Cell Type-dependent Regulation of the Hypoxia-responsive Plasminogen Activator Inhibitor-1 Gene by Upstream Stimulatory Factor-2

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Transcriptional regulation of the plasminogen activator inhibitor type-1 (PAI-1) inhibits conversion of the proenzyme plasminogen to the active fibrin-degrading protease plasmin by binding and inactivating both tissue type and urokinase type plasminogen activators (1, 2). In addition to its inhibitory effect on fibrinolysis (3, 4), PAI-1 appears to be a multifunctional protein modulating proteolytic processes associated with prethrombotic events, hemorrhage and thrombus formation, which are positively correlated with increased PAI-1 plasma levels and hypoxic conditions. Hypoxia was shown to induce rat PAI-1 gene expression via binding of the hypoxia-inducible factor-1 (HIF-1) to hypoxia-responsive element-1 (HRE-1) and hypoxia-responsive element-2 (HRE-2) within the rat PAI-1 promoter (5). While HRE-2 was shown to bind HIF-1 with high affinity, HRE-1 functioned as a low affinity HIF-1 binding site and a high affinity binding site for upstream stimulatory factors (USFs) (6). USF-1 and USF-2 are members of the basic helix-loop-helix leucine zipper transcription factor family and were first identified by their ability to bind to the adenovirus major late promoter (7). USFs can function as homodimers consisting of either USF-1 or USF-2 or as heterodimers of USF-1/USF-2 through E-box core sequences thereby acting as transcriptional activators or repressors (8, 9).

We previously found that USF-2 and HIF-1 had opposite effects on PAI-1 gene expression in primary rat hepatocytes. While HIF-1 induced USF-2 inhibited rat PAI-1 expression by outcompeting HIF-1 from binding to the HREs (6).

Although conservation was observed for HIF-1 binding to the HRE in the human PAI-1 promoter (10), a number of differences between the rat and human promoter exist. Especially, sequence analysis of the human PAI-1 promoter revealed that the USF-2 binding site was absent from the human promoter. Instead, two classical E-box sequences named E4 and E5 (−566/−559 and −681/−674) were found that might function as putative USF binding sites.

Thus, due to these differences between the rat and human PAI-1 promoter, it is conceivable that USF-2 might have a distinct effect on human PAI-1 gene transcription. Therefore, we analyzed the function of USF-2 in context of the classical E-boxes and the HRE in human hepatoma (HepG2), rat (H4IIE) hepatoma cells, and primary rat hepatocytes. Our results indicate that USF-2 enhances human PAI-1 expression and that the HRE in the human PAI-1 promoter significantly contributes to USF-mediated PAI-1 expression without binding it directly. We further demonstrate that the classical E-boxes E4 and E5 bind USFs and are required for USF-mediated PAI-1 expression. Furthermore, the inducible effect of USF-2 on human PAI-1 gene transcription is dependent on the presence of the USF-specific region within USF-2 and appears to be cell type-specific.

EXPERIMENTAL PROCEDURES

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers.

Animals—Male Wistar rats (200–260 g) were kept on a 12 h day/night rhythm with free access to water and food. Rats were anesthetized with pentobarbital (60 mg/kg of body weight) prior to preparation of hepatocytes.

Cell Culture—Hepatocytes were isolated by collagenase perfusion. Cells (1 × 10⁶ per dish) were cultured in a normoxic atmosphere of 16% O₂, 79% N₂, and 5% CO₂ (by volume) in medium M199 containing 0.5 nm insulin, 100 nm dexamethasone as permissive hormones and 4% fetal calf serum for the initial 5 h of culture. Cells were then cultured in serum-free medium from 5 to 24 h at normoxia. Then, the medium was changed, and cells were cultured under normoxia or hypoxia (8% O₂, 87% N₂, 5% CO₂ (by volume)) for the next 24 h. HepG2 and H4IIE cells were cultured under normoxia in minimal essential medium supplemented with 10% FCS for 24 h. Then, medium was changed and cells were further cultured under normoxia or hypoxia.
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Plasmid Constructs—The pG3–hPAI-806 plasmid, containing the human PAI-1 promoter 5'-flanking region from -806 to +19, pG3–hPAI-806–HRem, pG3–hPAI-806–M4, pG3–hPAI-806–M5, and hPAI-806–M45 have been described previously (11). The pG3–rPAI-766 plasmid containing the rat PAI-1 promoter was described before (5). The human USF-2 and ΔTDU2 as well as U2ΔUSR and U2ΔES plasmids were a kind gift from Dr. A. Kahn and Dr. M. Raymondjean and Dr. M. Sawadogo, respectively, and all plasmids have been described previously (12–14).

RNA Preparation and Northern Analysis—Isolation of total RNA and Northern analysis were performed as described (15). Digoxigenin-labeled antisense RNAs served as hybridization probes; they were generated by in vitro transcription from PBS-USF-1 using T3 RNA polymerase and pBS-β-actin using T7 RNA polymerase and RNA labeling mixture containing 3.5 mM 11-digoxigenin-UTP, 6.5 mM UTP, 10 mM KCl, 1 mM MgCl2, 1 mM EDTA, 5% glycerol, 7 mM GTP, 10 mM CTP, 10 mM ATP. Hybridizations and detections were carried out essentially as described before (15). Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Western Blot Analysis—PAI-1 and USF-2 Western blot analysis was carried out as described (6). In brief, media or cell lysates were collected, and 100 μg of protein were loaded on 10% SDS-polyacrylamide gels and after electrophoresis blotted onto nitrocellulose membranes. The primary mouse antibody against human PAI-1 (American Diagnostics, Pfungstadt, Germany), a primary rabbit antibody against USF-2 (Santa Cruz Biotechnology, Heidelberg, Germany), as well as primary mouse antibody against hemagglutinin-tag (Santa Cruz Biotechnology) or a goat anti-rabbit IgG (Santa Cruz Biotechnology) were used in dilution of 1:5000, respectively. The ECL Western blotting system (Amersham Biosciences, Freiburg, Germany) was used for detection.

Cell Transfection and Luciferase Assay—Freshly isolated rat hepatocytes (about 1 × 10⁶ cells per 60-mm dish), 4 × 10⁵ HepG2 and, 4 × 10⁵ H4IIe cells per 60-mm dish were transfected as described (16). In brief, 2 μg of the appropriate PAI-1 promoter Firefly luciferase (Luc) constructs were transfected together with 500 ng of USF-2a, U2ΔUSR, U2ΔES, and ΔTDU2 expression vectors or in the controls with 500 ng of an empty vector. After 5 h the medium was changed and the cells were cultured under normoxia for 18 h. Then, medium was changed again and the cells were further cultured for 24 h under normoxia or hypoxia.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared by modification of a standard protocol essentially as described (17, 18). The sequence of the human PAI-1 oligonucleotides used for EMSA are 5’-TTCTACA-CAGTACACACA-3’ (−199/−181); 5’-AAATACGGGGCTGCTT-3’ (−571/−552), and 5’-AGTCTGACATGGAGGAG-3’ (−689/−670). Equal amounts of complementary oligonucleotides were annealed and labeled with [γ-32P]ATP (Amersham Biosciences) using the 5’-end labeling kit (Amersham Biosciences). They were purified with the nucleotide removal kit (Qiagen, Hilden, Germany). Binding reactions were carried out in a total volume of 20 μl containing 50 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5% glycerol, 7 μg of nuclear extract, 1 μg of poly(dI-dC) and 5 mM dithiothreitol. After preincubation for 10 min on ice, 1 μl of the labeled probe (10⁶ cpm) was added, and the incubation was continued for an additional 10 min at room temperature. For super-shift analysis 0.4 μg of the USF-1, USF-2, and an ATF-1/CREB cross-reactive antibody (Santa Cruz Biotechnology) were added to the EMSA reaction, which was then incubated for 45 min on ice. The electrophoresis was then performed with a 5% non-denaturing polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 250 V. After electrophoresis, the gels were dried and exposed to a phosphorimaging screen.

RESULTS

Induction of Human PAI-1 mRNA and Protein Expression by USF-2 under Normoxia and Hypoxia—In HepG2 cells transfected with the empty control vector hypoxia enhanced PAI-1 mRNA by about 3.5-fold (Fig. 1). Transfection of the USF-2 expression vector induced human PAI-1 mRNA by about 6.5-fold under normoxia and hypoxia (Fig. 1). The hypoxia- and USF-mediated increase of human PAI-1 mRNA was followed by an increase of human PAI-1 protein levels. Hypoxia enhanced PAI-1 protein levels by about 2-fold in accordance with data from a previous study (5). Overexpression of USF-2 enhanced PAI-1 protein levels by about 4-fold under normoxia and hypoxia (Fig. 1).

USF-2 Activated Human PAI-1 Promoter Luc Gene Constructs in HepG2 Cells—Sequence analyses of the human PAI-1 promoter revealed five E-box-like sequences from which only four and five are classical E-boxes. Therefore, they were named E4 and E5. To investigate the involvement of the HRE as well as E4 and E5 in the USF-2-dependent induction of human PAI-1 expression, the −806 bp wild-type human PAI-1 promoter Luc construct (pG3-hPAI-806) and its derivatives mutated in the HRE as well as in the classical E-boxes were cotransfected with the USF-2 expression vector or an empty vector into HepG2 cells. When the wild-type PAI-1 promoter construct was cotransfected with the empty vector, hypoxia enhanced Luc activity by about 2-fold. Cotransfection of the wild-type human PAI-1 promoter Luc construct together with the USF-2 vector resulted in an about 20-fold increase of
Luc activity under both normoxia and hypoxia. The construct pG3hPAI-HREM containing the mutated HRE responded neither to USF-2 nor to hypoxia. Mutation of E-boxes E4 and E5 in the constructs pG3hPAI-M4 and pG3hPAI-M5 reduced induction under hypoxia and significantly decreased the USF-2-mediated induction of Luc activity to about 4- and 3-fold, respectively, compared with the control (Fig. 2). Double mutation of E5 and E4 in pG3hPAI-M45 also diminished induction of Luc activity by USF-2 to about 2-fold. Thus, these data indicate an involvement of the HRE as well as E4 and E5 in hypoxia- and USF-2-dependent PAI-1 expression.

**Binding of USF to E-box Sequences in the Human PAI-1 Promoter—**
To confirm the conclusion from the transfection experiments that USF-2 interacts with HRE, E4, and E5 within the human PAI-1 promoter, binding of nuclear proteins to oligonucleotides spanning the HRE, E4, and E5 was examined by EMSA. Furthermore, to investigate the presence of USF in these complexes, antibodies against USF-1 and USF-2 were included in the binding reaction (Fig. 3).

When the labeled oligonucleotide spanning the HRE was incubated with HepG2 nuclear extracts, three major DNA-protein complexes were detected, but the mobility of these complexes was not affected by incubation with antibodies against USF-1 and USF-2. The oligonucleotides spanning E4 and E5 bound also three major DNA-protein complexes. Addition of either USF-1 or USF-2 antibody supershifted the intermediate DNA-protein complexes bound to E4 and E5, confirming that this complex contains both USF-1 and USF-2. However, since it is known that proteins of the ATF/CREB family bind constitutively to HREs (19) it was tested whether the complexes formed with the HRE oligonucleotide contain ATF proteins. Addition of the ATF/CREB antibody to the reaction mixture resulted in a supershift not only with the HRE but also with E4 and E5 showing that the major DNA-protein complex contains ATF/CREB proteins. Thus, these data indicate that USFs can interact only with E4 and E5 within the human PAI-1 promoter.

**Regulation of PAI-1 Promoter Luciferase Gene Constructs by Wild-type and Mutant USF-2 in Different Cell Lines—** Since our results from the transfection studies and the results of our previous study with the rat PAI-1 promoter (6) implicate that the regulation by USF depends on the promoter context, different domains of USF, or the cell type, we investigated the different regulation of human and rat PAI-1 promoter Luc constructs together with plasmids expressing various USF-2 mutants in human (HepG2), rat (H4IIE) hepatoma cells, and primary rat hepatocytes. The USF mutants included the protein U2ΔUSR (ΔAA 208–230) lacking USF-specific region (USR), the protein U2ΔE5, which does not
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In this study, we have elucidated a complex role of the transcription factor USF-2 in the regulation of the human PAI-1 promoter in hepatoma cells and primary rat hepatocytes. We have demonstrated that in hepatoma cells USF-2 induced PAI-1 expression and that thereby the HRE and the classical E-boxes within the PAI-1 promoter played an essential role. Furthermore, USF-2 repressed PAI-1 expression in primary rat hepatocytes showing that the USF-2 effect on PAI-1 transcription is cell type-specific. In addition, we showed that mainly the USR domain within USF-2 appears to be essential for both the inducible and the repressive effect of USF-2 on PAI-1 expression.

**DISCUSSION**

USF Modulates PAI-1 Expression via Binding to E-box Motifs—USF was originally identified as a transcription factor activating the adenovirus major late promoter (7). USFs mainly function through E-box core sequences, but their ability to bind non-canonical E-boxes (9, 20, 21) as
well as pyridine-rich initiator (Inr) sites (22) has been reported. In our study, we showed that USF-2 activated the human PAI-1 promoter in HepG2 cells via the HRE, which contains a non-canonical E-box sequence and the classical E-boxes, E4 and E5 (Fig. 2). Mutations of E4, E5, or both decreased activation of the PAI-1 promoter by USF-2, while mutation of the HRE alone completely abolished it. In addition, in this study and in our previous one (6), we observed that the hypoxia-mediated response of the PAI-1 gene was abolished not only by mutation of the HRE but also after transfection of USF-2. Mutations of E4 and E5 also diminished the response of the PAI-1 promoter to hypoxia, and we have shown that they could be binding sites for HIF-1 under certain conditions (11). Thus, all these sites are critical for the action of USF and HIF-1 indicating that the competitive action between USF and HIF-1 as shown for the rat PAI-1 promoter (6) appears also to be likely within the human PAI-1 promoter. In all, these results proposed a model in which USF proteins may function as repressors of PAI-1 in certain cell types by binding to E-boxes thus preventing binding of proteins with strong transcriptional activity like HIF-1. A similar concept may hold true for other E-box binding proteins like Myc, which can displace HIF-1 from the p21cip1 promoter (23) or vice versa that HIF-1 can regulate the expression of some Myc target genes such as human telomerase reverse transcriptase (hTERT) and breast cancer anti-estrogen resistance 1 (BRCA1) (24, 25).

Our present data suggested that the HRE is the major USF binding site in human PAI-1 promoter. Interestingly, electrophoretic mobility-shift analyses demonstrated that E4 (−566/−559) and E5 (−681/−674) bind USFs, and although the HRE (−194/−187) contributed to the USF-dependent regulation of the human PAI-1 gene, it did not bind...
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USFs. Thus, these results again emphasize the role of the HRE, E4, and E5 in USF-2-dependent PAI-1 gene transcription and suggest a cooperative interaction among these elements within the promoter via a yet unknown cofactor. Similarly, a recent study proposed a cooperative model showing that E4 as a USF-1 binding site modulates the TGF-1β-dependent PAI-1 expression in human epidermal keratinocytes (26). Since we showed that a transcription factor from the ATF-1/CREB family binds constitutively to the HRE, it is tempting to speculate that CREB-binding protein is involved as a cofactor. It may then interact via the USR with USF proteins bound to E4 and E5 thus implicating that this complex cooperates with the general transcriptional machinery. This seems to be likely, since USF is related to the basal transcription factor TFII-I and both of them have been implicated in the recruitment of the general transcriptional complexes to TATA-less promoters and in stabilization of the general transcriptional machinery in TATA-box-containing promoters (22, 27, 28). The PAI-1 promoter contains a TATA box, and the USF action might be due to interaction with the basal machinery rather than with its stabilization, since luciferase assays showed that only mutation of the HRE is sufficient to abolish completely the USF effect. Furthermore, the cooperative mode is supported by a study showing that USF can act through both an E-box and a non-canonical E-box as both enhancer and initiator in the regulation of the vasopressin promoter (9). However, it is not known yet whether and to what extent USF, CREB-binding protein/p300, and the general transcriptional machinery interact, although preliminary evidence has been obtained (29). Thus, the details of this interaction need to be clarified in future studies.

USFs Act as Activators and Inhibitors—Among the USF-induced genes are those involved in cellular proliferation such as p53 (30), cyclin B1 (31), and transforming growth factor β2 (32) as well as glucose-controlled genes such as fatty acid synthase (33), L-type pyruvate kinase (12), and hormone-sensitive lipase (34). The inhibitor role of USF was reported for the immunoglobulin heavy chain enhancer gene (35), aor-specific preferentially expressed gene-1 (APEG-1) (36), the CYP1A1 gene (37), and CYP19 gene (38).

The findings of this study that USF-2 acted as an inducer of human PAI-1 expression appeared to be contrasted by our previous findings with the rat PAI-1 gene where USF-2 acted as inhibitor. First, we thought that these differences might be due to sequence variabilities within the promoter, since sequence analysis of the human and rat PAI-1 promoter revealed a number of differences. While complete conservation between the HRE in human and rat PAI-1 promoters was found, the two classical E-boxes, named E4 and E5, were found only in the human but not in the rat PAI-1 promoter. In addition, the USF-2 binding site in the rat promoter (6) is absent from the human PAI-1 promoter. However, our transfection experiments with wild-type human PAI-1 promoter pGI3hPAI-806 or rat PAI-1 promoter pGI3rPAI-766 Luc gene constructs together with a USF-2 expression vector in human (HepG2) and rat (H4IIE) cells as well as in primary rat hepatocytes showed that USF-2 induced both the human and the rat PAI-1 promoter constructs in either the human or the rat hepatoma cell lines (Fig. 4, 5). In addition, we found that USF-2 repressed both promoters to a different extend in primary rat hepatocytes (Fig. 6). Thus, these results show that the action of USF as activator or repressor of PAI-1 expression depends on the cell type rather than on differences between the promoters.

The USR Domain as a Critical Part within USF-2—Several functional domains of the USF-2 protein such as the USF specific region (USR) and especially the part of the transactivation domain that is encoded by exon 5 of the USF gene have been proposed to be important for USF activity (14, 39). The USR has been shown to be necessary and sufficient for transcriptional activation by USF-2 of promoters containing both a TATA-box and an initiator element (Inr), whereas the exon 5 is required together with USR for transcriptional activation of promoters containing only the TATA-box but no Inr element (14, 39).

In this work, we showed that in hepatoma cells the USR domain appears to contribute predominantly to the activity of USF-2 regulating the PAI-1 promoter, which contains E-box motifs, a TATA-box, but no Inr element. In addition, we obtained results showing that the USR is also required for PAI-1 gene repression by USF-2 in primary rat hepatocytes (Fig. 6). Furthermore, when the sequence corresponding to exon 5 was deleted from USF-2, no effects on the transcriptional activity of USF-2 were observed with one exception; enhanced transcriptional activity with the rat PAI-1 promoter in H4IIE cells (Fig. 5). This suggests that this part can inhibit the activity of USF-2 only in H4IIE cells, i.e. in a cell type-specific manner. Thus, our findings are in line with a study in which the transcriptional activity of USF proteins appeared also to be controlled by an unknown cofactor recognizing the USR domain (39), which might be either differently expressed or modified in a cell type-specific manner.

In summary, we found that the human PAI-1 promoter is regulated by USF via E4, E5, and the HRE. Thereby, the different regulation of the PAI-1 promoter occurred in a cell type-dependent manner where the USR domain of USF plays a crucial role implicating the interaction with a so far unknown cofactor.

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