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HES-1, a Novel Target Gene for the Aryl Hydrocarbon Receptor

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ABSTRACT

Known mainly for its role as a toxin sensor, the aryl hydrocarbon receptor (AhR) complex is also involved in homeostasis regulation and differentiation processes and activated by xenobiotic compounds like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Hairy and Enhancer of Split homolog-1 (HES-1) is a key regulator not only in differentiation, but also in the cell cycle, and we show here that HES-1 is a new target gene for AhR regulation. HES-1 is up-regulated by TCDD both at protein and mRNA levels in T47D human mammary carcinoma cells. Actinomycin D experiments have shown that the AhR-mediated up-regulation of HES-1 mRNA is caused by transcriptional activation of the HES-1 gene, and we have identified a functional AhR response element (XRE) at −48/−42 in the upstream regulatory region of human HES-1. The HES-1 protein down-regulates expression of its own gene, and the HES element overlaps the XRE. Our data indicate that HES-1 and the AhR complex compete for binding to the composite HES/XRE element. Also, we have previously shown that HES-1 is down-regulated by the estrogen receptor ligand 17β-estradiol (E_2). Up-regulation of HES-1 expression is correlated with suppression of cell proliferation, and the E_2-mediated down-regulation of HES-1 therefore increases cell proliferation. It is known that TCDD exerts antiestrogenic action in breast tissue both in vivo and in vitro. Our observation that both the estrogen receptor and AhR signaling pathways regulate HES-1, but with opposing effects, suggests the existence of a new pathway by which AhR represses E_2-signaling.

The balance between cell proliferation and cell differentiation affects cell fate; therefore, the proteins involved in these key biological processes must be tightly regulated. Many of the transcription factors involved constitute an intricate network of proteins belonging to the basic helix-loop-helix (bHLH) superfamily of DNA binding transcription factors. The Hairy and Enhancer of Split homolog-1 (HES-1) is known to play a role in neuronal differentiation (Kageyama and Nakanishi, 1997) and belongs to the family of bHLH transcriptional repressors related to the *Drosophila melanogaster* Hairy and Enhancer of Split proteins. HES-1 also inhibits cell proliferation in neuronal cells (Castella et al., 2000) and colon and mammary cancer cell lines (Strom et al., 2000; Muller et al., 2002). Interestingly, HES-1 is regulated by estrogens and is thought to be involved in the mitogenic effect of estrogens in breast carcinoma cell lines (Strom et al., 2000).

The bHLH-PAS proteins constitute a relatively large and diverse family. The only ligand-activated protein in that family is the aryl hydrocarbon receptor (AhR). Known mainly as a toxin sensor, AhR is also involved in embryonic development and cell proliferation and differentiation, although the mechanisms and target genes involved are largely unknown. AhR is activated by xenobiotic compounds such as polycyclic aromatic hydrocarbons, furans, and dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Currently, AhR is the only receptor known to bind TCDD, and the specificity of TCDD in combination with its high potency makes it a prototypical AhR ligand in cell experiments (Puga et al., 2002). Upon ligand binding, AhR becomes nuclear, complexes with its heterodimerization partner ARNT (a member of the bHLH-PAS family), and then binds to specific DNA sequences termed xenobiotic responsive elements (XREs). Among the genes induced by AhR are those encoding phase I and II detoxifying enzymes (Rowlands and Gustafsson, 1997). In addition to regulating drug-metabolizing proteins, AhR regulates the expression of a variety of estrogen-inducible genes and counteracts the mitogenic effects of estrogen in, for example, the T47D human mammary carcinoma cell line (Fernandez and Safe, 1992).

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ABBREVIATIONS: bHLH, basic helix-loop-helix; HES-1, Hairy and Enhancer of Split homolog-1; PAS, Per, ARNT, and Sim protein family; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; E_2, 17β-estradiol; WT, wild type; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; BrdU, bromodeoxy-uridine; HERP, HES-related repressor protein; cdk, cyclin-dependent kinase; DCC, dextran-coated charcoal.

HES-1 binds to the 5′-CACGCG-3′ sequence ("class C site") with high affinity (Ohsako et al., 1994); interestingly, the binding site for the AhR complex is 5′-TNGCGTG-3′ (Rowlands and Gustafsson, 1997), which is almost identical to a class C site in its inverse orientation. The remarkable similarity between the DNA binding elements for HES-1 and the AhR-ARNT complex, respectively, combined with the fact that both HES-1 and AhR block estrogen-induced proliferation of T47D cells, impelled us to look for a potential cross-talk between HES-1 and AhR. We found that HES-1 is regulated by the TCDD-induced AhR complex; furthermore, by using T47D cells as a test system to characterize the regulation of this new target gene for AhR in further detail, we found that the regulation occurs at the transcriptional level. Because HES-1 and the AhR complex are coexpressed in many tissues, this cross-talk could have implications both in differentiation processes and in cell proliferation. We have focused on the effects on cell proliferation and have found that both 17β-estradiol (E_2) and TCDD regulate HES-1, but with opposing effects. This observation suggests the existence of a new pathway by which the AhR complex represses E_2-induced proliferation in human mammary carcinoma cells in culture.

Materials and Methods

Chemicals and Biochemicals. TCDD was a gift from Dr. S. Safe (Texas A&M University, College Station, TX). All other chemicals and biochemicals were the highest quality available from commercial sources.

Cell Culture. T47D human mammary carcinoma cells were routinely cultured in a 1:1 mixture of Ham's nutrient mixture F-12 and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% PEST (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were seeded on six-well plates 24 h before transfection and 72 h before RNA extraction in a 1:1 mixture of phenol red-free Dulbecco's modified Eagle's medium and Ham's F-12 containing 10% dextran-coated charcoal-treated fetal bovine serum and 1% PEST (DCC medium).

Transient Transfection. Transient transfection experiments using the LipofectAMINE 2000 (Invitrogen) reagent were performed as described by the manufacturer, and luciferase activity was measured using a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Plasmid Constructs. 3× HES/XRE-LUC contains three repeats of a HES/XRE oligonucleotide (−58/−32) 5′ of human HES-1, ligated into a BglII site in the pGl3 simian virus 40 promoter vector (Promega, Madison, WI) driving the luciferase gene. 3× HES/mXRE-LUC contains a mutated XRE. The pCDNA3 vector encoding HES-1 has previously been described (Strom et al., 1997). DN2HES-1 is modified from previously described DNHES-1 (Strom et al., 1997) and is similar to B*ΔSHES-1 (Castella et al., 2000). Wild type (WT), 5′-CA CGA GCC GTT CGC GTG CAG TCC CAG A-3′ and 3′-GTG CTC GGC AAG CGC ACG TCA GGG TCT-5′; mXRE, 5′-CA CGA GCC GTG CGC GTG CAG TCC CAG A-3′, and 3′-GTG CTC GGC ACG CGC ACG TCA GGG TCT-5′.

Nuclear Extracts and in Vitro-Translated Proteins. Nuclear extracts were prepared as described by Schreiber et al. (1989). Recombinant phuAhR and pGEM7Z/ARNT (a gift from Dr. C. Bradfield, University of Wisconsin Medical School, Madison, WI), containing human AhR and ARNT cDNA, respectively, were used in vitro to transcribe and translate the corresponding protein in a TnT reticulocyte lysate system (Promega). Equal volumes of lysate containing the AhR and ARNT complex were transformed with 100 nM TCDD for 2 h at room temperature.

Cell Proliferation Assay. Cells were grown on 24-well plates in 5% DCC medium and treated with various compounds for 4 days before analysis of cellular ATP, which is proportional to the cell number. ATP was extracted from cells using 5% trichloroacetic acid, and the ATP was quantified using an ATP-kit (BioThema, Haninge, Sweden) and luminometer (Berthold Technologies) (Lundin, 2000).

Western Blot. Nuclear T47D extract (100 μg) resolved on a 12% SDS-PAGE gel was transferred onto a nitrocellulose filter (Amersham Biosciences Inc., Piscataway, NJ) and blocked with 5% milk powder in phospate-buffered saline-Tween 20 (0.1%). HES-1 antibody (Strom et al., 2000), PCNA antibody (Santa Cruz), and the AhR antibody (Affinity BioReagents, Golden, CO) were used at a dilution of 1:1000. Rabbit and mouse horseradish peroxidase-conjugated secondary anti-IgG antibody (Amersham Biosciences Inc.) was used at 1:10,000. Antibody binding was visualized using Super Signal enhanced chemiluminescence (Pierce).

Electrophoretic Mobility Shift Assay and UV Cross-Links. Electrophoretic mobility shift assays (EMSAs) were performed using the 26-bp HES/XRE composite site (−57/−32) 5′ of human HES-1: ACG AGC CGT TCG CGT GCA GTC CCA GA. Ten picomoles of synthetic double-stranded oligonucleotide were end-labeled using [γ-^{32}P]ATP and a 5′-end labeling kit (Amersham Biosciences Inc.). In the binding reaction, in vitro-translated AhR-ARNT protein (2 μl of lysate) or nuclear extracts (20 μg) from various treatment groups were incubated with 0.50 μg of poly(dAdT) (Amersham Biosciences Inc.) for 15 min at room temperature. After addition of γ-^{32}P–labeled probe, the mixture was incubated for an additional 15 min at room temperature. In competition experiments, a 50-fold excess of unlabeled wild-type probe or unrelated probe (5′-ACA CTT ATG TGA GTC AGG GGC TGA GG-3′) was incubated with the lysate for 5 min at room temperature before addition of radiolabeled probe. The reaction mixtures were loaded onto a 5% polyacrylamide gel and electrophoresed at 225 V in 1× Tris-borate and EDTA. After gel drying, retarded protein-DNA complexes were visualized by autoradiography. For UV cross-linking, a 2× EMSA binding reaction was performed using a bromodeoxyuridine-substituted HES/XRE probe labeled with [α-^{32}P]dCTP exposed to short-wave UV light using a Stratalinker 2400 (Stratagene, La Jolla, CA).

RNA Extraction and cDNA Synthesis. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and digested with DNase I (Roche Diagnostics, Indianapolis, IN). Two micrograms of total RNA were then reverse-transcribed using the SuperScriptII reverse transcriptase (Invitrogen).

Real-Time PCR. cDNA (150 ng) was amplified in a real-time PCR using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 200 nM primer for HES-1, and 300 nM fluorigenic HES-1 probe. The reactions were performed in an ABI PRISM model 7700 sequence detector (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 min, and 60°C for 1 min. The sequences of HES-1 primers and probes were as follows: forward primer, AGG CGG ACA TTC TGG AAA TG; reverse primer, CGG TAC TTC CCC AGC ACA CTT; and probe, TGT GCT CAG CGC AGC CAT CTG C. The 18S rRNA (PDAR, Applied Biosystems) was used as a reference.

Statistical Analysis. Results are expressed as means ± S.D. for at least three separate determinations for each experiment. Statistical significance has been determined by usage of Student's t test, and the levels of probability are noted.

Results

The amount of HES-1 protein in human T47D mammary carcinoma cells was investigated by Western blot analysis using nuclear extracts from cells treated with 10 nM TCDD or vehicle alone. As shown in Fig. 1, the level of HES-1 protein was significantly increased in cells treated with 10

nM TCDD (Fig. 1, lane 2) compared with cells treated with vehicle only (Fig. 1, lane 1).

To clarify whether HES-1 regulation occurs at the transcriptional or translational level, we measured the level of HES-1 mRNA by real-time PCR. As shown in Fig. 2, TCDD significantly up-regulated the level of HES-1 mRNA after 2 h of treatment (Fig. 2A). To investigate the mechanism behind this effect, we treated the cells with actinomycin D, thereby blocking transcriptional initiation. Real-time PCR analysis revealed a HES-1 mRNA half-life of approximately 25 min (Fig. 2B), which is very similar to the 24.1-min half-life of HES-1 found in fibroblasts (Hirata et al., 2002). Cotreatment with actinomycin D and TCDD had no effect on the stability of HES-1 mRNA (half-life approximately 23 min, Fig. 2C). These results indicate that TCDD regulation of HES-1 mRNA is transcriptional; they also suggest the presence of XREs in the 5′-regulatory region of the human HES-1 gene. Indeed, an intact XRE was found at −48/−42 (Table 1).

We used the −48/−42 HES/XRE probe in gel mobility shift experiments to investigate the ability of TCDD-bound AhR complexes to bind to DNA. Figure 3A shows a typical gel shift experiment using in vitro-translated AhR-ARNT lysate transformed with 100 nM TCDD (lane 2) or incubated with DMSO only (lane 1). The results show that reticulocyte lysate enriched in AhR and ARNT proteins binds to the HES/XRE probe in a TCDD-inducible manner (compare lane 2 with lane 1). Moreover, the binding is specific because the specific TCDD-inducible band (see arrow) decreases in intensity after incubation with 50-fold excess of unlabeled probe (lane 3), whereas this TCDD-retarded band is not affected by coincubation with a 50-fold excess of unlabeled, unrelated probe (lane 4). To investigate whether the AhR-ARNT complex in T47D cells can also recognize the XRE element upstream of the HES-1 gene, we isolated nuclear extract of T47D cells treated with vehicle only (C) or with 10 nM TCDD (T) (Fig. 3B). A TCDD-induced nuclear protein complex bound to the XRE, as the arrow indicates. To reveal the identity of this complex, a UV cross-linking experiment was conducted. The

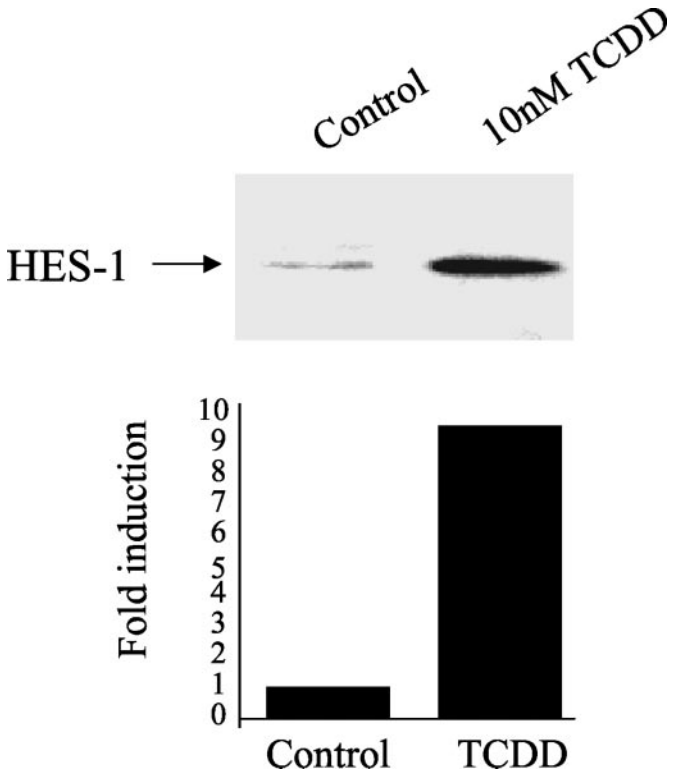
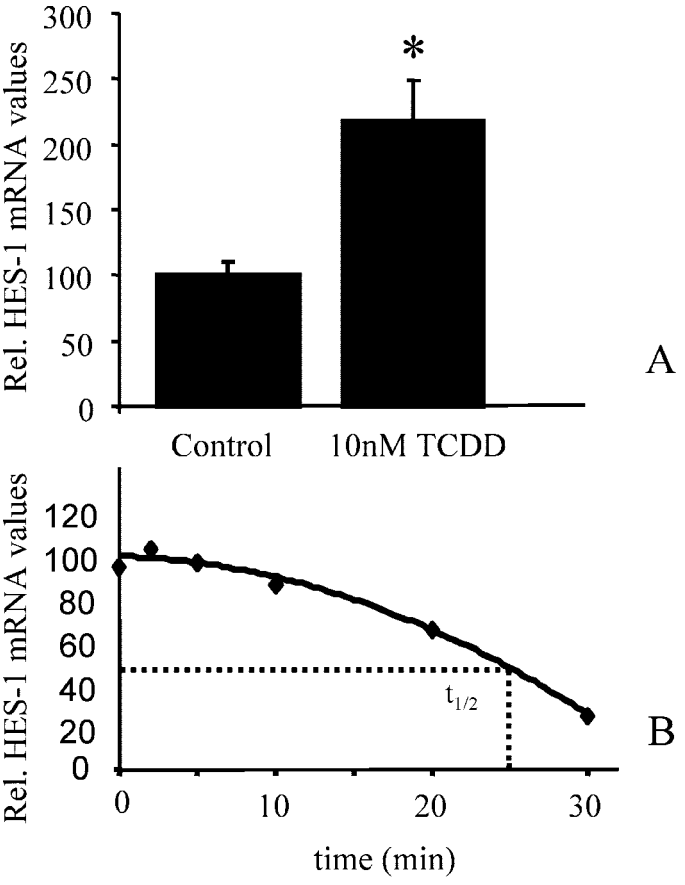


Fig. 1. TCDD treatment up-regulates the HES-1 protein. Nuclear extract (100 μg) from T47D cells treated with vehicle alone (0.1%, DMSO) or TCDD (10 nM) for 3 days was subjected to 12% SDS-PAGE. Western blotting of endogenous HES-1 in T47D cells was carried out using HES-1-specific antibody at a dilution of 1:1000. HES-1 expression levels were expressed quantitatively as bars using the NIH Image program.



	$t_{1/2}$
actinomycin D+vehicle	25 ±6min
actinomycin D+10nM TCDD	23 ±5min

C

Fig. 2. A, HES-1 mRNA is up-regulated by TCDD-treatment. The level of quantitative HES-1 mRNA in T47D cells was analyzed by real-time PCR, and the 18S rRNA was used as internal standard. At each time point, the amount of mRNA after TCDD treatment is shown relative to the control group receiving vehicle only. The asterisk represents a value that is significantly different ($P < 0.05$) from control values. B and C, the stability of HES mRNA is not affected by ligands. The half-life of HES-1 mRNA in T47D cells was investigated using real-time PCR with the 18S rRNA as internal control. Cells were treated with 10 μg/ml actinomycin D (B) or actinomycin D in combination with 10 nM TCDD (C) at the indicated time points.

TABLE 1

Composite HES/XRE sequence in the 5′-flanking region of the human *HES-1* gene

XRE	−48/−42	TCGCGTG
Inverse HES-1	−48/−42	TCGCGTG
HES/XRE	−48/−42	TCGCGTG
HES/mXRE	Transfection	CCGCGTG

thymidine bases in the DNA were replaced with bromodeoxyuridine (BrdU), and the proteins were then cross-linked to DNA after exposure to UV-light. The cross-linking made it possible to study the complex even after boiling the extracts for SDS-PAGE analysis. Incubation of cell extracts with a radiolabeled probe provides important information regarding the molecular size of the protein complex binding to DNA. Thus, by cross-linking control- or TCDD-treated nuclear T47D extracts with the [^{32}P]BrdU-XRE probe, a complex of approximately 220 kDa binding to the XRE in a TCDD-dependent manner was identified (Fig. 3C). The human AhR-ARNT complex migrates in this size range (Wang et al., 1995). To further confirm the identity of the XRE-binding proteins, a UV cross-linking experiment was conducted using a nonradioactively labeled BrdU-XRE probe followed by Western blotting using an AhR antibody. As shown in Fig. 3D, an AhR antibody signal appeared in the TCDD-treated extract but not in the control cell extract. Also, the AhR alone runs as a 105-kDa protein in a regular Western blot (Rowlands and Gustafsson, 1997), and the absence of this band in the UV-crosslinking experiments and the appearance of a 220-kDA band support the view that AhR has been covalently bound to ARNT and DNA.

To investigate whether the AhR complex is transcriptionally active when bound to the HES-1 XRE sequence, we made a reporter construct containing three repeats of HES-1 XRE fused to the reporter gene luciferase (HES/XRE-LUC). Transiently transfected T47D cells showed a more than 13-fold induction of luciferase activity after treatment with 10 nM TCDD (see Fig. 4A, left), indicating that the HES/XRE is functional. Interestingly, when mutating the XRE by exchanging the first T with a C (HES/mXRE-LUC) (Table 1), the TCDD-induced luciferase activity was almost eliminated (Fig. 4A, right), showing the importance of an intact XRE for the TCDD-effect. The HES-1 gene is auto-regulated; i.e., HES-1 protein represses the transcriptional activity of its own gene (Takebayashi et al., 1994). Notably, the binding sites for HES-1 and AhR overlap at the −48/−42 position (Table 1). To study whether HES-1 and the ligand-activated AhR complex compete for binding to the HES/XRE composite site, we changed the HES/AhR ratio either by over-expressing HES-1 or by eliminating endogenous HES-1 function. The DN2HES-1 plasmid encodes a dominant-negative HES-1 protein in which the repressor domain has been deleted and is mutated in the DNA binding domain. When this protein dimerizes with endogenous HES-1, it blocks the function of HES-1 (Strom et al., 1997). TCDD-treated T47D cells transfected with DN2HES-1 protein (Fig. 4B, lane 6) expressed the same level of HES/XRE-LUC luciferase activity as TCDD-treated cells transfected with empty vector (Fig. 4B, lane 2), whereas transfection of TCDD-treated T47D cells with a vector encoding WT HES-1 resulted in significantly lower HES/XRE-LUC luciferase activity (Fig. 4B, lane 4). Interestingly, the same pattern was observed in untreated cells transfected with empty vector, WT HES-1 expression vector, or DN2HES-1 (Fig. 4B, lanes 1, 3, and 5). Overall, these results indicate that HES-1 and AhR might compete for binding to HES/XRE and that the ratio between the two factors is an important determinant for the transcriptional activity of the HES-1 gene.

HES-1 is a novel target for E_2 regulation, and the current hypothesis is that HES-1 suppresses cell division. Thus, treatment of T47D cells with the mitogen E_2 results in a significant decrease in HES-1 protein correlating with increased proliferating activity (Strom et al., 2000). The mitogenic effects of E_2 treatment in human T47D mammary carcinoma cells can be antagonized by cotreatment with TCDD (Fernandez and Safe, 1992). When we repeated this experiment in our T47D cells, we could confirm these described opposite effects of E_2 and TCDD on cell proliferation (data not shown). Because of the antiestrogenic effects of TCDD, it could be anticipated that TCDD would increase the level of HES-1 protein; therefore, we performed a Western blot analysis in T47D cells. As shown in Fig. 5A, treatment with 0.1 nM E_2 (lane 2) resulted in an almost 20-fold decrease in HES-1 protein compared with cells treated with vehicle alone (Fig. 5A, lane 1), whereas combined treatment with 0.1 nM E_2 and 10 nM TCDD resulted in a less than 4-fold decrease in HES-1 protein compared with the control group (Fig. 5A, lane 3). The same nuclear extract was then analyzed for expression of PCNA, a marker of cell proliferation. Treatment with 0.1 nM E_2 increased the expression of PCNA

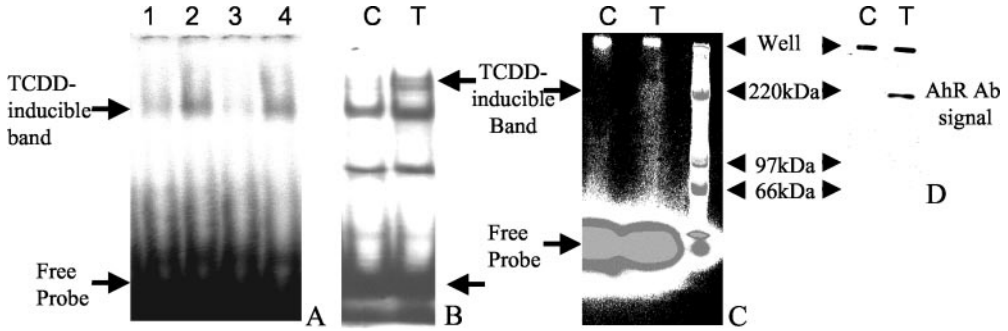


Fig. 3. A and B, a TCDD-inducible protein binds the XRE element present in the HES-1 flanking region. Electrophoretic mobility shift assays were performed using a 26-bp ^{32}P-labeled oligonucleotide containing the XRE site from the 5′-regulatory region of human HES-1. For the binding assays, 2 μl of in vitro transcribed and translated AhR and ARNT protein were loaded onto a 5% polyacrylamide gel after transformation with 100 nM TCDD (lane 2) or DMSO (lane 1) for 2 h at room temperature. For competition experiments, TCDD-transformed lysate was incubated with 50-fold excess of unlabeled HES/XRE probe (lane 3) or 50-fold excess of unlabeled unrelated probe (lane 4) (A). B, a TCDD-inducible retarded band is observed using T47D nuclear extracts (20 μg) from control (C) or 10 nM TCDD (T) treatments. C and D, UV cross-linking experiments identify the TCDD-inducible XRE-binding protein as the AhR complex. [^{32}P]BrdU-XRE was incubated with T47D nuclear extracts treated with vehicle alone (C) or 10 nM TCDD (T) for 2 h. Photoinduced cross-linking was carried out as described under *Materials and Methods*. Upon loading the samples onto an 8% SDS-PAGE gel, a 220-kDa band appeared in T (C). BrdU-XRE was incubated with T47D nuclear extract as described in B. After cross-linking, the samples were subjected to Western blot analysis using a human AhR antibody. A specific band appears in T at 220 kDa (D).

almost 10-fold (Fig. 5B, lane 2) compared with control cells (Fig. 5B, lane 1), whereas cotreatment of E_2 with TCDD decreased the PCNA signal to less than 6-fold (Fig. 5B, lane 3). The negative correlation between HES-1 and PCNA expression indicates that TCDD might antagonize the mitogenic effect of E_2 by up-regulating the level of HES-1.

Discussion

We have identified a novel gene regulated by TCDD in T47D cells, namely HES-1. This observation indicates the existence of a cross-talk between the bHLH factor, HES-1, and the bHLH-PAS proteins AhR and ARNT. An interaction between these two different families of bHLH factors opens up exciting possibilities in regard to developmental regulation, differentiation, and cell proliferation, because both HES-1 and the AhR complex are reported to play key roles in these biological processes.

In line with fibroblast cells, where the half-life of HES-1 mRNA is 24.1 min (Hirata et al., 2002), the half-life of HES-1 mRNA is relatively short, approximately 25 min (Fig. 2B). The short half-life of HES-1 allows a rapid tuning of the level of this protein in response to changes in environmental stimuli, an essential characteristic of a protein with a key role in many cellular functions.

Both AhR and ARNT contain a bHLH and PAS homology domain where the PAS domain and HLH motif are involved in dimerization, with the basic region determining DNA binding specificity (Rowlands and Gustafsson, 1997). HES-1 is a member of the Hairy-related bHLH proteins that confer transcriptional repression; the basic region in HES-1 recognizes the DNA motif, CACGCG, called the class C site (Ohsako et al., 1994). Interestingly, as shown in Table 1, the XRE present at −48/−42 upstream of the HES-1 gene contains a class C site in the inverse orienta-

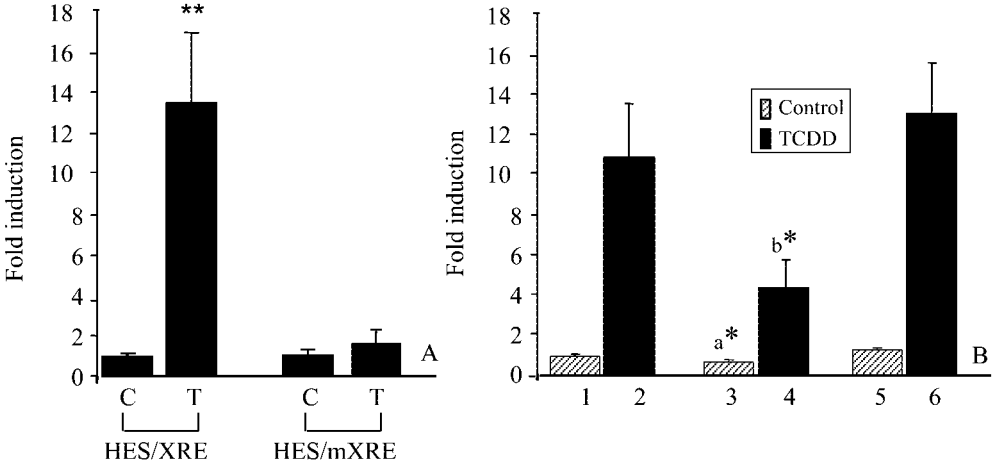


Fig. 4. A, the AhR complex binding to HES/XRE is transcriptionally active. T47D cells were transiently transfected with 3× HES/XRE-LUC (lanes 1 and 2) or 3× HES/mXRE-LUC (lanes 3 and 4), and treated with vehicle alone (C) or 10 nM TCDD (T) for 24 h before harvesting and analysis of luciferase activity. Results are represented as relative values and the cells transfected with 3× HES/XRE-LUC and treated with vehicle alone were set as reference. Significant ($P < 0.01$) induction by TCDD treatment compared with DMSO alone is indicated by **. B, HES-1 and the AhR complex bind to HES/XRE in a competitive manner. T47D cells were transiently transfected with 3× HES/XRE-LUC and 1 μg of HES-1-encoding vector (lanes 3 and 4) or 1 μg of DN2HES-1 encoding vector (lanes 5 and 6). PCDNA3 empty vector was used as control (lanes 1 and 2). Lanes 1, 3, and 5 were treated with vehicle only, whereas lanes 2, 4, and 6 were treated with 10 nM TCDD for 24 h before analysis. Significant ($P < 0.05$) reduction by HES-1 encoding vector compared with PCDNA3 empty vector for DMSO-treated cells (lanes 3 and 1)(a*) and for TCDD-treated cells (lanes 4 and 2)(b*) are indicated by an asterisk.

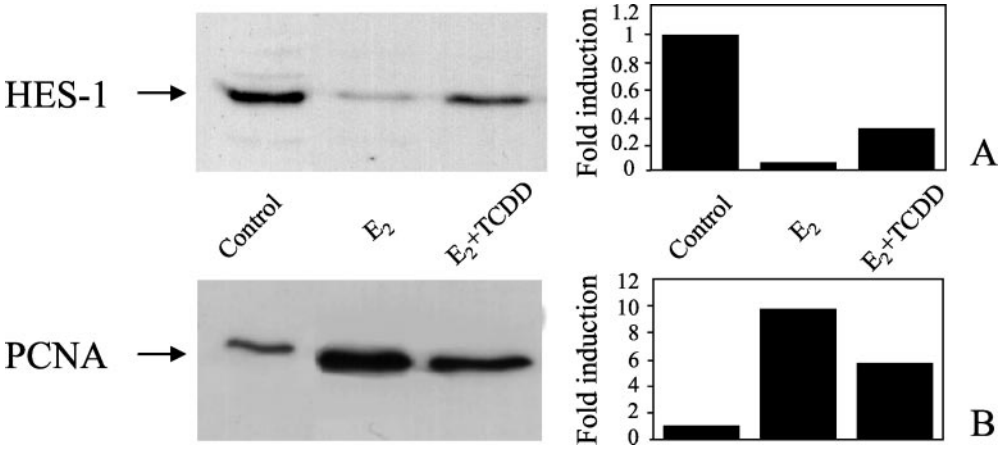


Fig. 5. TCDD counteracts the E_2-induced down-regulation of HES-1 protein. Nuclear extract (100 μg) from T47D cells treated with vehicle alone (0.1% DMSO), E_2 (0.1 nM), or a combination of E_2 (0.1 nM) and TCDD (10 nM) for 3 days was subjected to 12% SDS-PAGE. Western blotting of endogenous HES-1 in T47D cells was carried out using HES-1-specific antibody at a dilution of 1:1000 (A). The same extract was then subjected to analysis using the PCNA antibody at a 1:10,000 dilution (Fig. 5B). HES-1 and PCNA expression levels were expressed quantitatively as bars using the NIH Image program.

tion (−47/−42) and is thus a composite element with the potential to bind not only AhR, but also HES-1. Because AhR up-regulates transcription of HES-1 and HES-1 down-regulates its own gene (Takebayashi et al., 1994), competition between the two transcription factors for DNA binding is probably critical in regulation of the HES-1 gene. The overlapping response elements suggest that binding of one factor precludes binding of the other by steric hindrance. Also, the transfection data shown in Fig. 4B indicate that the ratio between HES-1 and the AhR complex is important, because over-expression of HES-1 can counteract the transcriptional activation of HES-1 mediated by the TCDD-bound AhR complex. Conversely, over-expression of a HES-1 protein deleted of its repressor domain, DN2HES-1, has no effect on AhR activity.

The recently discovered HERP (HES-related repressor protein) family of transcriptional repressors, also known as HEY/HesR/HRT/CHF/Gridlock, is closely related to the HES family (Iso et al., 2003). It has been reported that HES-1 and HERP can form heterodimers and that the HES-HERP heterodimer binds to the class C DNA sequence with higher affinity than the respective homodimers (Iso et al., 2001). The repression mechanisms of HERP seem to differ from those of the HES family in that the bHLH domain of HERP is sufficient for recruitment of a corepressor complex including NCoR, mSin3A, and HDACl (Iso et al., 2001), whereas the C-terminal YQPW motif plays a crucial role in the HES family by recruiting members of the groucho family of corepressors (Fisher et al., 1996). The HERP family currently consists of three members, of which HERP1 has been shown to interact with ARNT in a yeast two-hybrid screen. The HERP1-ARNT interaction inhibits DNA binding of the ARNT/EPAS-1 heterodimer to a HIF1-binding site in the vascular endothelial growth factor promoter (Chin et al., 2000). The observation, that the cross-talk between HES-1 and AhR-ARNT is not restricted to these specific proteins but might represent a more general phenomenon involving members of the Hairy bHLH family of transcriptional repressors interacting with members of the bHLH-PAS family, could have widespread implications for key cellular processes. Also, one could speculate that the existence of a composite HES/XRE binding site is not restricted to the HES-1 promoter but might occur in other genes involved in HES-1 or AhR signaling. Indeed, the *CYP1A1* gene that is strongly up-regulated by the liganded AhR-ARNT complex contains a composite HES/XRE binding site in the upstream regulatory region. It is therefore possible that HES-1 (or the HES/HERP heterodimer) might work as a transcriptional repressor on this gene by blocking DNA binding of the AhR-ARNT complex. We are currently investigating this exciting possibility.

E_2 and TCDD have opposing effects at the level of HES-1 protein, with E_2 down-regulating and TCDD up-regulating HES-1 levels. That TCDD and other AhR ligands possess antiproliferative potential has been observed both in vivo and in vitro in a variety of tissues and species. For instance, benzo[*a*]pyrene-resistant T47D cells containing nonfunctional AhR have a higher rate of cell proliferation than observed for wild-type cells at both basal and E_2-induced levels (Moore et al., 1996). TCDD inhibits E_2-induced growth of rat uterus (Romkes et al., 1987) and causes a decrease in the occurrence of spontaneous mammary and uterine tumors in female Sprague-Dawley rats (Kociba et al., 1978). So far, only a few proteins have been identified as AhR-regulated targets in the cell cycle. For example, the $p27^{kip1}$ cyclin-dependent kinase (cdk) inhibitor that keeps the cells quiescent in the G_1 phase is up-regulated by TCDD (Kolluri et al., 1999). Also, TCDD blocks E_2-induced cyclin D1- and cdk2- and cdk4-dependent kinase activities (Wang et al., 1998), and AhR interaction with the retinoblastoma protein might delay cell cycle progression by repressing E2F-dependent transcription (Wang et al., 1998; Puga et al., 2000). The identification of HES-1 as an AhR target gene raises the question of how HES-1 affects the cell cycle, and our future experiments will be aimed at identifying genes downstream from HES-1 signaling.

AhR is not the only receptor known to antagonize estrogen action. The retinoic acid receptor α has been shown to inhibit proliferation in human breast cancer cell lines (Raffo et al., 2000), and we have previously shown that treatment of MCF-7 human breast cancer cells with the retinoic acid receptor α ligand all-*trans*-retinoic acid inhibits E_2-induced cell proliferation by preventing E_2-mediated down-regulation of HES-1 protein (Muller et al., 2002). The mechanisms underlying the regulation of HES-1 are currently poorly understood, and although real-time PCR analysis in combination with actinomycin D experiments have shown that E_2 treatment regulates HES-1 at the transcriptional level (Muller et al., 2002), a DNA binding element that mediates the E_2-induced down-regulation of HES-1 transcription has yet to be identified.

The observation that HES-1 protein is up-regulated approximately 10-fold by TCDD, whereas HES-1 mRNA is up-regulated only 2-fold, reflects the fact that HES-1 protein down-regulates expression of its own gene. This negative autoregulatory loop means that there is no direct relationship between mRNA and protein levels. Furthermore, it is possible that TCDD treatment affects the stability of the HES-1 protein by an as-yet unidentified pathway. HES-1 protein is degraded by the ubiquitin-proteasome pathway (Hirata et al., 2002), and this pathway is also involved in E_2-induced down-regulation of the estrogen receptor (Nirmala and Thampan, 1995). Interestingly, the insulin receptor substrates 1 and 2, which are also degraded by the ubiquitin-proteasome pathway, are up-regulated by treatment with E_2 (Morelli et al., 2003), suggesting that the ubiquitination levels of proteins can be both up- and down-regulated by E_2 depending on the target protein. TCDD uses the ubiquitin-proteasome pathway to down-regulate AhR and estrogen receptor proteins (Wormke et al., 2003). So far, TCDD has only been reported to increase turnover of proteins, but it is possible that TCDD can act in some cases to decrease the level of ubiquitination of proteins and thereby stabilize them. Research in this field would provide essential information into whether, for example, TCDD uses this mechanism to regulate HES-1.

In conclusion, the data presented here show that a regulator of cell proliferation, HES-1, is controlled by both E_2 and TCDD with opposing effects. The antiproliferative activity associated with HES-1 suggests that it might work as a tumor suppressor, and one could envision that up-regulation of HES-1 by appropriately designed ligands might be exploited as a novel strategy to combat breast cancer. We are currently looking into the consequences of the cross-talk between HES-1 and AhR on development and differentiation,

because the two proteins seem to be temporally coexpressed in many tissues.

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