ratliff TL (2003) Structure/function analysis of the murine CD95L promoter reveals the identification of a novel transcriptional repressor and functional CD28 response element. *J Biol Chem* **278:**35950–35958.

Davis IJ and Lau LF (1994) Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nurr-1 in the adrenal glands. *Mol Cell Biol* **14:**3469–3483.

Denis MG (1998) Characterization, cloning and expression of the Tage4 gene, a member of the immunoglobulin superfamily. *Int J Oncol* **12:**997–1005.

Denison MS and Heath-Pagliuso S (1998) The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull Environ Contam Toxicol* **61:**557–568.

Denison MS, Pandini A, Nagy SR, Baldwin EP, and Bonati L (2002) Ligand binding and activation of the Ah receptor. *Chem Biol Interact* **141:**3–24.

Dertinger SD, Nazarenko DA, Silverstone AE, and Gasiewicz TA (2001) Aryl hydrocarbon receptor signaling plays a significant role in mediating benzo[a]pyrene- and cigarette smoke condensate-induced cytogenetic damage in vivo. *Carcinogenesis* **22:**171–177.

Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM, and Gonzalez FJ (1996) Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol Appl Pharmacol* **140:**173–179.

Fisher MT, Nagarkatti M, and Nagarkatti PS (2004) Combined screening of thymocytes using apoptosis-specific cDNA array and promoter analysis yields novel gene targets mediating TCDD-induced toxicity. *Toxicol Sci* **78:**116–124.

Gonzalez FJ and Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos* **26:**1194–1198.

Griffith TS, Brunner T, Fletcher SM, Green DR, and Ferguson TA (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science (Wash DC)* **270:**1189–1192.
Gronbaek K, Straten PT, Ralfkiaer E, Ahrenkiel V, Andersen MK, Hansen NE, Zeuthen J, Hou-Jensen K, and Guldberg P (1998) Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. *Blood* **92:**3018–3024.
Gu YZ, Hogenesch JB, and Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40:**519–561.
Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35:**307–340.
Holsapple MP, Morris DL, Wood SC, and Snyder NK (1991) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: possible mechanisms. *Annu Rev Pharmacol Toxicol* **31:**73–100.
Hu JH, Jiang J, Ma YH, Yang N, Zhang MH, Wu M, Fei J, and Guo LH (2003) Enhancement of germ cell apoptosis induced by ethanol in transgenic mice overexpressing Fas Ligand. *Cell Res* **13:**361–367.
Kabelitz D (1993) The primary immune response. *Dtsch Med Wochenschr* **118:**1575–1576.
Kamath AB, Camacho I, Nagarkatti PS, and Nagarkatti M (1999) Role of Fas-Fas ligand interactions in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity: increased resistance of thymocytes from Fas-deficient (lpr) and Fas ligand-defective (gld) mice to TCDD-induced toxicity. *Toxicol Appl Pharmacol* **160:**141–155.
Kamath AB, Nagarkatti PS, and Nagarkatti M (1998) Characterization of phenotypic alterations induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on thymocytes in vivo and its effect on apoptosis. *Toxicol Appl Pharmacol* **150:**117–124.
Kamath AB, Xu H, Nagarkatti PS, and Nagarkatti M (1997) Evidence for the induction of apoptosis in thymocytes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in vivo. *Toxicol Appl Pharmacol* **142:**367–377.
Kazlauskas A, Poellinger L, and Pongratz I (1999) Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. *J Biol Chem* **274:**13519–13524.
Kim DW, Gazourian L, Quadri SA, Romieu-Mourez R, Sherr DH, and Sonenshein GE (2000) The RelA NF-kappaB subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-myc promoter in mammary cells. *Oncogene* **19:**5498–5506.
Kobata T, Takasaki K, Asahara H, Hong NM, Masuko-Hongo K, Kato T, Hirose S, Shirai T, Kayagaki N, Yagita H, et al. (1997) Apoptosis with FasL+ cell infiltration in the periphery and thymus of corrected autoimmune mice. *Immunology* **92:**206–213.
Kovacs B, Liossis SN, Dennis GJ, and Tsokos GC (1997a) Increased expression of functional Fas-ligand in activated T cells from patients with systemic lupus erythematosus. *Autoimmunity* **25:**213–221.
Kovacs B, Szentendrei T, Bednarek JM, Pierson MC, Mountz JD, Vogelgesang SA, and Tsokos GC (1997b) Persistent expression of a soluble form of Fas/APO1 in continuously activated T cells from a patient with SLE. *Clin Exp Rheumatol* **15:**19–23.
Ma Q and Whitlock JP Jr (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Biol Chem* **272:**8878–8884.
Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai T, Korsmeyer SJ, Casper RF, et al. (2001) Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat Genet* **28:**355–360.
Mimura J and Fujii-Kuriyama Y (2003) [Xenobiotics and transcriptional regulation]. *Tanpakushitsu Kakusan Koso* **48:**2261–2266.
Nazarenko DA, Dertinger SD, and Gasiewicz TA (2001) In vivo antagonism of AhR-mediated gene induction by 3′-methoxy-4′-nitroflavone in TCDD-responsive lacZ mice. *Toxicol Sci* **61:**256–264.
Olnes MJ, Verma M, and Kurl RN (1994) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-mediated gene expression in the immature rat thymus. *Exp Clin Immunogenet* **11:**102–109.
Poland A and Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22:**517–554.
Puga A, Barnes SJ, Chang C, Zhu H, Nephew KP, Khan SA, and Shertzer HG (2000) Activation of transcription factors activator protein-1 and nuclear factor-kappaB by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem Pharmacol* **59:**997–1005.
Rhile MJ, Nagarkatti M, and Nagarkatti PS (1996) Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicology* **110:**153–167.
Safe S (2001) Molecular biology of the Ah receptor and its role in carcinogenesis. *Toxicol Lett* **120:**1–7.
Schlezinger JJ, Blickarz CE, Mann KK, Doerre S, and Stegeman JJ (2000) Identification of NF-kappaB in the marine fish *Stenotomus chrysops* and examination of its activation by aryl hydrocarbon receptor agonists. *Chem Biol Interact* **126:**137–157.
Schmidt JV and Bradfield CA (1996) Ah receptor signaling pathways. *Annu Rev Cell Dev Biol* **12:**55–89.
Sharma K, Wang RX, Zhang LY, Yin DL, Luo XY, Solomon JC, Jiang RF, Markos K, Davidson W, Scott DW, et al. (2000) Death the Fas way: regulation and pathophysiology of CD95 and its ligand. *Pharmacol Ther* **88:**333–347.
Stassi G, Di Liberto D, Todaro M, Zeuner A, Ricci-Vitiani L, Stoppacciaro A, Ruco L, Farina F, Zummo G, and De Maria R (2000) Control of target cell survival in thyroid autoimmunity by T helper cytokines via regulation of apoptotic proteins. *Nat Immunol* **1:**483–488.
Straus SE, Sneller M, Lenardo MJ, Puck JM, and Strober W (1999) An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann Intern Med* **130:**591–601.
Sulentic CE, Holsapple MP, and Kaminski NE (2000) Putative link between transcriptional regulation of IgM expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin and the aryl hydrocarbon receptor/dioxin-responsive enhancer signaling pathway. *J Pharmacol Exp Ther* **295:**705–716.
Sulentic CE, Kang JS, Na YJ, and Kaminski NE (2004) Interactions at a dioxin responsive element (DRE) and an overlapping kappaB site within the hs4 domain of the 3′alpha immunoglobulin heavy chain enhancer. *Toxicology* **200:**235–246.
Tian Y, Ke S, Denison MS, Rabson AB, and Gallo MA (1999) Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J Biol Chem* **274:**510–515.
Tian Y, Rabson AB, and Gallo MA (2002) Ah receptor and NF-kappaB interactions: mechanisms and physiological implications. *Chem Biol Interact* **141:**97–115.
Whitlock JP Jr (1999) Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* **39:**103–125.
Zheng Y, Ouaaz F, Bruzzo P, Singh V, Gerondakis S, and Beg AA (2001) NF-kappa B RelA (p65) is essential for TNF-alpha-induced fas expression but dispensable for both TCR-induced expression and activation-induced cell death. *J Immunol* **166:**4949–4957.

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