The adenoviral E1A oncoprotein activates the Smad7 promoter: Requirement of a functional E-box

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Abstract. DNA tumorviruses like adenoviruses (AdV) or human papillomaviruses (HPV) have adopted various strategies to interfere with antiproliferative transforming growth factor-ß (TGF-ß) signalling. Here we report that the AdV E1A oncoprotein is sufficient to induce Smad7 expression, an inhibitor of TGF-ß signalling. E1A but not HPV oncoproteins activated the Smad7 promoter. A promoter proximal E-box was crucial for E1A-mediated transcriptional activity. E1A but not HPV oncoproteins induced specific binding activity at this E-box, which was identified as upstream stimulatory factor. In conclusion, these results unravel a novel mechanism of how the AdV E1A oncoprotein induces a cellular inhibitor of TGF-ß signalling.

Introduction

In epithelial and hematopoietic cells transforming growth factor-ß (TGF-ß) inhibits proliferation. TGF-ß signals through heteromeric complexes of type I and type II serine/threonine kinase receptors (TGF-ßR). Upon ligand binding, receptor-regulated R-Smad proteins (i.e., Smad2 and 3) are recruited to the receptor complex and phosphorylated. Complexes formed between activated R-Smads and the common mediator Co-Smad4 translocate to the nucleus, where they activate transcription in concert with interacting transcription factors and p300/CBP co-activators (reviewed in refs. 1,2).

Inhibitory Smads (Smad6 and 7) associate with type I receptors and interfere with recruitment and phosphorylation of R-Smads (3,4), or lead to proteasomal degradation of the receptor complex (5). TGF-ß/Smad3 signalling itself induces Smad7 expression, which mediates an inhibitory feedback loop (4,6-8). Smad7 is also up-regulated by various other stimuli (9-12).

The TGF-ß response element of the Smad7 promoter is located in a region highly conserved among different species. It consists of a functional Smad binding element (SBE), a 3-bp adjacent E-box sequence (CACGTG) and an overlapping activator protein-1 (AP-1) site (6,13). E-boxes are recognised by members of the basic-helix-loop-helix leucine-zipper transcription factor family. These include Myc proteins, microphthalmia-associated transcription factor and the highly related factors TFEB, TFEC and TFE3, as well as the upstream regulatory factors USF-1 and -2 (14). In hepatocarcinoma HepG2 cells, USF is present in constitutive and TGF-ß-inducible protein complexes binding to the Smad7 promoter (6). Also, TFE3 can bind to the E-box of the Smad7 promoter. It synergises with Smad3 in TGF-ß-mediated transcriptional activation of the Smad7 gene (15).

Proliferating host cells are prerequisites for the life cycles of DNA-tumor viruses such as adenoviruses (AdV) and human papillomaviruses (HPV). They have acquired strategies to counteract TGF-ß signalling, which release epithelial cells from TGF-ß-mediated growth inhibition. In this regard, AdV E1A is the best studied oncoprotein, which interferes with TGF-ß signalling at various levels. It down-regulates TGF-ßRII, interacts with R-Smads and competes for the important transcriptional co-activator p300 (16-20). Similarly, E7 oncoproteins from various HPV as well as HPV5E6 directly interact with Smad proteins and inhibit their activity (21-23).

Here, we describe a novel strategy of the AdV E1A oncoprotein, which affects a TGF-ß antagonising pathway. Our

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data show for the first time that E1A but not HPV oncoproteins induce Smad7 expression, a cellular inhibitor of TGF-ß signalling.

Materials and methods

Cell lines. The cervical carcinoma cell lines SiHa (ATCC HTB-35), CaSki (ATCC CRL-1550) (both HPV16-positive), C33A (ATCC HTB-31, HPV and AdV-negative), as well as the AdV-transformed cell line 293 (ATCC CRL-1573) and its derivative 293T (24) were cultured in DMEM (Invitrogen, Carlsbad, CA). All media were supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-alanyl-L-glutamin, and 10 or 0.5% heat inactivated fetal calf serum (FCS) as indicated (all from Invitrogen).

Plasmids and plasmid construction. The pcDNA-16E6 expression vector was obtained by introducing a 630-bp fragment encoding HPV16E6 into EcoRV sites of pcDNA3.1$^+$ (Invitrogen). The pcDNA-16E7 plasmid was constructed as previously described (25). AdV2 E1A in pXJ41 (pXJ41-E1A) and respective empty vector were kindly provided by Dr Gertrud Steger. For stable transfection the E1A coding sequence was re-cloned from pXJ41-E1A into pDNA3.1$^+$. Briefly, E1A was released from pXJ41 with *Eco*RI and *Bgl*II, cloned into *Eco*RI and *Bam*HI sites of pBS II SK$^+$ (Stratagene, La Jolla, CA), cut with *Eco*RI and *Not*I and subcloned into pcDNA3.1$^+$. pCMV-EGFP encoding the enhanced green fluorescent protein gene under the control of the human CMV-immediate early-promoter was kindly provided by Dr W. Krauss.

The rat Smad7 promoter p(-1280)-Smad7prom-Luc construct (S7-Luc), the deletion constructs p(-625)-Smad7prom-Luc (S7-625) and p(-469)-Smad7prom-Luc (S7-469), as well as p(-1280)-Smad7prom-Luc constructs with disrupted SBE (S7-SBE*) or E-box elements (S7-Ebox*, replacing the CACGTG by GAATTC or S7-Ebox-del with deleted Ebox) have previously been described (6).

Transient transfection and luciferase assay. C33A cells were seeded at a density of 1.2x10^5 cells/well in 12-well culture plates (Renner, Dannstadt, Germany). After 24 h the medium was changed to 0.5 ml containing 0.5% FCS. Next, cells were transfected using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany) with 0.2 μg of the respective luciferase reporter construct and 30 or 120 ng of pXJ41-E1A or the same amounts of pcDNA-16E6 or pcDNA-16E7 expression plasmids (25). pCMV-EGFP (0.1 μg) was used to control transfection efficiency. Total amount of DNA was adjusted to 0.8 μg with respective empty expression vector. Twenty-four hours after transfection medium was changed again. Twenty-four hours later cells were detached and transfection efficiencies were determined by flow cytometry of EGFP (FACSCalibur, Becton-Dickinson, Heidelberg, Germany). Cellular lysates were prepared as described previously (26) and assayed for luciferase activity using a model LB9501 luminometer (Berthold, Wildbad, Germany). The values were normalised to the percentages of EGFP-positive cells and protein contents of the lysates.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from C33A cells transiently transfected with pXJ41-E1A, pXJ41, pcDNA-16E6, pcDNA-16E7 or pcDNA3.1$^+$ vector. Six hours post transfection cells were processed as described previously (6). Double stranded oligonucleotides from the rat Smad7 promoter designated S7wt (964-998, according to GenBank accession no. AF156727) comprising the SBE, AP-1 and E-box elements and mutated versions thereof (SBE*, E-box* and AP1*) (6) were end-labeled with [γ-^{32}P]-ATP using polynucleotide kinase. Binding reactions were performed for 20 min at room temperature in a 20 μl volume containing 10 μg of nuclear extracts, 200 pg of labeled oligonucleotides, 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05 M EDTA, 15% glycerine, 25 ng/ml poly(dIdC), 1 mg/ml bovine serum albumin, 25 μg/ml aprotinin, 0.05 mg/ml ssDNA, 12 mM dithiothreitol, 1.5 mM phenylmethylsulphonylfluoride (Sigma-Aldrich, St. Louis, MO). In competition experiments unlabeled oligonucleotides were added at 50-fold excess. In supershift experiments 0.5 μg of monoclonal anti-TFE3 (kindly provided by Dr B. Lüscher) or MOPC-21 antibodies (Sigma-Aldrich) or 2 μg of the polyclonal antibodies anti-USF1, anti-Smad2, anti-Smad3, anti-Smad4 (sc-8983, sc-6200, sc-6202, sc-7154; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit immunoglobulin (rabbit Ig, Sigma-Aldrich) were pre-incubated with nuclear extracts for 40 min on ice. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5x TBE.

RNA preparation and RT-PCR. C33A cells (1x10^6) were seeded in 10 cm plates. After 24 h they were transfected with 10 μg of linearised pcDNA3.1$^+$ or pcDNA-E1A expression plasmid and selected in 1000 μg/ml G418 for 3 weeks. Total RNA was isolated from these cells as well as from SiHa, CaSki, 293 and 293T cells (Qiagen, Hilden, Germany). First strand synthesis was performed using oligo-dT primers and the Omniscript RT kit (Qiagen). A commercial primer set was used for ß-actin cDNA specific RT-PCR according to the manufacturer's instructions (Stratagene). E1A was amplified with the 5'-ttggaccagctgatcgaag-3' and 5'-ctgccgggaaaggtg agg-3' primers. Smad7 specific RT-PCR was performed in 50 μl according to Koinuma *et al* (27) applying the following PCR conditions: 25 cycles with denaturation at 94˚C for 45 sec, annealing at 60˚C for 45 sec and extension at 72˚C for 45 sec.

RT-PCR products from the different cell types were separated on the same 2.5% agarose gel and photographs were taken. RT-PCR product intensities of the virally transformed cells were compared with those of C33A cells, which had been analysed on the same gel.

Results

Smad7 is constitutively expressed in HPV- and AdV-transformed cell lines. The cervical carcinoma cell lines SiHa, CaSki (both HPV16-positive) and C33A (HPV-negative and AdV-negative), as well as the adenovirus-transformed cell line 293, and its derivative 293T additionally expressing the large T antigen of polyomavirus SV40, were investigated for Smad7 expression by RT-PCR. Strong signals were obtained for all virally transformed cell lines (Fig. 1A), whereas HPV and AdV-negative C33A cells displayed much lower Smad7

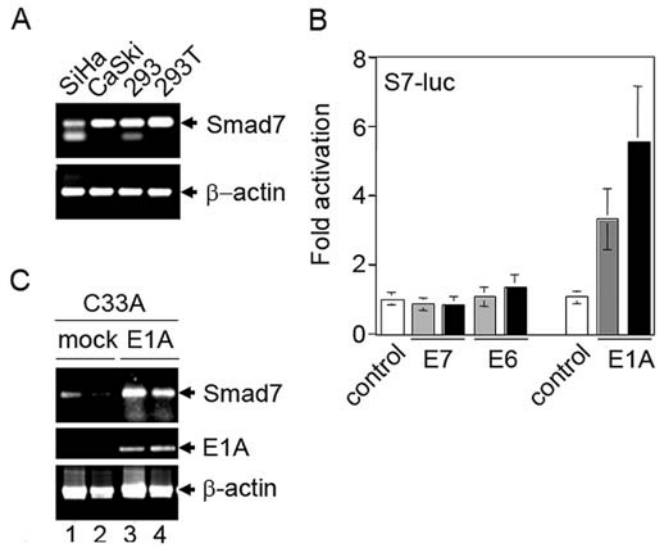

Figure 1. Smad7 expression in AdV- or HPV-transformed cells. (A) Total RNA was prepared from SiHa, CaSki, 293 and 293T cells. RT-PCR was performed for Smad7 (upper panel) and ß-actin as control (lower panel). Ten μl of each reaction were separated on a 2.5% agarose gel (same gel as C33A cells shown in (C)). (B) Effects of HPV oncoproteins and AdV E1A on transactivation of the Smad7 promoter. C33A cells were transfected with 0.2 μg of the wild-type rat Smad7-luciferase reporter construct S7-Luc, 0.1 μg pCMV-EGFP and empty vector control (white column), 30 ng (grey column), or 120 ng (black column) of pDNA3.1$^+$ plasmids encoding HPV16E7, HPV16E6 or pXJ41 encoding AdV2 E1A. The total amount of DNA was adjusted with respective empty expression vector. Normalised luciferase activities of the control (empty expression vector only) were set to one. At least three independent experiments were conducted in triplicates and averaged. (C) Expression of Smad7 in C33A cells stably transfected with E1A. C33A cells were transfected with E1A in pcDNA3.1$^+$ (right panel) or empty expression vector (mock, left panel) and investigated by RT-PCR for Smad7 (upper panel), E1A (middle panel) or ß-actin mRNA expression (lower panel). Lanes 1 and 3 show 20 μl, lanes 2 and 4 10 μl of a representative RT-PCR reaction. To compare results from C33A cells with virally transformed cells, all RT-PCR products were separated on the same gel as in (A).

RT-PCR signals. This is shown in Fig. 1C lane 2, where identical amounts of cDNA were analysed on the same gel.

E1A activates the Smad7 promoter. Since all virally transformed cell lines expressed Smad7, we were interested, whether individual oncoproteins were able to directly activate the Smad7 promoter. C33A cells were transiently transfected with a reporter construct under the control of the Smad7 promoter. Co-transfection of expression vectors encoding HPV16E6 and E7 oncogenes did not alter Smad7 promoter activity. Functionality of the HPV constructs was verified in independent assays, where regulation of other cellular genes was tested [(22) and unpublished data]. In contrast, 120 ng of E1A expression plasmid led to an almost 6-fold induction of Smad7 promoter activity (Fig. 1B).

In order to investigate whether E1A was able to activate the endogenous Smad7 gene, C33A cells were stably transfected with pcDNA-E1A expression plasmid or empty vector as a control. As shown in Fig. 1C (lanes 3 and 4), endogenous Smad7 mRNA levels were up-regulated by E1A in C33A cells. These data demonstrated for the first time that E1A can activate the Smad7 promoter and induce Smad7 expression.

Mutation within the E-box abolishes E1A-mediated activation of the Smad7 promoter. To narrow down the E1A responsive promoter region, deletion constructs and mutated versions of the Smad7 promoter were investigated in C33A cells transfected with or without E1A expression plasmid. Basal activities of deleted constructs, starting from position -625 (S7-625) or position -469 (S7-469), as well as mutated constructs, in which SBE or E-box elements had been inactivated by point-mutations (S7-SBE*, S7-Ebox*) (6), were slightly elevated by 2-2.5-fold in comparison to the wild-type construct (starting from position -1280) (Fig. 2A). Only a construct containing sequences from positions -1280

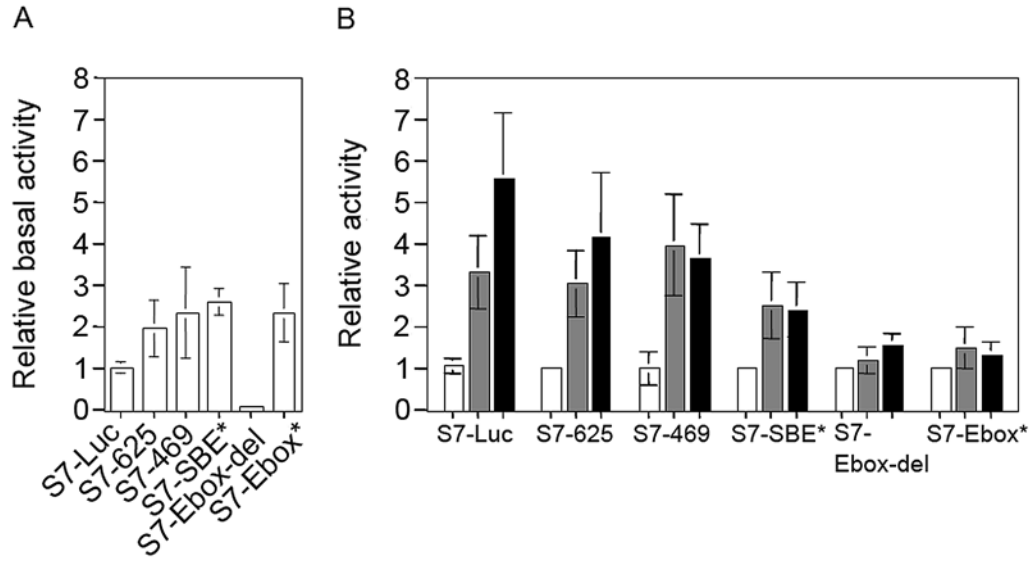

Figure 2. Basal (A) and E1A-inducible (B) activities of deletion constructs and mutated versions of the Smad7 promoter. The S7-Luc wild-type reporter construct (same as in Fig. 1B), S7-625, S7-469, S7-SBE*, S7-Ebox-del or S7-Ebox* and pCMV-EGFP vector were transfected into C33A cells without (white column) or with 30 ng (grey column) or 120 ng (black column) of E1A-pXJ41 expression plasmid. The total amount of DNA was adjusted with empty expression vector pXJ41. Relative luciferase activities were determined as in Fig. 1B. In (A), the activity of S7-Luc was set to 1. In (B), the basal activity of each reporter construct (with empty expression vector pXJ41 only) was set to one.

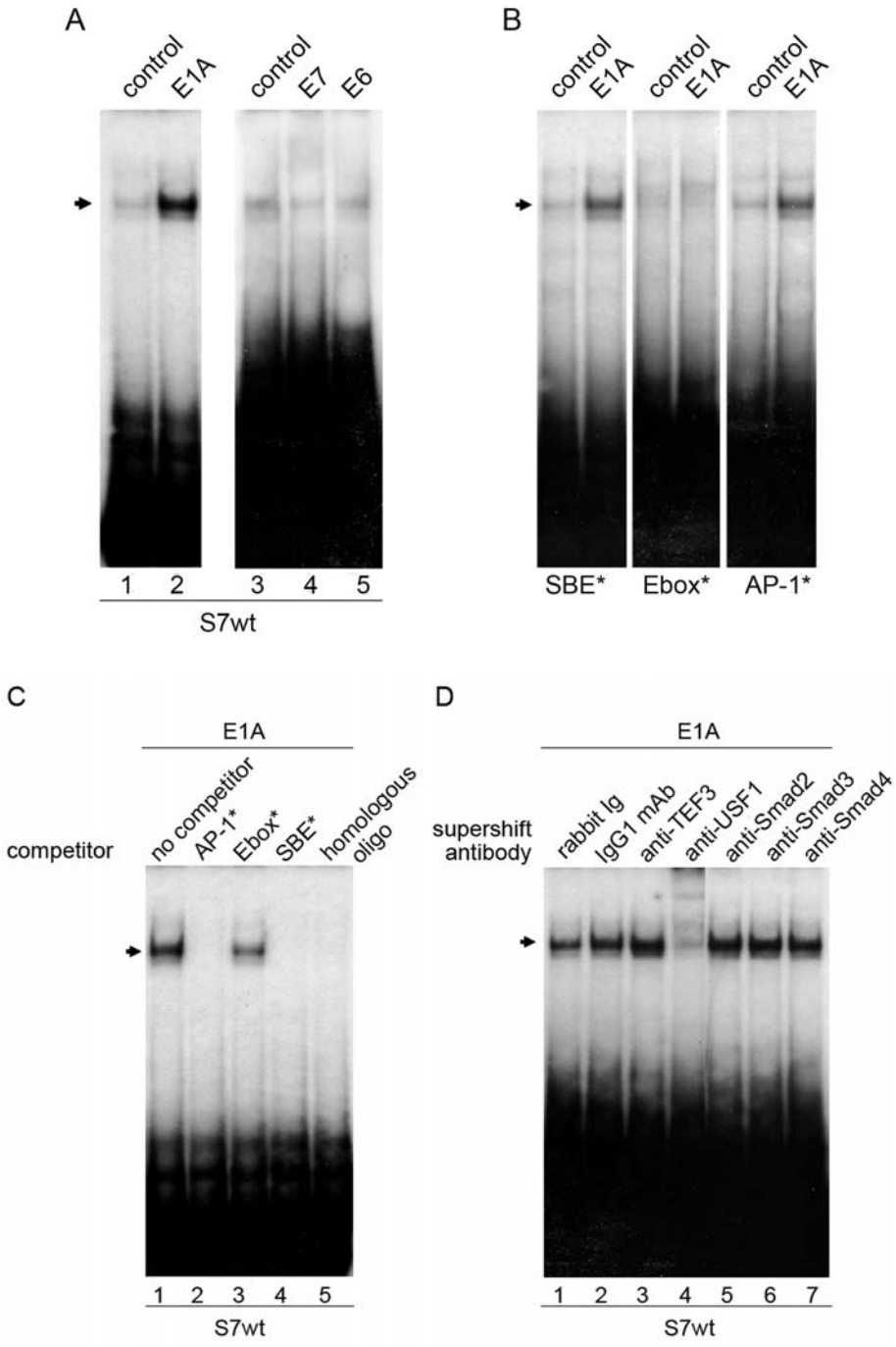

Figure 3. Characterisation of the E1A-inducible binding activity at the S7wt oligonucleotide comprising SBE, Ebox and AP-1 binding sites. (A) Nuclear extracts from C33A cells transiently transfected with pXJ41 (lane 1), E1A in pXJ41 (lane 2), pcDNA3.1$^+$ (lane 3), 16E7 (lane 4) and 16E6 in pcDNA3.1$^+$ (lane 5) were prepared and analysed by EMSA using the [^{32}P]-labelled S7wt oligonucleotide. The arrow indicates the most prominent binding activity present in nuclear extracts of E1A transfected cells. (B) Nuclear extracts from C33A cells transiently transfected with E1A in pXJ41 or vector alone were analysed by EMSA using [^{32}P]-labelled S7 oligonucleotides comprising mutated SBE (SBE*), mutated Ebox (Ebox*) or mutated AP-1 (AP-1*) sites, respectively. (C) Competition of E1A-inducible binding activity at the S7wt oligonucleotide (lane 1) with 50-fold excess of AP-1* (lane 2), Ebox* (lane 3), SBE* (lane 4) or homologous S7wt oligonucleotides (lane 5). (D) Identification of the E1A-inducible binding activity by supershift analysis. Nuclear extracts from C33A cells transiently expressing E1A were pre-incubated for 40 min on ice with 2 μg of non-specific rabbit Ig (lane 1), 0.5 μg MOPC-21 (lane 2, isotype control for anti-TFE3) as controls, 0.5 μg anti-TFE3 (lane 3), 2 μg of the polyclonal rabbit anti-USF-1 (lane 4) or anti-Smad2-4 (lanes 5-7). Binding to the S7wt oligonucleotide was determined by EMSA. The arrow indicates the major binding activity induced in E1A transfectants.

to -335 with deletion of the E-box and its 3'-flanking promoter sequences (S7-Ebox-del) had lost basal activity in C33A cells, in a similar way as previously reported for HepG2 cells (6).

In order to assess inducibility by E1A and to facilitate comparison of the different constructs, their basal activities were set to 1 each (Fig. 2B). Ectopic E1A expression still induced activation of the -625 and -469 deletion constructs up to 4-fold. Activation of the construct with an SBE element mutation was reduced to 2.5-fold. Responsiveness to E1A was almost completely lost in E-box deficient and E-box mutated constructs (Fig. 2B) indicating that E1A-mediated activation critically depends on an intact E-box.

E1A induces USF-1 binding activity at the Smad7 promoter. To determine the E1A-dependent mechanism of Smad7 gene regulation, nuclear extracts from C33A cells transfected with E1A, HPV16E6 and E7 expression plasmids or empty vectors were investigated by EMSA. Wild-type Smad7 oligonucleotides (S7wt) comprised the proximal SBE, AP-1 and E-box elements of the Smad7 promoter (-356 to -322). Strong induction of binding activities was observed in extracts from E1A transfected cells but not from E6 or E7 transfectants (Fig. 3A). One prominent band was observed, which was accompanied by a less intense slightly faster migrating band. Since E1A does not bind to DNA itself, these data suggested that E1A led to induction or to activation of a cellular factor binding to the Smad7 promoter.

To characterise binding activities further, EMSAs were performed with S7 promoter oligonucleotides mutated in either the SBE, AP-1 binding site or the E-box. Oligonucleotides comprising mutated SBE or AP-1 elements still displayed strong E1A-mediated binding activity. However, no DNA binding activity was detected, when the E-box was mutated (Fig. 3B). In competition experiments binding activities were completely abolished when unlabeled homologous S7wt oligonucleotides or S7 oligonucleotides with mutated AP-1 or SBE sites were employed. In contrast, binding activity was preserved when an S7 oligonucleotide comprising a mutated E-box element was used as competitor (Fig. 3C). This confirmed the results obtained in Fig. 3B.

In order to define the nature of the E1A-inducible protein complex binding to the Smad7 promoter, supershift analyses were performed. As shown in Fig. 3D, E1A-mediated binding activity at the S7wt oligonucleotide was neither supershifted by antibodies directed against Smads2-4, nor by anti-TFE3 or control antibodies. Only antibodies directed against the E-box binding protein USF-1 led to a complete supershift.

Discussion

In this study we show that both HPV- and AdV-transformed cells may constitutively express Smad7. The AdV E1A oncoprotein activated endogenous Smad7 transcription. Activation of the Smad7 promoter by E1A was dependent on a functional E-box, where E1A induced binding activity of the transcription factor USF-1. This was in contrast to HPV16 encoded oncoproteins, which neither directly activated the Smad7 promoter, nor induced USF-1 binding activity.

USF proteins are key components of the transcription machinery. They are important factors in gene regulation networks, including stress and immune responses, cell cycle and proliferation. USF factors are ubiquitously expressed with different cell-specific ratios of USF homo- and heterodimers (28). In transformed cells, mostly antiproliferative USF properties were studied (29). Their transcriptional activity is promoter and context specific, depending on factors expressed in the respective cell type (30). USF factors can mediate TGF-ß responses depending on an E-box within the regulatory region of activated genes, as previously shown for the plasminogen activator inhibitor-1 (31).

Constitutive or TGF-ß-inducible USF binding activity has also been demonstrated for the Smad7 promoter (6). Both, USF and Smad3 mediate a negative feedback response to TGF-ß stimulation, thereby limiting the antiproliferative activity of this cytokine. E1A has adopted a similar strategy to induce expression of the TGF-ß inhibitor Smad7. We show that it employs an E-box-dependent mechanism and the most prominent binding activity induced by E1A at the Smad7 promoter has been identified as USF-1. Thus, E1A like TGF-ß uses a primarily anti-proliferative transcription factor for Smad7 gene activation.

Additional analysis revealed that E1A did not increase USF-1 protein expression levels (data not shown). Interestingly, it was previously reported that E1A may associate with USF (previously termed as major late transcription factor) (32) and enhance gene transcription from USF binding sites (33). Several studies have demonstrated that multiple signal transduction pathways including the p38 stress activated kinase pathway (34), the protein kinase A and C pathways (35), the cdk1 pathway (36) and the PI3-kinase pathway (37) can modulate USF DNA-binding activity. The molecular mechanism, how the E1A oncoprotein leads to induction of USF binding activity at the Smad7 promoter, is currently unclear and will be subject of future studies.

Smad7 up-regulation may represent a novel strategy, how virally transformed cells counteract anti-proliferative TGF-ß-mediated signalling. In this study we observed constitutive Smad7 expression in both AdV and HPV transformed cell lines. HPV oncoproteins share a variety of functions with AdV E1A (38-40), including binding and inhibition of R-Smads (21-23). The mechanism identified here appears to be unique for E1A and was not directly attributable to HPV oncoproteins. Thus, it can be speculated that other cellular factors contribute to increased Smad7 expression in HPV-positive tumor cells.

Together, our results describe a novel mechanism of how E1A induces a cellular inhibitor of TGF-ß signalling, a function apparently not shared by HPV oncoproteins.

Acknowledgements

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