ACCELERATED PUBLICATION

Regulation of TG-interacting factor by transforming growth factor-β

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TG-interacting factor (TGIF) is a transcriptional co-repressor that directly associates with Smad (Sma- and Mad-related protein) proteins and inhibits Smad-mediated transcriptional activation. By using Affymetrix (Santa Clara, CA, U.S.A.) oligonucleotide microarray analysis, we found that TGIF mRNA level was elevated by transforming-growth-factor-β (TGF-β) treatment in a human T-cell line, HuT78. Subsequent reverse-transcription PCR assays indicated that TGF-β1 and activin were able to induce a rapid and transient increase in the level of TGIF in both HuT78 and HepG2 hepatoma cells. To analyse whether or not the regulation of TGIF mRNA occurs at the transcriptional level, a 2.4 kb human TGIF promoter was isolated. A primer extension assay was performed to localize the putative transcription initiation site of the promoter. When transiently expressed in HepG2 cells, this promoter was stimulated by TGF-β1 and activin treatment in a time-dependent manner. A series of deletion mutants of the TGIF promoter were also generated to further characterize the TGF-β responsive region of the promoter. In addition, expression of TGIF was able to cause a dose-dependent inhibition of TGF-β and activin signalling. Taken together, these experiments indicated that TGIF is a novel transcriptional target of TGF-β and activin signalling and is likely involved in a negative feedback loop to desensitize TGF-β/activin action.

Key words: activin, microarray, TG-interacting factor (TGIF), transcription, transforming growth factor-β (TGF-β).

INTRODUCTION

Transforming growth factor-βs (TGF-βs) are a group of pleiotrophic extracellular factors implicated in a variety of biological processes, including regulation of cell proliferation, apoptosis, immune modulation and early development [1]. TGF-β and activin exerts their biological functions by interacting with two transmembrane receptors, type I and type II, that both possess serine/threonine kinase domains in their intracellular regions [2]. After ligand binding, the activated type I receptor relays the signal to a group of proteins called Smad (Sma- and Mad-related protein) [3,4]. Both functional and biochemical studies have indicated that Smad2 and Smad3 are the pathway-specific Smads activated by TGF-β and activin receptors, via phosphorylation at their C-terminal ends by the kinase activity of the type I receptor [3,4]. On phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with Smad4, the common mediator Smad [5,6]. These complexes then migrate to the nucleus and activate gene transcription, either through direct DNA binding by the Smad proteins or through association with other sequence-specific transcription factors [3]. On the other hand, Smad6 and Smad7 comprise another group of Smad proteins, the inhibitory Smads, that are able to inhibit signalling of TGF-β family members [3,4].

TG-interacting factor (TGIF) functions as a transcriptional co-repressor that directly associates with Smad proteins and inhibits Smad-mediated transcriptional activation [7]. TGIF belongs to the three-amino-acid loop extension ('TALE') subfamily of homeodomain proteins. TGIF preferentially binds to the sequence CTGTCAA, with the central five nucleotides being most important for the binding [8]. Recent studies have indicated that TGIF functions as a transcriptional co-repressor through two mechanisms [9]. TGIF may repress the transcription of promoters when it directly binds the cognate site via the homeodomain, or serve as a repressing partner when associating with other transcription factors such as Smad [10]. TGIF is able to associate with TGF-β-activated Smad proteins Smad2 and Smad3 and repress TGF-β-activated transcription through these Smad proteins [7]. TGIF is constitutively localized in the nucleus, and the interaction of TGIF with Smads leads to recruitment of TGIF into the Smad-responsive elements, thereby repressing TGF-β-activated genes. The repressive activity of this TGIF–Smad complex is partly dependent on the histone deacetylase activity recruited by TGIF as well as the competition with transcriptional co-activators such as p300 [9].

In humans, mutations of the TGIF gene have been linked to holoprosencephaly (HPE) [11]. HPE is a genetic disorder that affects brain and craniofacial development with a variable penetrance and phenotypes ranging from severe defects to normal individuals [12]. In HPE, TGIF mutation was found in a single allele, indicating that the phenotype of HPE is caused by the haploid insufficiency of the gene product [11]. These studies suggest that the subtle change to the amount of TGIF could have a dramatic effect on the developmental programming of the body, providing further evidence that the fine-tuning of TGF-β signalling is crucial for the biological function mediated by TGF-β receptors. In an effort to delineate genes regulated by TGF-β, we used cDNA microarray analysis and found that TGIF is one

Abbreviations used: (CA-) ALK, (constitutively active) activin receptor-like kinase; FBS, fetal-bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HEK-293, human embryonic kidney 293 cells; HPE, holoprosencephaly; NIH, National Institutes of Health; RT-PCR, reverse-transcription PCR; SBE, Smad-binding element; Smad, Sma- and Mad-related protein; TGF-β, transforming growth factor-β; TGIF, TG-interacting factor.

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of the transcriptional targets of TGF-β signalling. We report here the initial characterization of the transcriptional regulation of TGIF by TGF-β.

MATERIALS AND METHODS

Cell culture and cell transfection

The human embryonic kidney 293 (HEK-293) and human HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium ('DMEM') containing 10% (v/v) fetal-bovine serum (FBS) supplemented with penicillin and streptomycin. HuT78 cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% (v/v) FBS. Transient cell transfection was performed by the calcium phosphate method for HEK-293 cells and a SuperFect® reagent (Qiagen) for HuT78 and HepG2 cells.

Plasmids

The Smads, the constitutively active TGF-β type I receptor CA-ALK5 (constitutively active activin receptor-like kinase 5), the constitutively active activin type I receptor (CA-ALK4) and the TGF-β-responsive Smad-binding element (SBE) luciferase reporter have been previously described [13–15]. The $-2393/+119$ bp human TGIF promoter was cloned by PCR using two primers 5′-GAATTGTGCCAGTGTTTCTCTTTG-3′ and 5′-CGGCGCTGTCAGAGTGAGAGAGGC-3′ (primer A) with the human genomic DNA as template. The resultant PCR fragment was first cloned into pCRII-TOPO (Invitrogen). The PCR insert was confirmed by DNA sequencing and released by *Eco*RV and *Hin*dIII digestion, followed by cloning into the *Sma*I and *Hin*dIII site of pGL3-basic (Promega). The $-2023/+119$ bp TGIF promoter was cloned by digestion of the $-2393/+119$ bp promoter construct with *Xho*I (which cuts at an internal site of the PCR fragment) and *Hin*dIII and subcloned into pGL3-basic. The $-1329/+119$ bp construct was generated by PCR with 5′-GAGGGAGGGTGGCAGGCTGGC-3′ (primer C) and primer A and then cloned into the *Kpn*I- and *Xho*I-digested pGL3-basic. The $-1329/-579$ bp construct was cloned by PCR with primer C and 5′-GCGAGTTCCGGA-CTGGATTGCAAG-3′ (primer B) and then cloned into the *Kpn*I/*Xho*I sites of pGL3-basic. All other promoter constructs were generated by *Sma*I partial digestion of the $-1329/+119$ promoter construct and full digestion with *Xho*I and then cloned into the *Sma*I/*Xho*I sites of pGL3-basic.

Microarray analysis

HuT78 cells were cultured in a 100-mm-diameter dish in the presence or absence of TGF-β1 (1.25 ng/ml) for 90 min. Total RNA from these cells was isolated using RNeasy® (Qiagen). Preparation of cRNA, hybridization, and scanning of the human gene U95A arrays were performed according to the manufacturer's (Affymetrix, Santa Clara, CA, U.S.A.) protocol. The arrays were scanned at 3 mm with the GeneArray scanner (Affymetrix). The chips of Human Genome U95Av2 Array were used and they represent about 12000 individual genes.

Reverse-transcription PCR (RT-PCR)

RNA from HuT78 and HepG2 cells was isolated using RNeasy® (Qiagen). A 1 μg portion of the recovered RNA was treated with RNase-free DNase I (Invitrogen) to remove the residual DNA, and reverse-transcribed in a 25 μl volume reaction with oligo(dT) primer using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) to generate first-strand cDNA. The products were diluted and used in PCR to detect the expression of TGIF, Smad7 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The sequences of the PCR primers are as follows: 5′-GTCTTCACCACCATGGAGAAGG-3′ and 5′-TCGCTGT-TGAAGTCAGAGGAGA-3′ for G3PDH; 5′-GTGTTGCTGT-GAATCTTACGGG-3′ and 5′-TTCACAAAGCTGATCTGC-ACGG-3′ for Smad7; 5′-CAGAGCAAGAAAAAGCGTTG-3′ and 5′-GACCACTCTGTGTATTCGTACTTG-3′ for TGIF. The PCR products were separated on 2%-(w/v)-agarose gels and revealed with ethidium bromide. The band intensity was quantified using densitometric analysis by the NIH *Image* program.

Promoter assay and primer extension

HepG2 or HEK-293 cells were transfected with different combinations of plasmid DNA. A *Renilla* luciferase vector driven by a simian-virus-40 promoter was co-transfected to serve as an internal control for transfection efficiency. The cells were harvested at 36 h after transfection by lysis with 20 μl of TNEN lysis buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/2.0 mM EDTA/1.0% Nonidet P40/1.0 μg/ml each aprotinin and leupeptin/1.0 mM PMSF/10.0 mM NaF/1.0 mM Na_3VO_4].). A 10 μl portion of the lysate was used for the Dual-Luciferase Assay (Promega). The samples were counted for 10 s in an FB12 luminometer (Zylux, Oak Ridge, TN, U.S.A.), and the data were represented as the relative light unit/s. Variance analysis was used to determine the statistical difference among experimental groups. The primer extension assay was performed using the total RNA isolated from HuT78 and HepG2 cells with a method previously described by us [14]. Both oligonucleotide primers, primer A and primer B as described in the 'Plasmids' subsection above, were used in this assay.

RESULTS AND DISCUSSION

TGF-β augments TGIF mRNA level in HuT78 and HepG2 cells

Initially we used a cDNA microarray method to analyse genes regulated by TGF-β in a human T-cell line, HuT78. The HuT78 cell line was originally established from a patient with mycosis fungoides and has been characterized as a model cell line for studying human T-cell function [16]. HuT78 cells produce several cytokines typical for human Th0 cells in response to anti-CD3 and PMA stimulation, such as interleukin-4 and interferon-γ. To catch the 'immediate' response to TGF-β treatment, these cells were treated with TGF-β1 (1.25 ng/ml) for 90 min and the isolated total RNA was applied to microarray analysis. The Human Genome U95Av2 Array chips from Affymetrix were used and these chips contain about 12000 sequences previously characterized in terms of function or disease association. To assure the accuracy of the data, four individual sample preparations were used for either control or TGF-β-treated cells. The relative expression level of these $\approx$ 12000 genes was detected and used in statistical analysis (Student's t test) to reveal genes significantly changed by TGF-β. The up-regulated genes include those previously identified to be transcriptional targets of TGF-β signalling, such as JunB [17], plasminogen activator inhibitor 1 [18], and Smad7 [14]. Interestingly, the expression level of TGIF was also significantly elevated by TGF-β treatment in these cells (results not shown).

We next used RT-PCR to confirm the results obtained by cDNA microarray. HuT78 cells were treated with TGF-β1 and activin for different amount of times and total RNA was isolated from these cells for RT-PCR analysis. Activin is another member of the TGF-β superfamily and shares the same Smad proteins as

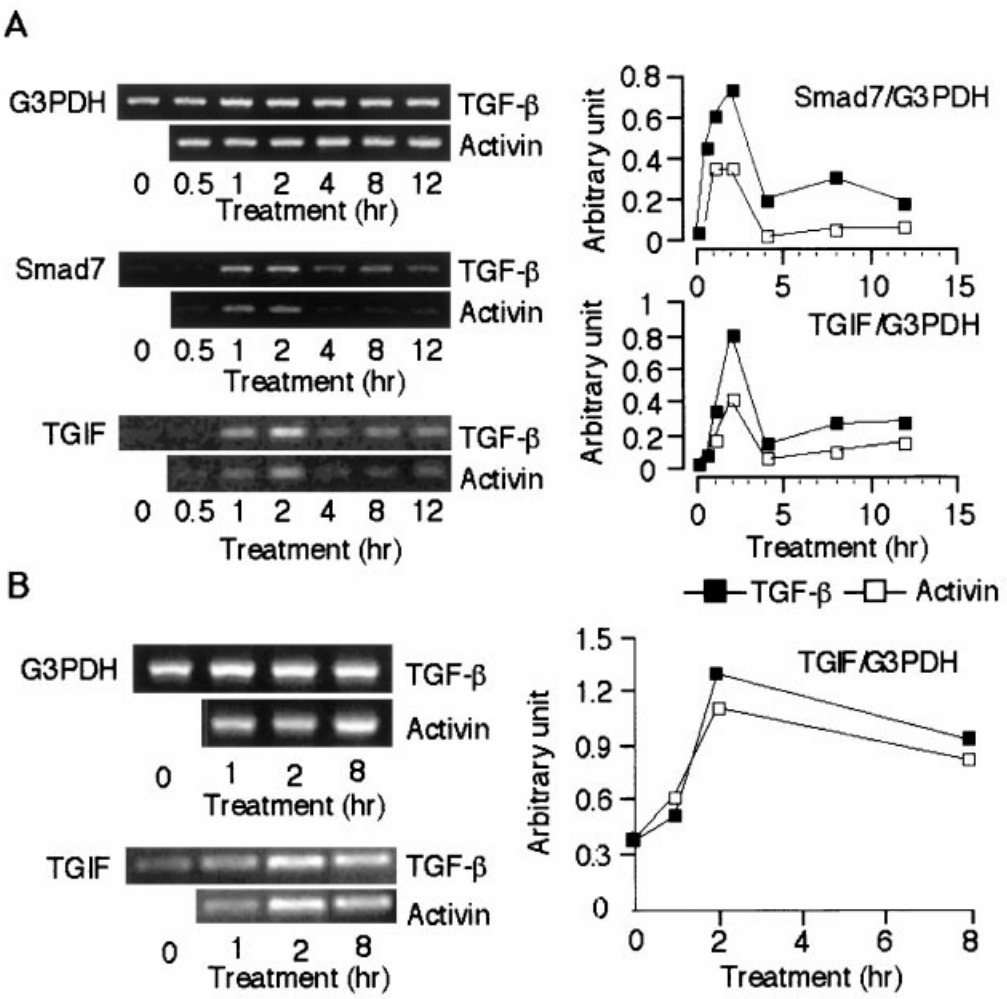

Figure 1 TGIF is up-regulated by TGF-β and activin in HuT78 and HepG2 cells by RT-PCR assay

(**A**) HuT78 cells were treated with TGF-β1 (1 ng/ml) or activin (10 ng/ml) for the different amounts of time indicated. Total RNA isolated from these cells was used in RT-PCR with primers specific for G3PDH, TGIF and Smad7. The relative amounts of PCR products were analysed using the NIH *Image* program and the resulting intensity was plotted against the values of G3PDH at each time point. (**B**) A similar experiment was performed in HepG2 cells. Only the data for G3PDH and TGIF are shown here.

TGF-β. As shown in Figure 1(A), TGF-β1 and activin treatment led to a rapid and transient increase in the TGIF transcript, with a maximal stimulation at 2 h after treatment. As a positive control, we also found that Smad7 transcripts were rapidly up-regulated by TGF-β and activin with a maximal stimulation at 1–2 h. These data, therefore, have corroborated our microarray findings and demonstrated that TGIF is one of the target genes regulated by TGF-β in HUT78 cells. To determine if TGIF is also regulated by TGF-β in other cell types, we did a similar experiment in HepG2 cells originally derived from human hepatoma. As shown in Figure 1(B), TGF-β and activin treatment in these cells were also able to elevate the mRNA level of the TGIF gene.

Characterization of the TGIF promoter

Because direct measurement of transcriptional rate is required to actually demonstrate that TGF-β elevates TGIF mRNA level at the transcriptional level, we isolated and characterized the putative promoter region of the TGIF gene. A pair of oligonucleotide primers based on the human genomic sequence (National Human Genome Research Institute, NIH, Rockville Pike, Bethesda, MD, U.S.A.) was used in PCR to clone a 2393 bp sequence 5′ upstream of the TGIF coding region. A partial sequence of the putative TGIF promoter is shown in Figure 2(A). To determine the transcription initiation site, we used two primers about 300 bp (primer A) and 1000 bp (primer B) away from the first ATG codon in a primer extension assay. We chose to use primer B because there is a putative TATA box about 1100 bp away from the start codon. The primer extension result is shown in Figure 2(B). Both primers were able to detect a major transcription initiation site. We hypothesized that the site identified with primer A that is 300 bp away from the start coden is likely the legitimate initiation place for TGIF gene on the basis of the following considerations: (1) the putative TATA box in the human TGIF gene is not conserved in the mouse genome; (2) there are multiple putative Sp1 sites around the initiation site detected by primer A and this is common for TATA-less promoters [19]; (3) the promoter constructs with a deletion of the putative TATA-box-containing region had a

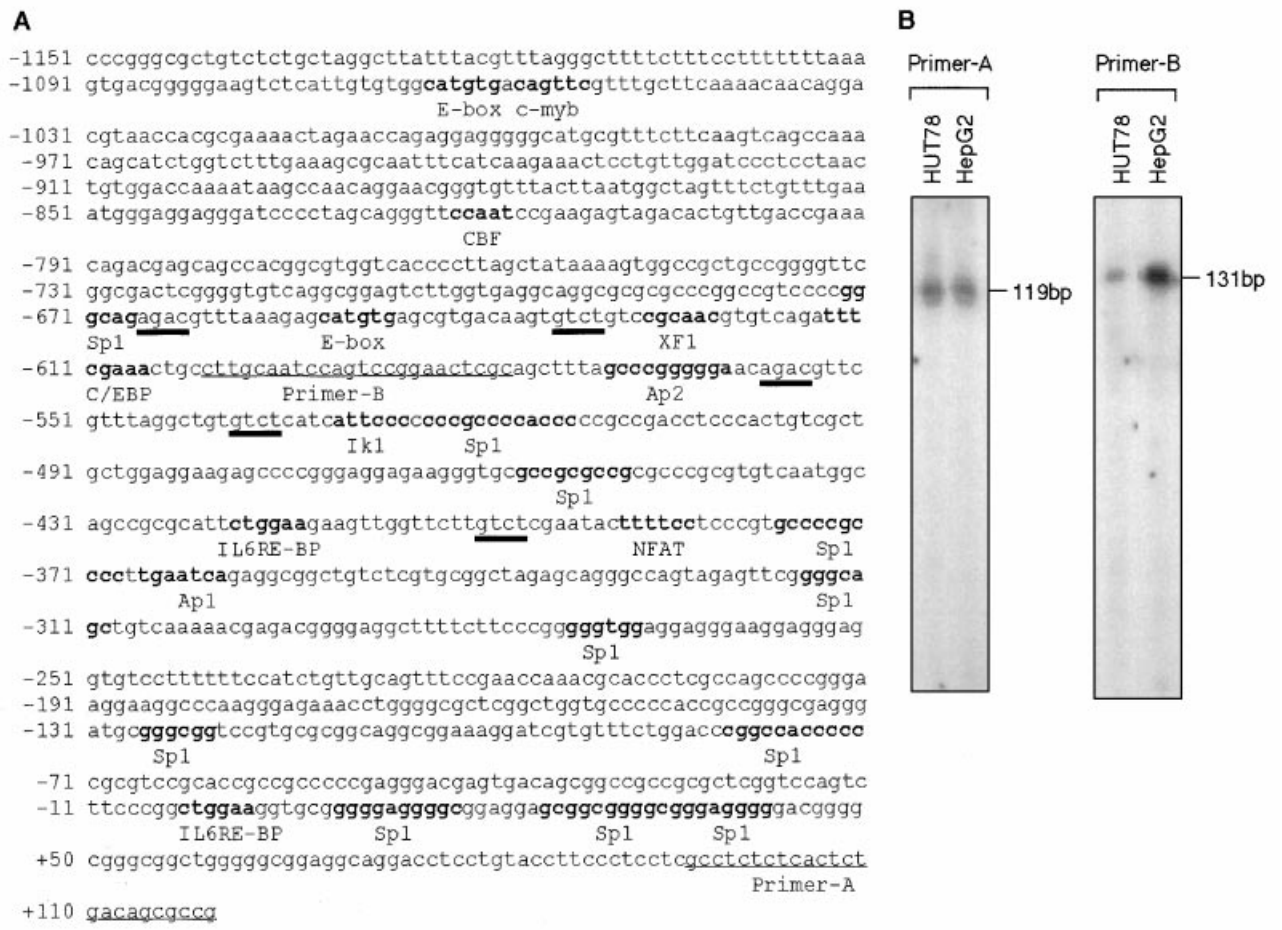

Figure 2 Human TGIF promoter sequence and primer extension assay

(**A**) Partial sequence of the TGIF promoter. The consensus transcription-factor-binding sites that are conserved in both human and mouse genomic sequences are marked by bold letters. The sequences corresponding to the two primers used in primer extension assay are underlined. The consensus SBEs are indicated by bold underlining. (**B**) Primer extension assay to determine the transcription initiation site. Total RNA from HuT78 and HepG2 cells were used in a primer extension assay with two different primers. DNA sequencing was performed using the same primers with TGIF promoter DNA as a template to determine the length of the extension products.

basal activity comparable with that of the full-length (2393 bp) promoter, as will be discussed subsequently. On the basis of these considerations, the transcription initiation site detected by primer A is denoted as +1. To determine the putative transcription-factor-binding sites, we used the online Transcription Element Search Software (TESS) from University of Pennsylvania (http://www.cbil.upenn.edu/tess) to search the putative promoter regions of both human and mouse genomic sequence (available from the NIH) on the assumption that only the sites conserved in both human and mouse are potentially significant in the regulation of TGIF transcription. The search results, as well as the putative SBEs (GTCT or AGAC) conserved in both species, are shown in Figure 2(A).

TGF-β is able to stimulate TGIF promoter in HuT78 cells

To determine whether or not the putative TGIF promoter is regulated by TGF-β, we made a reporter construct that contains a firefly luciferase gene downstream of the isolated 2393 bp TGIF genomic sequence. When this reporter was transfected into HepG2 cells, both TGF-β and activin treatment were able to stimulate the luciferase activity as compared with the untreated samples. As shown in Figure 3, TGF-β and activin treatment for 4 h appeared to reach the maximal stimulation of the promoter activity by 1.7- and 2.9-fold respectively. At 15 h after treatment, these ligands were still able to elevate the activity by 1.5- and 2.2-fold respectively. Interestingly, activin stimulated the TGIF promoter more potently than TGF-β, likely reflecting a difference in either the expression level of the corresponding receptors on the plasma membrane or the secretion of these ligands by the cells at basal state. Nevertheless, this experiment clearly demonstrated that TGF-β and activin are able to up-regulate the TGIF promoter, and this stimulation may, at least in part, contribute to the elevated TGIF transcript level observed in our microarray analysis and RT-PCR studies.

Regulation of the TGIF promoter by Smad proteins downstream of TGF-β receptors

To determine whether or not Smad proteins regulate the isolated TGIF promoter, and if so, which region of the promoter confers such regulation, we made systematic deletions of the TGIF promoter. These deletion mutants were linked to a luciferase reporter and transfected into HEK-293 cells for transcriptional

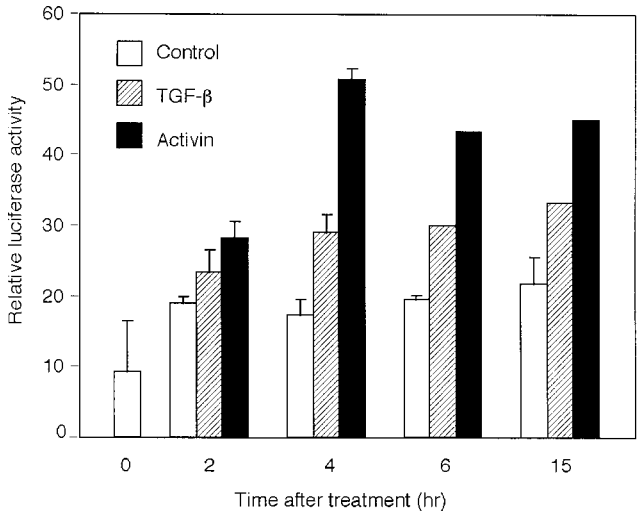

Figure 3 TGF-β and activin are able to stimulate the TGIF promoter in HepG2 cells

A 2393 bp putative TGIF promoter sequence was linked to a firefly luciferase reporter and transfected into HepG2 cells, followed by treatment with TGF-β1 or activin for different amounts of time. A *Renilla* luciferase plasmid driven by the simian-virus-40 promoter was used to normalize the transfection efficiency. A dual luciferase assay was performed to detect the relative activity of both luciferases. The relative change of luciferase activity as compared with the untreated sample (control) is shown here as the mean $\pm$ S.D. At least three independent experiments were performed with similar results.

assays. HEK-293 cells were chosen because they are highly transfectable and have been extensively used to characterize the transcriptional regulation by Smad proteins as well as other transcription factors [20,21]. The basal activity and the activity induced by cotransfection with a constitutively active TGF-β type I receptor (CA-ALK5), Smad3 and Smad4 are shown in Figure 4. Most of the deletion mutants, except for the shortest one, were able to give rise to comparable basal transcriptional activities. Interestingly, the constructs $-676/+119$ bp, $-570/+119$ bp, $-477/+119$ bp and $-279/+119$ bp, which lack the putative TATA box found in the human genome (but not in the mouse genome), were associated with a high basal activity, indicating that this TATA box is not required for transcriptional initiation of the TGIF promoter. Except for the constructs of $-1329/-579$ bp and $-198/+119$ bp, all of the promoter fragments were highly responsive to Smad-mediated activation with a stimulation of 4–40-fold. To our surprise, we were not able to determine a narrowly defined region that mediates the Smad activation in this experiment, indicating that the TGF-β-responsive regions are scattered along the whole length of the promoter sequence used here. In addition, we performed a similar experiment in HepG2 cells with these deletion constructs of TGIF promoter to determine the effect of TGF-β treatment. As shown in Figure 5, TGF-β treatment was able to stimulate various deletion mutants of the promoter, similar to what was found in HEK-293 cells. Taken together, these data further strengthened our observation that the TGIF promoter is regulated by TGF-β signalling.

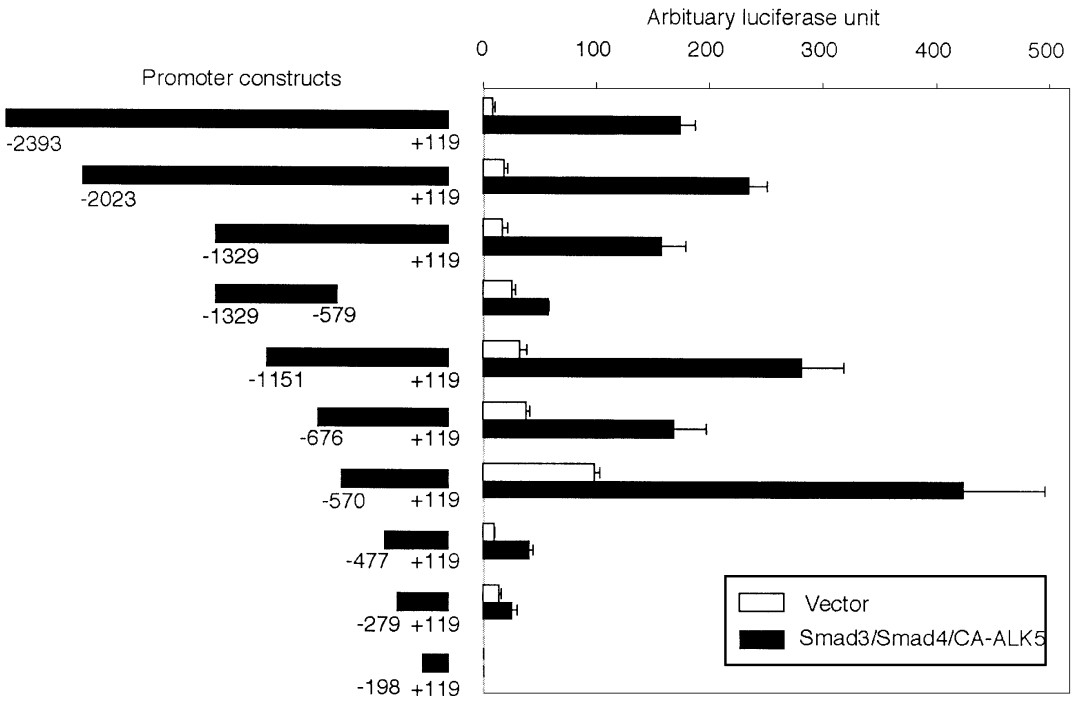

Figure 4 TGF-β-activated Smad proteins are able mediate stimulation of the TGIF promoter

Various deletion mutants of the TGIF promoter were linked to a firefly luciferase reporter and used in a transcriptional assay in HEK-293 cells. Smad3, Smad4 and a constitutively active TGF-β receptor (CA-ALK5) were co-transfected to activate the TGF-β signalling pathway. Both the basal (without Smads and CA-ALK5) and the Smad-stimulated activities are shown as means $\pm$ S.D. A simian-virus-40-promoter-driven *Renilla* luciferase was co-transfected to normalize the transfection efficiency. Three independent experiments were performed with similar results.

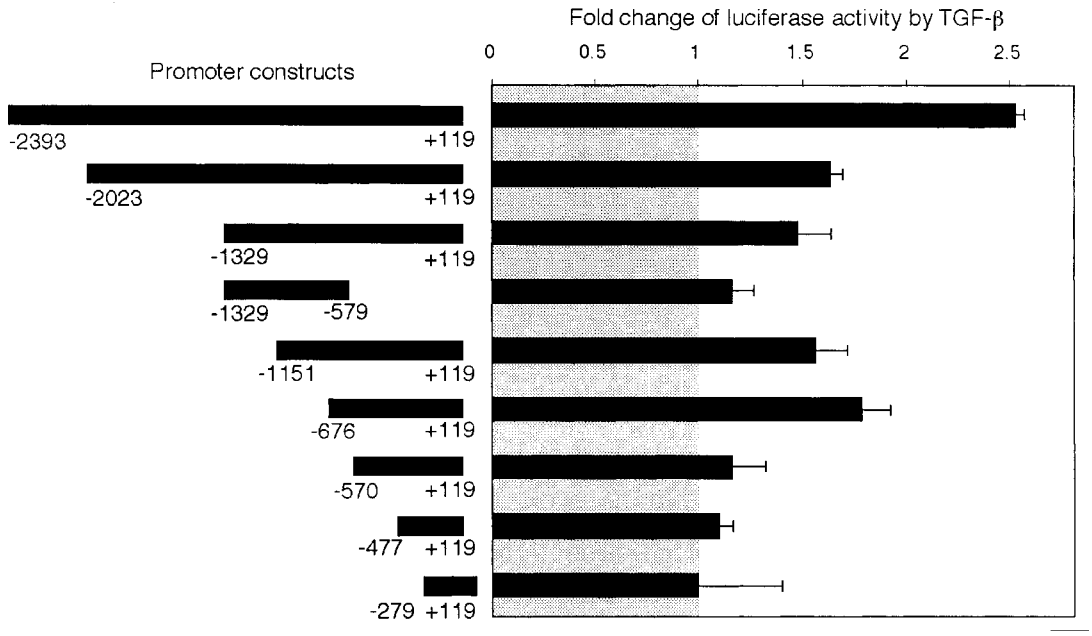

Figure 5 Response of various TGIF promoter deletions to TGF-β treatment

Various deletions of the TGIF promoter as in Figure 4 were transfected into HepG2 cells and treated with TGF-β1 for 4 h. The fold changes of the luciferase activity after TGF-β treatment as compared with the untreated control are shown as means ± S.D. A total of four independent experiments were performed with similar results.

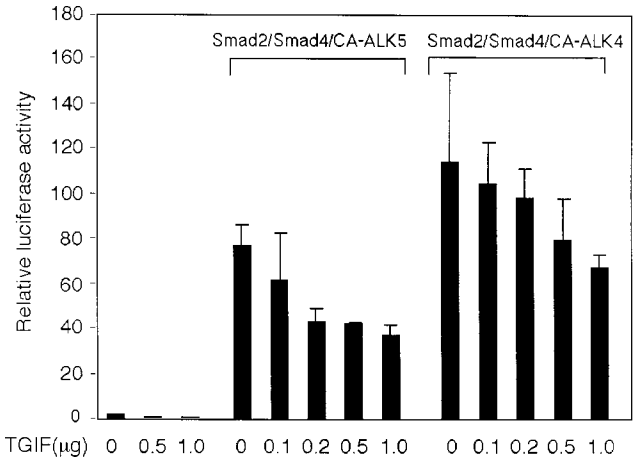

Figure 6 TGIF is able to inhibit TGF-β- and activin-mediated transcriptional regulation

Different amounts of TGIF plasmid were transfected as indicated into HEK-293 cells with or without activation of the TGF-β or activin signalling pathway by co-expression of Smad2, Smad4, a constitutively active TGF-β type I receptor (CA-ALK5) or a constitutively active activin type I receptor (CA-ALK4) (0.2 μg each). The fold change of luciferase activity as compared with the untreated samples (set to 1) is shown as the mean ± S.D. At least three independent experiments were performed with similar results.

TGIF is able to inhibit TGF-β- and activin-mediated transcriptional response

Our studies revealed that the transcription of TGIF itself is under the control of TGF-β and activin signalling, indicating a novel feedback loop for the rapid termination of the signalling at the transcriptional level. To provide further evidence that the elevated TGIF expression is able to repress Smad-mediated gene transcription, we expressed different amounts of TGIF in HEK-293 cells and analysed its effect on Smad-activated transcription. As shown in Figure 6, Smad2 and Smad4 in the presence of CA-ALK5 or CA-ALK4 were able to strongly stimulate a TGF-β-responsive promoter that contains a luciferase reporter driven by two repeats of the SBE from the Smad7 promoter [14]. Co-expression of TGIF was able to cause a dose-dependent inhibition of the Smad-mediated transcriptional stimulation, with the highest level of TGIF being able to decrease the stimulation by more than 50%. Therefore these data provided further evidence that the transcriptional capacity regulated by TGF-β and activin signalling can be modulated by the relative level of TGIF in a cell.

In summary, our experiments indicated that TGIF mRNA level is up-regulated by TGF-β and activin signalling and this regulation appears to occur at the transcriptional level. The regulation is likely involved in a negative feedback loop to timely terminate TGF-β and activin signalling after initial activation. Previous studies have also indicated that Smad7 and SnoN (another Smad repressor) are involved in such a feedback regulation. TGF-β has been found to regulate the transcription of Smad7 gene by direct binding of TGF-β-restricted Smads to the SBE of the Smad7 promoter region [14]. In addition, SnoN is also transcriptionally up-regulated by TGF-β signalling [22]. Taken together, these data indicate that different strategies can be employed by the cell to desensitize TGF-β and activin signalling. Initial activation of the TGF-β/activin signalling pathway leads to activation of Smad2 and Smad3, which stimulate gene transcription through interaction with general transcription co-activators such as p300. However, transcription of Smad7, TGIF and SnoN are turned on afterwards and these molecules would in turn bring about the down-regulation of TGF-β and activin signalling at different levels.

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