0026-895X/01/5904-716–724$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:716–724, 2001

Vol. 59, No. 4
499/891374
Printed in U.S.A.

Cell-Specific Regulation of Human Aryl Hydrocarbon Receptor Expression by Transforming Growth Factor-β_1

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Received August 17, 2000; accepted December 20, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Previous studies showed that TGF-β down-regulates aryl hydrocarbon (AhR) expression in human lung carcinoma cells A549. Here we analyzed the molecular mechanisms by which TGF-β modulates AhR expression. A 5799-nucleotide 5′-flanking region of human AhR gene was isolated. Transient transfection studies of full-length (hAhRP) and deletion promoter constructs indicate the requirement of a *cis*-regulatory element encompassing −1980 to −1892 for full constitutive activity. Basal hAhRP activity occurs in a cell-specific manner; human hepatoma HepG2 cells possess a 10-fold higher activity compared with A549 cells. TGF-β exerts cell-specific effects on hAhRP activity. Treatment of cells with 100 pM TGF-β leads to a 50% inhibition in A549 and a 3-fold induction in HepG2 cells. Deletion mutagenesis identified a TGF-β-responsive sequence containing a functional conserved Smad-binding element. Transient overexpression of Smad 2, 3, and 4 indicates that these signal transducers modulate hAhRP activity. The down-regulation of AhR by TGF-β is modulated by 5′-TG-3′-interacting factor (TGIF). Transient overexpression of TGIF in MDA-MB231 and HepG2 cells led to inhibition of hAhRP activity and a similar decrease of AhR mRNA expression. Our findings indicate that Smad proteins are involved in the cell-specific regulation of AhR expression by TGF-β.

The Ah receptor (AhR) is a ligand-activated member of basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) transcription factor family, which is characterized by the bHLH DNA-binding domain and the PAS region of dimerization. In absence of a ligand, the AhR is localized in the cytosol and complexed with two 90-kDa heat-shock proteins. After binding of a ligand, 90-kDa heat-shock proteins dissociate from AhR and the receptor forms a heterodimer with AhR-nuclear translocator (Arnt) via the bHLH and PAS domains. The AhR/Arnt complex then binds to specific DNA recognition sequences termed xenobiotic responsive elements, which have been found in several drug metabolizing enzymes such as cytochromes P450 1A1, 1B1, and 1A2; UDP glucuronosyltransferase 1A6; NADPH-quinone-oxidoreductase; and glutathione-*S*-transferase Ya. Numerous xenobiotics, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are ligands of AhR, and the expression of AhR is necessary for the teratogenic, immunotoxic, carcinogenic, and biochemical effects of TCDD (Okey et al., 1994; Rowlands and Gustafsson, 1997).

Whereas the function of AhR in dioxin toxicity is well characterized, the physiological functions of AhR are poorly understood. AhR-deficient mice displayed reduced liver weights, transient microvesicular fatty metamorphosis, disturbed extramedullary hematopoesis, and portal hypercellularity with thickening and fibrosis (Fernandez-Salguero et al., 1995). From gene targeting experiments and in vitro findings, a functional importance of AhR in cell-cycle regulation has been suggested. In human keratinocytes, TCDD-induced cell proliferation and terminal differentiation (Milstone and LaVigne, 1984; Gaido et al., 1992; Gaido and Maness, 1994). In rat hepatocytes, TCDD has been reported to inhibit or stimulate cell growth (Wiebel et al., 1991; Wölfle et al., 1993). Other studies implicate a possible role of AhR in modulating or mediating apoptotic processes. Immunohistochemical analysis of embryonic tissues showed that AhR expression is developmentally regulated and occurs in regions undergoing remodeling processes (Abbott et al., 1995). Stimulation of resting T cells with mitogens resulted in a

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 503/A5 and by a grant from the National Cancer Institute of Canada to P.A.H.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded); Arnt, aryl hydrocarbon receptor nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; SBE, Smad binding element; TGIF, 5′-TG-3′-interacting factor; BMS, basal medium supplement; FCS, fetal calf serum; nt, nucleotides; AhRP, aryl hydrocarbon receptor promoter; kb, kilobase pair(s); SBS, Smad binding sequence; SBCE, Smad binding core element; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; TβR, transforming growth factor-β receptor; RT, reverse transcription; Cdk, cyclin-dependent kinase.

marked increase of AhR expression, and the kinetics of this increase is parallel to the onset of apoptosis (Lowrence et al., 1996; Crawford et al., 1997). The reduced liver size in AhR null mice was associated with apoptotic processes (Zaher et al., 1998).

Little is known about the regulation of AhR expression by exogenous and endogenous factors. In previous studies, we have found that TGF-β modulates the expression of AhR and genes of *Ah* gene battery in human A549 lung carcinoma cells. We could show that the down-regulation of AhR by TGF-β is transcriptionally controlled whereas the effect of TGF-β on genes of *Ah* gene battery like CYP1A1, CYP1B1, and NMO1 is independent from the down-regulation of AhR mRNA (Döhr et al., 1997; Döhr and Abel, 1997). The present study focuses on the mechanisms by which TGF-β modulates AhR expression.

TGF-β exerts its cellular effects by interacting with its cognate receptor (TβR). After binding of its agonist, this membrane serine/threonine kinase receptor activates intracellular mediators, the Smad proteins. These proteins share a common three-domain structure, a conserved N- and C-terminal region, called MH1 and MH2 domain, respectively, flanked by a variable proline-rich linker domain. Five different Smad species have been shown to be involved in TGF-β signaling. Smad 2 and 3 are activating Smads that are phosphorylated by the activated TβR. After phosphorylation, association between Smad 2/3 and Smad 4 occurs, followed by nuclear translocation and binding to target promoters. Smad 6 and 7 act as inhibitors of TGF-β signaling, because they are phosphorylated by the activated TβR without subsequent association with Smad 4. The Smad complexes are capable of recruiting specific transcription factors such as FAST-1 or FAST-2 via their MH2 domain, thus mediating ligand-specific responses (Verschueren and Huylebroeck, 1999; Miyazono, 2000; Wrana and Attisano, 2000). The MH1 domain of Smad 3 was shown to bind a specific DNA element, called Smad-binding element (SBE), that was identified in JunB (Jonk et al., 1998) and PAI-1 promoters (Dennler et al., 1998). Thus, activated Smads are able to specifically regulate target promoters through physical and/or functional interaction with the transcription machinery. Usually, TGF-β signaling leads to an induction of gene transcription; in some cases, however, it was found that TGF-β can act as a transcriptional inhibitor. For example, the oncoprotein Evi-1 can prevent binding of Smad 3 to DNA, thereby blocking TGF-β-stimulated growth arrest in certain cell lines (Kurokawa et al., 1998). Recently, an interaction of activated Smad 2 with the ubiquitously expressed homeodomain protein 5′-TG-3′-interacting factor (TGIF) was shown to result in transcriptional repression of Smad-regulated genes (Wotton et al., 1999). TGIF was first described as transcription factor that competitively binds to the retinoid X receptor response element mediating the down-regulation of retinoid X receptor expression (Bertolino et al., 1995). It was proposed that TGIF can recruit histone deacetylase through interaction with activated Smad 2, resulting in a specific down-regulation of gene transcription (Wotton et al., 1999).

Because the AhR has an important impact on the biological responses evoked by a broad range of environmental chemicals such as TCCD or related compounds, it is likely that changes of AhR expression in a particular tissue could dramatically influence the biological outcome of these xenobiotics. TGF-β has been found to be a modulator of AhR expression and it is therefore of considerable interest to identify the signaling components that control the AhR transcription. Here, we report a cell-specific effect of TGF-β on AhR expression and identify a 5′-TGTCTG-3′ element within the 5′-flanking region of AhR gene that mediates TGF-β responsiveness.

Experimental Procedures

Materials. Recombinant TGF-β_1 was supplied by Sigma (Taufkirchen, Germany). Total RNA preparation kit and Reverse Transcription System were from Roche (Mannheim, Germany). Oligonucleotides were synthesized from Amersham Pharmacia Biotech (Freiburg, Germany). Transfectam and Luciferase Reporter Assay System were from Promega (Heidelberg, Germany). Media for cell culture were purchased from Sigma and PAA (Linz, Austria). Penicillin/Streptomycin, BMS, FCS, $NaHCO_3$, and glutamine were from Seromed (Berlin, Germany).

Cloning of Human AhR-Promoter. The promoter region of human AhR was generated by PCR amplification of genomic DNA isolated from the human colon carcinoma cell line LS180. Appropriate oligonucleotide sequences (forward primer, 5′-ACT GAA AGG TGG CTT ACT GAG G-3′, reverse primer, 5′-CAA GTC CTC TGT CTC CCA GC-3′) were designed on human AhR-promoter identified by Takahashi et al. (1994). PCR was carried out in 20 mM Tris/HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton-X 100, 100 mg/ml BSA, and 10 nM each dNTP. *Taq* DNA Polymerase, 2.5 units (Life Technologies, Grand Island, NY) and 2.5 U *Pfu* (Stratagene, La Jolla, CA) were used for PCR. Dimethyl sulfoxide in a final concentration of 10% (v/v) was included. Cycling conditions were 35 cycles with denaturation at 95°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 3 min. PCR products were separated by agarose gel electrophoresis and subcloned onto pCRII (Invitrogen, Carlsbad, CA). Positive clones were identified by restriction enzyme digestion mapping and termed hAhRP-pCRII. Final identity was confirmed by automated sequencing (HSC Biotech Service Center, Ontario, Canada) and agreed with that of the published sequence (Takahashi et al., 1994). For isolation of human AhR 5′-flanking region, hAhRP-pCRII was digested with *Eco*RI and *Eco*N1, yielding a hAhRP homolog fragment of about 700 nt that was used to screen a lymphocyte genomic DNA library (Stratagene). This identified a clone of about 14 kb of genomic DNA encompassing hAhRP at the 3′ end. This fragment was digested with *Sac*I to generate a fragment of about 6 kb that was inserted into the *Sac*I site of Bluescript SK (Stratagene). Sequence analysis verified that this fragment did indeed encompass the human AhR-promoter, including 5640 nt upstream of the putative start site and 159 nt of the nontranslated region. Subsequently, this *Sac*I fragment was inserted into the *Sac*I site of pGL3 basic (Promega, Madison, WI). Clones with the appropriate orientation were identified by restriction enzyme digestion. This luciferase expression plasmid under control of the human AhR-promoter was termed pGL3-hAhRP.

Generation of Deletion Constructs of pGL3-hAhRP. To produce deletion constructs of pGL3-hAhRP, the clone was digested with *Kpn*I and several endonucleases (*Eco*RV, *Eco*RI, *Nde*I, *Sau*I, *Apa*I) that possess singular recognition sequences within the full-length construct and are termed AhRΔ(−2510), AhRΔ(−1980), AhRΔ(−1892), AhRΔ(−881), and AhRΔ(−120), respectively (Fig. 1). The fragments were separated by agarose gel electrophoresis followed by standard cleanup methods. Blunt ends were generated by treatment with Klenow enzyme and were subsequently used for T4-DNA ligation (Sambrook et al., 1989). To produce a deleted AhRP construct containing a Smad-binding sequence (SBS), we inserted the −2510 to −1980 AhRP element upstream of the AhRΔ(−120) minimal promoter termed AhRΔSBS. In addition, two copies of the Smad-binding core element (SBCE: 5′-CAGACA-3′) were fused to

the 5′-end of AhRΔ(−1980) using the following primers to generate a double stranded SBCE oligonucleotide: forward primer, 5′-CCA CAG **TGT CTG** GTA TAT CCA CAG **TGT CTG** GTA TAT TG-3′; reverse primer, 5′-CAT GGG TGT C**AC AGA C**CA TAT AGG TGT C**AC AGA C**CA TAT AAC TTA A-3′ (SBCE in bold letters). This construct was termed 2SBCEΔ(−1980). The appropriate sequence of deletion and Smad-binding constructs was confirmed by sequencing using pGL3basic-specific primers: forward primer, 5′-CTA GCA AAA TAG GCT GTC CC-3′, reverse primer, 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′.

Site Directed Mutagenesis of AhRΔ(−2510). The QuickChange site directed mutagenesis kit from Stratagene was used to produce a set of different pointmutated CAGA-box containing hAhRP constructs. According to the manufacturer's instructions, the oligonucleotides SBCE-1 (5′-CTA CTG TAA CCC ACA GT**T TT**T GGT ATA TTA TAG GGG CTA AAA TC-3′), SBCE-2 (5′-CTA CTG TAA CCC ACA GTG **AC**C GGT ATA TTA TAG GGG-3′), SBCE-FR (5′-GTA ACC CAC AGT GTC TGG TA**G CGC** ATA GGG GCT AAA ATC TTG C-3′), and their corresponding antisense oligonucleotides were used to mutate the SBCE recognition sequence and flanking region. The mutated bases are indicated in bold letters and were verified by automated sequencing (SDR Biotech Service Center, Oberursel, Germany).

Cell Culture and Treatment. The human cell lines A549 (lung carcinoma), HepG2 (hepatocellular carcinoma), and MDA-MB231 (breast carcinoma) were supplied by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). A549 and MDA-MB231 cells were cultured in DMEM (Sigma, Taufkirchen, Germany) with 2.5 g/l glucose, and 2 mM glutamine. HepG2 cells were cultured in RPMI 1640 (PAA) and 3.7% (w/v) $NaHCO_3$. Media were supplemented with 10% FCS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained under standard conditions at 37°C in 6.5% CO_2. Cells were treated with 100 pM TGF-β_1 in media containing 5% BMS (v/v) instead of 10% (v/v) FCS. TGF-β_1 (80 nM stock solution) was dissolved in 4 mM HCl/0.1% (w/v) bovine serum albumin. Control cells received the respective solvent vehicle.

Nuclear Extracts. Nuclear extracts were prepared from control and TGF-β_1-treated HepG2 cells. Cells were harvested 30 to 60 min after treatment and processed according to Dennler et al. (1998). Confluent cells from a 75-cm^2 flask were washed with PBS and scraped. Cells were suspended in 2 ml of ice-cold buffer A (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 125 nM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM $(NH_4)_2MoO_4$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and were allowed to swell on ice for 10 min. They were then lysed by 30 strokes with a Teflon pestle. Nuclei were pelleted by centrifugation and resuspended in 250 μl of buffer B (buffer A, 420 mM NaCl, and 20% glycerol). The nucleus membrane was lysed by 15 strokes with a Teflon pestle. The resulting suspension was agitated for 30 min at 4°C. After centrifugation, the buffer of supernatant was exchanged (buffer C: EMSA buffer without spermidine and poly dI-dC) by gel filtration using NAP5 Pharmacia columns according to the manufacturer's instructions. Nuclear extracts were aliquoted and stored at −80°C until use.

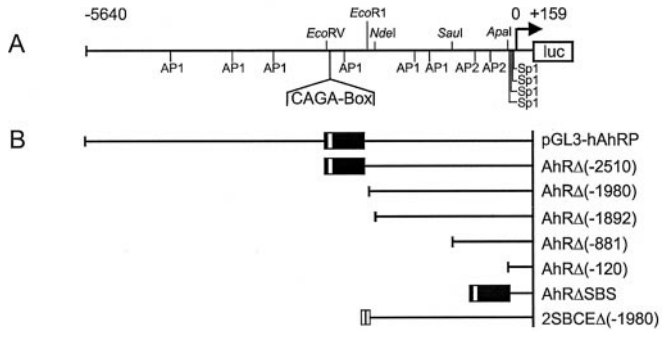

Fig. 1. Diagram of 5′-flanking region and deletion mutagenesis of human AhR. A, scheme of 5′-flanking region of human AhR: 5799 bp encompassing 5640-bp upstream transcriptional start site (indicated by arrow) and 159-bp untranslated region cloned into a luciferase (luc) reporter vector. Position of recognition sites for transcription factors AP1, AP2, and Sp1, and Smad-binding CAGA-box are presented. Endonucleases used for generation of deletion constructs are in italics. B, set of deletion constructs used in this study. Deletion constructs were prepared as described under *Experimental Procedures* and verified by sequencing. The solid bar represents the TGF-β-responsive sequence (−2510 to −1980), the open bar represents the Smad-binding core element/CAGA-box (5′-CAGACA-3′).

Electrophoretic Mobility Shift Assays (EMSA). The oligonucleotide was end-labeled with [γ-^{32}P]dATP using the T4 Polynucleotide kinase labeling kit (Pharmacia, Heidelberg, Germany). Binding reactions containing 12 μg of nuclear extracts and 10 to 20 fmol of labeled SBCE oligonucleotide (for sequence, see *Generation of Deletion Constructs*) were performed for 30 min at 37°C in 30 μl of binding buffer C [20 mM HEPES, pH 7.9, 30 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.8 mM KP_i, 20% glycerol, 4 mM spermidine, and 3 μg of poly(dI-dC)]. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5x TGE (25 mM Tris-base, 190 mM Glycine, 1 mM EDTA, pH 8.3).

Transfection Experiments. Cells (1.5–2 × 10^5/well) were seeded onto six-well plates and maintained for 16 h in 10% FCS (v/v) supplemented media under standard conditions. Cells were then transiently transfected with 1 to 2 μg of AhR-promoter constructs and 0.15 μg of pRL-TK Renilla luciferase control plasmid using 5 to 10 μg of the cationic lipopolyamine Transfectam (Promega) in 1 ml of DMEM/5% BMS (v/v) or RPMI/5% BMS (v/v) according to the manufacturer's instructions. The cells were incubated with the DNA/liposome mixture for 5 h and subsequently treated with 100 pM TGF-β_1 in DMEM/5% BMS (v/v) or RPMI/5% BMS (v/v) for 24 to 40 h or the respective vehicle control followed by cell lysis in 350 μl of passive lysis buffer. Luciferase activities in cell lysates were determined using the dual-luciferase assay system in a Berthold Multi-Bioluminat LB 9505C luminometer. Finally, firefly luciferase activity was normalized to Renilla luciferase activity.

RT-PCR. Total RNA was prepared using the RNA preparation kit from Roche followed by treatment with the Reverse Transcription System according to the manufacturer's instructions, including DNaseI digestion. PCR amplifications were performed using a DNA thermal cycler (Biometra, Göttingen, Germany) for the indicated cycles with the following profile: 4 min at 94°C before the first cycle, 1 min for denaturation at 94°C, 1 min for primer annealing, 30 sec to 1.5 min for primer extension at 72°C, and 7 min at 72°C after the last cycle. The following annealing temperatures and cycle numbers were used for gene-specific amplification: β-actin 60°C, 24 cycles; AhR 61°C, 28 cycles (Döhr et al., 1997); TGIF, forward primer, 5′-CTG AGA AAG GAT GGC AAA GAT-3′, reverse primer, 5′-TGG AGT AGG GGG AGG AGT GTT-3′, 57°C, 24 cycles; p300, forward primer, 5′-ATG GGT CAA CAG CCA GCC CCG CAG GTC-3′, reverse primer, 5′-TGG GTC AGG TAG AGG GCC ATT AGA AGT-3′, 68°C, 28 cycles.

Results

Oligonucleotide Sequence and Luciferase Activities of AhR-Promoter and Deletion Constructs. The sequence analysis of a 5.8-kb 5′-region of AhR gene revealed identity to the published sequence of AhR-promoter (GenBank Accession number 001621). No TATA box was found within the promoter, and the human AhR-promoter displays characteristics of a TATA-less promoter. The basal promoter region is GC-rich and contains multiple Sp-1 binding sites. Additionally, several AP-1 and AP-2 binding sites were identified. A putative Smad-binding core element was found at position −2495 to −2490 in the AhR-promoter (Fig. 1A). To

assess basal promoter activity of 5′-flanking region of the *hAhR* gene, the full-length fragment and different deletion constructs (Fig. 1B) were transfected into A549 and HepG2 cells. The luciferase activities of full-length pGL3-hAhRP and deleted promoter constructs were about 50- to 1100-fold higher than background, defined as the activity of the promoterless pGL3basic plasmid. Luciferase activities of pGL3-hAhRP and its different deletion constructs in transfected A549 and HepG2 cells are shown in Fig. 2. Generally, the luciferase activities in transfected HepG2 cells were about 5- to 10-fold higher than in A549 cells. In A549 cells, the constructs AhRΔ(−2510) and AhRΔ(−1980) exhibited similar luciferase activities compared with the full-length construct, whereas luciferase activities decreased dramatically in A549 cells when nucleotides upstream −1980 were removed (Fig. 2, open columns). The luciferase activities of AhRΔ(−1892), AhRΔ(−881) and AhRΔ(−120) constructs were about 20-fold lower compared with the activity of full-length promoter. In HepG2 cells, the luciferase activities of AhRΔ(−1892), AhRΔ(−881), and AhRΔ(−120) constructs were only 3- to 4-fold lower compared with the full-length promoter. The results of transfection studies indicate a cell-specific regulation of AhR expression and suggest that a region upstream −1892 is essential for basal expression of AhR.

Effect of TGF-β on hAhR-Promoter Activity and Identification of TGF-β-Responsive Region. To investigate the effect of TGF-β on hAhR-promoter activity, A549 and HepG2 cells were transfected with pGL3-hAhRP and then treated with increasing concentrations (0–200 pM) of TGF-β for 40 h. Although TGF-β led to dose-dependent decrease of luciferase activities in A549 cells, the luciferase activities were enhanced in TGF-β-treated HepG2 cells (Fig. 3). Treatment of cells with 200 pM TGF-β resulted in a reduction of luciferase activity in A549 by 45%, whereas in HepG2 cells, the promoter activities were elevated by a factor of ~3 under the same conditions. These results illustrate a cell-specific action of TGF-β on AhR expression. For identification of a TGF-β-responsive region within the AhR-promoter, A549 cells and HepG2 cells were transfected with different promoter constructs and treated with 100 pM TGF-β for 40 h. The analysis (Fig. 4) showed that a TGF-β-responsive region is located between nucleotides −2510 and −1980, and the TGF-β responsiveness was lost in constructs lacking the promoter region upstream of nucleotide −1980 (Fig. 4). Sequence analysis of this TGF-β-responsive promoter fragment revealed a putative 5′-CAGACA-3′-box at position −2490, which has been identified by Jonk et al. as an essential core feature of the Smad 3-binding element (Jonk et al., 1998). To investigate whether the effect of TGF-β on AhR-promoter is mediated by an SBS, we fused one copy of SBS (region −2510 to −1980) to the 5′-end of the AhRΔ(−120) minimal promoter (for an overview, see Fig. 1) and tested the resulting AhRΔSBS construct in A549 and HepG2 cells. The transfection data (Fig. 4) show that the SBS can confer the inhibition of luciferase activity in A549 cells or the stimulation of luciferase activity in HepG2 cells. Furthermore, two copies of SBCE were cloned upstream of AhRΔ(−1980), which originally lacks TGF-β responsiveness. The resulting 2SBCEΔ(−1980) construct mediates TGF-β-induced up-regulation in HepG2 cells and down-regulation in A549 cells (Fig. 4). To analyze whether the SBCE is involved in TGF-β mediated modulation of AhR transcription, site-directed mutagenesis of the core element was performed. Point mutations of the CAGA-like reverse complementary 5′-TGTCTG-3′ sequence reduced TGF-β-induced luciferase activities to control levels whereas point mutation within the CAGA 3′-flanking regions had only a minor effect on luciferase activities (Table 1). These results indicate that the 5′-flanking region of the AhR gene contains a TGF-β-responsive site and suggest that Smads are involved in TGF-β-dependent modulation of AhR expression.

Effect of Smad Proteins on AhR-Promoter Activity. To assess whether Smad proteins can modulate AhR-promoter activity, Smad 2 or Smad 3 expression plasmids (kind gift of G. Gross, GBF, Braunschweig, Germany) were co-transfected with AhRΔ(−2510), AhRΔ(−1980) or pGL3-hAhRP in A549 cells. As shown in Fig. 5A, coexpression of increasing amounts of Smad 2 and Smad 3 resulted in marked repression of AhRΔ(−2510) luciferase activities. Both proteins exhibited identical inhibitory activities (Fig.

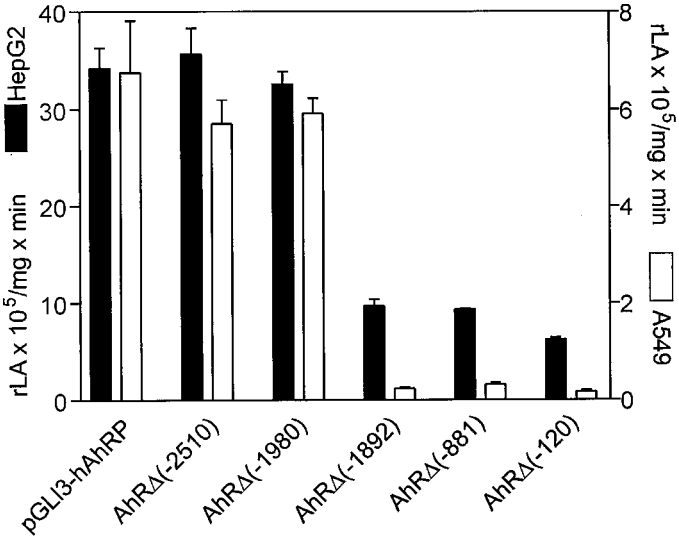

Fig. 2. Constitutive activity of human AhR-promoter full-length and deletion constructs in human hepatoma cells HepG2 and human lung cancer cells A549. AhR-promoter constructs were transiently transfected into HepG2 (1 μg/well, solid bars) and A549 (2 μg/well, open bars) cells. Relative luciferase activity (rLA) was determined after 40 h as described under *Experimental Procedures*. Bars represent the mean of luciferase activity ± S.D. (n = 3) normalized to protein content. For construct overview, see Fig. 1B.

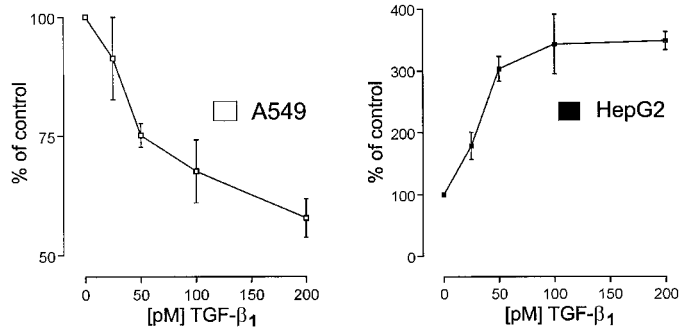

Fig. 3. Dose-dependent effect of TGF-β on hAhR-promoter activity. A549 (□) and HepG2 (■) cells were transiently transfected with pGL3-hAhRP (A549 2 μg, HepG2 1 μg) and treated with the indicated concentrations of TGF-β. Relative luciferase activity was determined after 40 h as described under *Experimental Procedures*. Bars represent the mean of luciferase activity ± S.D. (n = 3) normalized to protein.

5A), and similar results were obtained when the full-length promoter construct pGL3-hAhRP was cotransfected (data not shown). Smad 2 and Smad 3 had no influence on AhRΔ(−1980) transcription (Fig. 5A). Transfection of Smad 4 alone resulted in the same inhibitory effect on AhRΔ(−2510) activity as Smad 2 and Smad 3 (Fig. 5B). When Smad 4 was cotransfected together with Smad 2 or Smad 3, the inhibitory activities were slightly increased, indicating a cooperative function on AhR expression (Fig. 5B). TGF-β did not further influence the Smad activities (data not shown). Thus, transient transfection of Smad 2 or Smad 3 and in combination with Smad 4 led to a strong repression of AhR-promoter transcription in A549 cells.

TGF-β Induces Binding of a Nuclear Protein to SBCE. To investigate whether TGF-β induces binding of nuclear proteins to the putative Smad-responsive region of AhR-promoter, HepG2 cells were treated with 100 pM TGF-β for 30 and 60 min and nuclear proteins were extracted for EMSA. The double-stranded oligonucleotide probe SBCE was end-labeled with ^{32}P and incubated with the nuclear protein extracts. As shown in Fig. 6, TGF-β induces binding of nuclear proteins to the SBCE oligonucleotide in a time-dependent manner. The specific complex could be identified by displacing the label with an excess of unlabeled probe (Fig. 6, lane 5). However, when performing supershift analyses with anti-Smad antibodies, we failed to detect a retarded band (data not shown).

TGIF Regulates TGF-β- and Smad-Dependent Transcription of AhR. Recently, it was reported that the Smad corepressor TGIF and the coactivator p300 tune TGF-β signaling (Wotton et al., 1999). Therefore we were interested whether TGIF is involved in TGF-β-mediated down-regulation of AhR expression. At first, we studied the TGIF and p300 mRNA expression in A549, HepG2 and MDA-MB231 cells. The latter cell line was selected because previous studies have shown that the AhR expression is unaffected by TGF-β treatment (Döhr and Abel, 1997). As shown in Fig. 7A, A549 cells and HepG2 cells exhibited similar pattern of TGIF expression, whereas the amount of TGIF mRNA in MDA-MB231 cells was considerably lower compared with A549 cells. In contrast, the expression of p300 was lowest in A549 cells, intermediate in HepG2 cells, and highest in MDA-MB231 cells (Fig. 7A). From these mRNA-expression studies, we suggest that the low level of TGIF expression in MDA-MB231 cells might be responsible for the lacking response of

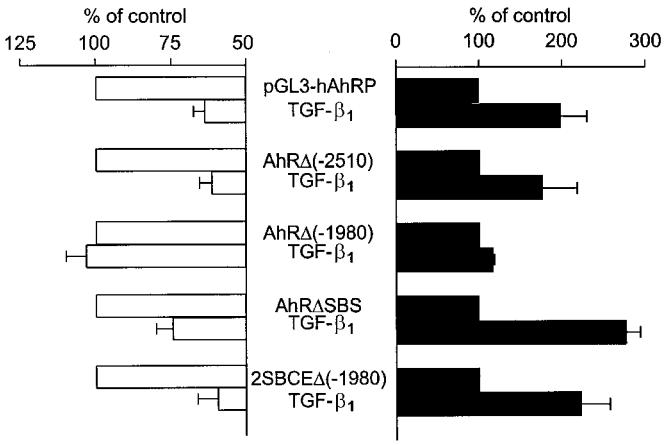

Fig. 4. Cell-specific effect of TGF-β on different deletion constructs. A549 (open bars) and HepG2 (solid bars) cells were transiently transfected with equimolar amounts of the designated deletion constructs as described under *Experimental Procedures* and treated with 100 pM TGF-β for 40 h where indicated. Means of three independent experiments are given.

TABLE 1

Effect of SBCE mutagenesis on TGF-β_1 responsiveness.

HepG2 cells were transiently transfected with 1 μg of the indicated constructs and treated with 100 pM TGF-β for 40 h. Promoter activity was determined as described under *Experimental Procedures* (mean ± S.D.; $n = 3$).

The core motive is underlined. Mutated bases are indicated in bold letters.

	Relevant Motive	Relative TGF-β_1 Responsiveness
AhRΔ(−2510)	5′-CAG<u>TGTCTG</u>GTATATTAT-3′	1[a]
SBCE-1Δ(−2510)	5′-CAG**TTTT**GGTATATTAT-3′	0.53 ± 0.08
SBCE-2Δ(−2510)	5′-CAGT**GACC**GGTATATTAT-3′	0.58 ± 0.07
SBCE-FRΔ(−2510)	5′-CAGTGTCTGGTA**GCGC**AT-3′	0.78 ± 0.15

[a] The relative luciferase activity in untreated HepG2 cells transfected with AhRΔ(−2510) was 0.4 to 0.5.

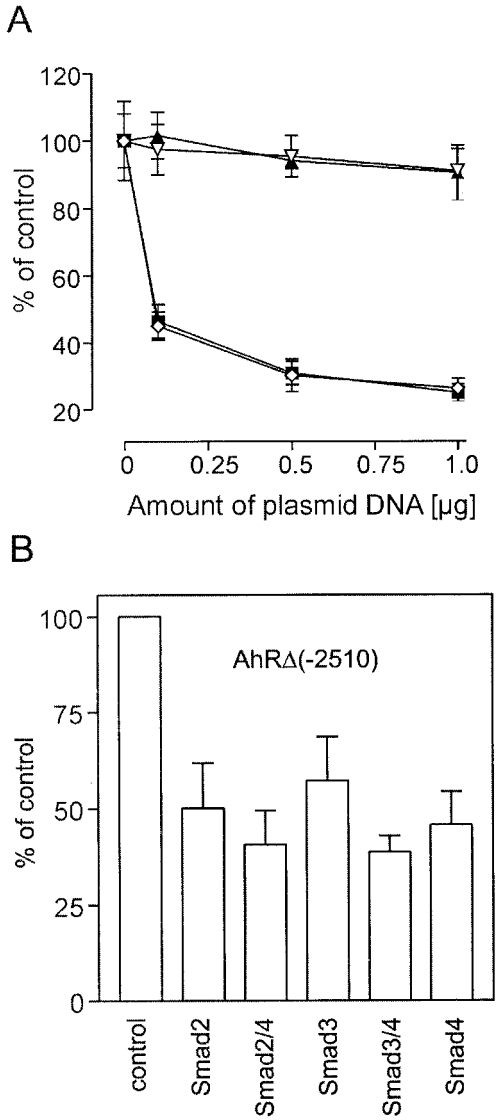

Fig. 5. Smad proteins repress TGF-β–dependent hAhR-promoter activity in human lung cancer cells A549. A, 2 μg of the TGF-β-responsive AhRΔ(−2510) or TGF-β-nonresponsive AhRΔ(−1980) construct were transiently cotransfected with the indicated increasing amounts of Smad 2 or Smad 3. B, 2 μg of the TGF-β-responsive AhRΔ(−2510) construct were cotransfected with 0.1 μg of each indicated Smad expression plasmid. Luciferase activity was determined 40 h after transfection as described under *Experimental Procedures*.

AhR-mRNA expression toward TGF-β in this cell line. To test this hypothesis, we examined the effect of ectopically expressed TGIF on AhR-promoter activity in MDA-MB231 cells. The cells were transiently transfected with a TGIF expression vector (Wotton et al., 1999) together with the AhRΔ(−2510) or AhRΔ(−1980) construct. Coexpression of TGIF resulted in a distinct repression of AhRΔ(−2510) luciferase activity, whereas AhRΔ(−1980) activity remained unaffected (Fig. 7B). Similar results were obtained in HepG2 cells transiently transfected with AhRΔ(−2510). TGIF strongly inhibited TGF-β-mediated stimulation of luciferase activity (Fig. 8A) This inhibition was accompanied by a decrease in AhR mRNA expression (Fig. 8B). TGF-β treatment of nontransfected HepG2 cells resulted in an increase in AhR mRNA content (Fig. 8B).

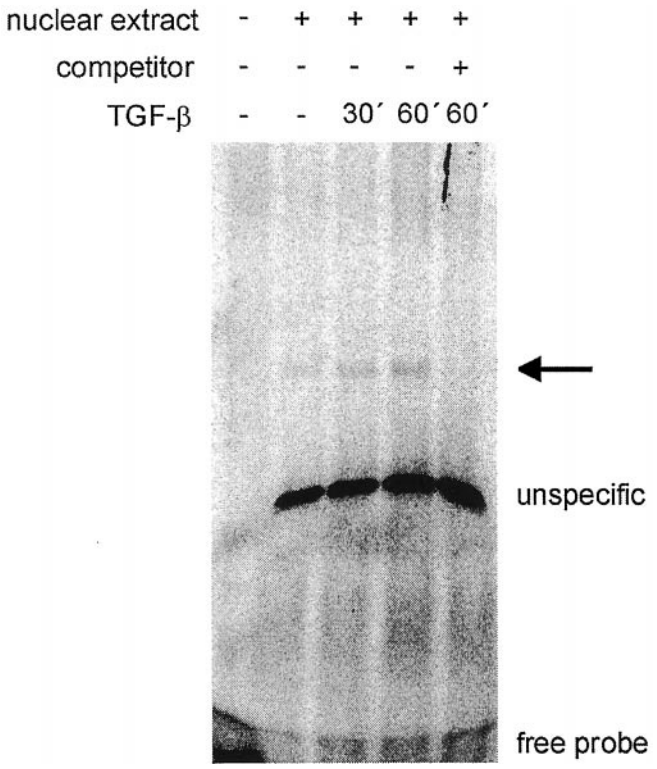

Fig. 6. TGF-β induces nuclear protein binding to the Smad-binding core element (SBCE). Nuclear extracts from TGF-β (100 pM) treated and untreated HepG2 cells were used for EMSA. EMSA was performed using a double-stranded, ^{32}P-labeled oligonucleotide containing two CAGA-box core sequence. The band corresponding to the specific TGF-β-induced complex is indicated by an arrow. Excess (100-fold) of an unlabeled oligonucleotide was added as competitor.

Discussion

In the present study, we show that the constitutive expression of human AhR varies as a function of cell type, and that TGF-β can modulate the expression of the AhR gene in a cell-specific fashion. In addition, we have identified a *cis*-acting element in the human AhR-promoter responsible for modulation of AhR expression by TGF-β. A tissue- and cell-specific regulation of AhR expression has been published from AhR-promoter analysis in murine cell lines of different origin. Like its murine ortholog, the human AhR-promoter bears multiple transcription initiation sites that are clustered in a GC-rich region and contains neither TATA nor CCAAT boxes (Schmidt et al., 1993; FitzGerald et al., 1996; Fitzgerald et al., 1998). The GC-rich region includes four consensus sequences for Sp-1 bindings sites, which seem to be necessary for basal expression of the AhR-promoter construct. However, in contrast to findings with the murine AhR-promoter (FitzGerald et al., 1996), an additional regulatory domain seems to be important for maximum constitutive expression of human AhR gene. A 88-bp region spanning from −1980 to −1892 in the human promoter is required to ensure maximum constitutive expression. In addition to this regulatory domain, consensus sequences for six AP-1 and two AP-2 binding sites could be identified, which suggests that other signaling pathways could be important for controlling AhR expression.

In this study, we have identified a TGF-β-sensitive DNA element within the human AhR-promoter, termed CAGA-box, and several lines of evidence support the idea that the CAGA-box is crucial for TGF-β signaling on the AhR gene.

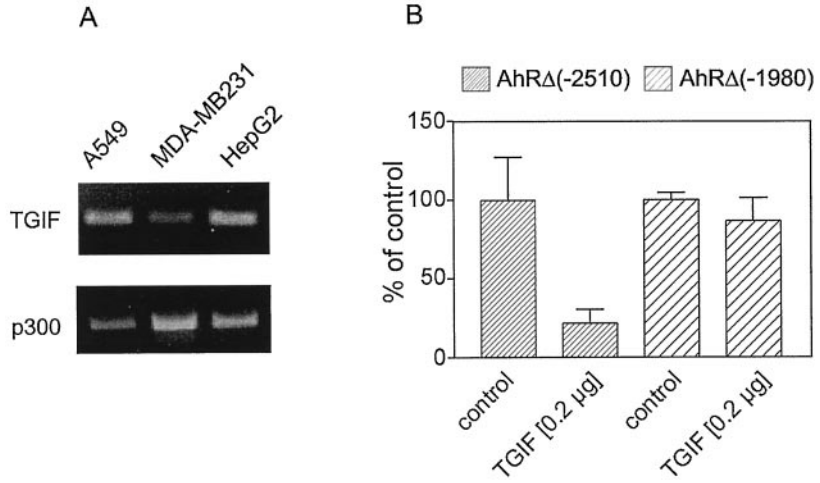

Fig. 7. A, basal expression of TGIF and protein 300 (p300) in different cell lines. RT-PCR was carried out on total RNA (1 μg) extracted from A549, MDA-MB231, and HepG2 cells (90% confluence) using TGIF- or p300-specific oligonucleotides. Results are representative of three separate experiments. B, TGIF inhibits the basal expression of TGF-β-dependent hAhR-promoter activity. MDA-MB231 cells were transiently cotransfected with 2 μg AhRΔ(−2510) or AhRΔ(−1980) and 0.2 μg of TGIF expression plasmid. Luciferase activity was determined 40 h after transfection as described under *Experimental Procedures*.

First, these sequences, when fused to luciferase reporter gene, mediate a transcriptional response toward TGF-β. Second, AhR deletion constructs lacking this DNA element did not display this response; third, the modulation of CAGA sequences by site directed mutagenesis abolished the TGF-β effect on AhR expression. The involvement of the CAGA-box in TGF-β-mediated transcriptional effects was shown for PAI-1 (Dennler et al., 1998) and JunB gene (Jonk et al., 1998), and CAGA sequences have been identified in promoters of various TGF-β-inducible genes, such as TGF-β_1, human α2(I) collagen, and human germline Igα constant region (Dennler et al., 1998).

TGF-β signaling to nucleus is particularly mediated by Smad 2, Smad 3, and the common mediator Smad 4. Overexpression of Smad 2 or Smad 3 led to a marked suppression of AhR transcription in A549 cells. Smad 2 exhibited the same inhibitory activity as Smad 3 and coexpression of Smad 4 acted cooperatively. A CAGA-box seems to be necessary for Smad-mediated modulation of AhR expression because changes of AhR-promoter activities were not observed in studies with the CAGA-less AhRΔ(−1980) promoter construct. Smad 2 is structurally related to Smad 3, but a DNA binding activity of Smad 2 has not yet been reported. Nuclear extracts from untreated and TGF-β-treated HepG2 cells exhibited constitutive and TGF-β-inducible binding activity toward a CAGA-box-containing probe, but obviously anti-Smad 2- or Smad 3-antibodies did not recognize the complex because no supershifted complex was observed (data not shown). Possibly, the Smad-DNA complex is associated with additional factors that render the Smad proteins inaccessible to the respective antibody. Thus, our data cannot rule out the possibility that the Smad-containing complex cooperates with other transcription factors that bind to the CAGA-box and modulate the rate of transcription of the AhR gene. Such cooperative action of Smad 3/Smad 4 complexes has been found for TGF-β–induced AP-1 activation through interaction of Smad 3 with c-Jun and c-Fos (Zhang et al., 1998).

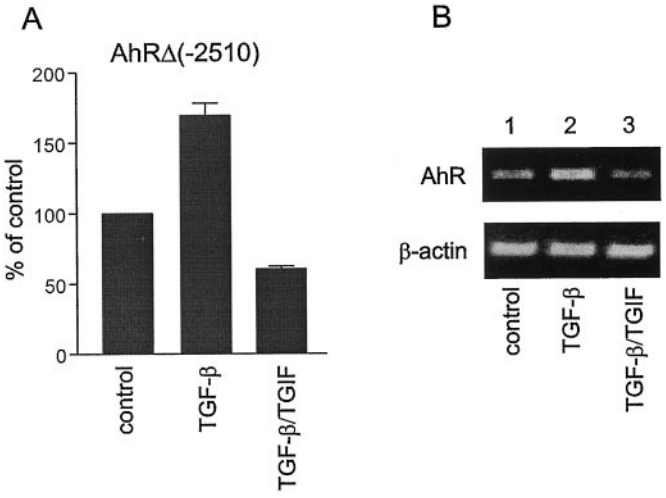

Fig. 8. A, TGIF inhibits TGF-β-induced AhR-promoter activity. HepG2 cells were transiently transfected with 1 μg AhRΔ(−2510) or cotransfected with 1 μg AhRΔ(−2510) and 0.2 μg TGIF expression plasmid. After transfection, cells were treated with 100 pM TGF-β for 40 h and promoter activity was determined as described under *Experimental Procedures*. B, Effect of TGF-β and TGIF on expression of hAhR mRNA in HepG2 cells. RT-PCR was carried out on total RNA (1 μg) extracted from 90% confluent cells treated with 100 pM TGF-β for 40 h. Lane 1, untreated; lane 2, 100 pM TGF-β treatment; lane 3, TGF-β treatment after transfection with 0.2 μg TGIF expression plasmid. β-Actin was included as internal control. Results are representative of three separate experiments.

The effect of TGF-β on the promoters of PAI-1 and JunB was specifically restricted to the Smad 3/Smad 4 pathway, whereas in our study, Smad 2 and Smad 3 exhibited similar effects on AhR transcription. This indicates a possible difference in TGF-β-mediated transcriptional regulation between AhR and PAI-1 or JunB, respectively. Smad 2 and Smad 4 were found to participate with FAST-1 in an active DNA-binding complex termed activin response factor (ARF). FAST-1 is the principle DNA-binding component of ARF and Smad 4 was shown to promote the binding of ARF complex. (Chen et al., 1997; Liu et al., 1997). There are two CAGA-like sequences flanking the 6-bp repeats of ARF to which FAST-1 binds. But it is still unknown whether Smad 2 or Smad 4 cooperate with FAST in binding these CAGA-like sequences. In HepG2 cells, FAST-1 does not seem to be expressed and transient transfection with FAST-1 had no effect on basal and TGF-β-induced transcription of a CAGA reporter (Chen et al., 1997). Hence, it seems unlikely that FAST-1 is involved in mediation of TGF-β-induced changes of AhR transcription.

The interaction of Smad proteins with the transcription machinery is complex, and various auxiliary factors like p300, TGIF, or Evi-1 have been found to determine the cell-specific actions of TGF-β-activated Smad proteins on target genes (Verschueren and Huylebroeck, 1999). It has been proposed that the cellular levels of transcriptional coactivators p300 and corepressor TGIF are discriminants for either positive or negative TGF-β signaling (Wotton et al., 1999). As shown here, TGIF is highly expressed in A549 cells and TGF-β was found to down-regulate the AhR expression in this cell line. MDA-MB231 cells exhibited significant levels of p300 mRNA, whereas the expression of TGIF was low. This cell line was found to lack TGF-β-induced effects on genes that in other cell lines are negatively regulated by TGF-β such as c-myc, cyclin A and AhR (Döhr and Abel, 1997). In contrast, HepG2 cells expressed high levels of both p300 and TGIF. TGF-β induced AhR expression in this cell line. Transient overexpression of TGIF in MDA-MB231 and HepG2 cells suggests that the corepressor TGIF takes part in TGF-β-mediated down-regulation of the AhR gene. We did not study these interactions in detail, but it was recently found that both Smad 2 and Smad 3 can interact with TGIF, resulting in recruitment of histone deacetylases, thus forming a transcriptional repressor complex (Wotton et al., 1999).

Until today, there are only few hints on specific endogenous and exogenous regulators of AhR expression. Serum, platelet-derived growth factors, and tyrosine kinase phosphorylation were shown to induce the activity of AhR-promoter driven reporter gene in the murine 3T3 cell line (Vaziri et al., 1996). Treatment of murine MLE 15, 41–5, and F9 cells with TCDD, retinoic acid, cAMP, or TPA resulted in a repression of AhR transcription (FitzGerald et al., 1996). Recently, it was found that the histone deacetylase inhibitors *n*-butyrate and trichostatin A increase the murine AhR-promoter activity. Deletion analyses of the upstream region of the AhR gene attribute the inhibitory effects on histone deacetylase to a 167-bp region covering nucleotides −90 to +77 of the AhR gene promoter (Garrison et al., 2000; Garrison and Denison, 2000).

There is increasing evidence for a functional activity of

AhR in regulation of cell growth; e.g., the activation of AhR by TCDD has been shown to block estrogen-induced cell proliferation in MCF-7 cells by inhibition of Cdk2-, Cdk4-, and Cdk7-dependent kinase activities (Wang et al., 1998). AhR has been found to interact with $p27^{Kip1}$cyclin/Cdk inhibitor and inhibits proliferation of rat hepatoma 5L cells and fetal thymocytes (Kolluri et al., 1999). Recently, an interaction of AhR with the retinoblastoma protein was reported. Ectopical expression of AhR and retinoblastoma protein in human osteosarcoma SAOS2 cells demonstrated synergistic effects on repression of E2F-dependent transcription and on induction of cell cycle arrest (Puga et al., 2000).

Similar to AhR, TGF-β is implicated in numerous cellular processes from the early state of development to a variety of normal and abnormal cellular functions. One of the major biological events governed by TGF-β signaling is cellular growth. For example, TGF-β modulates the phosphorylation levels of nuclear transcription factors such as retinoblastoma protein, CREBP and c-Jun, and TGF-β affects various G_1 cell cycle components. TGF-β inhibits the expression of Cdk2- and Cdk4-kinase and cyclins E and D mRNA expression by elevating the expression of Cdk inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ (Choi et al., 1999; Nagahara et al., 1999; Depoortere et al., 2000).

Our study and data from gene targeting experiments imply a cross talk between AhR and the TGF-β signaling pathway but the mechanisms of interactions are still unclear. In livers of AhR null mice, a higher expression of TGF-β_1 and TGF-β_3 was found compared with wild-type animals (Zaher et al., 1998). AhR-deficient murine embryo fibroblast cells secreted higher levels of latent and active TGF-β protein into conditioned medium than cells derived from control animals (Elizondo et al., 2000). However, the findings that AhR null mice and control mice exhibit similar levels of TGF-β_1 and TGF-β_3 mRNA suggest that the receptor does not control TGF-β expression at the transcriptional level (Zaher et al., 1998). Thus, the role of AhR in maintaining TGF-β expression remains to be elucidated.

In conclusion, our data show that TGF-β regulates AhR transcription in a cell-specific manner by interaction with a TGF-β-responsive element located in the AhR-promoter region. Furthermore, it was found that Smad proteins, especially Smad 2, 3, and 4, mediate TGF-β signaling on the AhR and the transcriptional repressor TGIF seems to be involved in the down-regulating processes, which needs to be further clarified.

Acknowledgments

We thank Birgit Neumann for excellent technical assistance; Dr. Gerhard Gross (GBF, Braunschweig, Germany) for providing pcDNA3.1 Smad 2, 3, 4; and Dr. Joan Massagué for providing pCMV5/TGIF-Flag.

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