

Tenascin-C upregulation by transforming growth factor- β in human dermal fibroblasts involves Smad3, Sp1, and Ets1

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In cultured human dermal fibroblasts, transforming growth factor (TGF)- β induced the mRNA expression of tenascin-C (TN-C). The molecular mechanism(s) underlying this process is not presently understood. In this study, we performed serial 5' deletion and a transient transfection analysis to define a region in the TN-C promoter mediating the inducible responsiveness to TGF- β . This region contains an atypical nucleotide recognition element for the Smad family of transcriptional regulators. A DNA affinity precipitation assay revealed that Smad2/Smad3 bound to this site in a transient and specific manner. Overexpression of Smad3 or Smad4 activated the TN-C promoter activity and superinduced the TN-C promoter activity stimulated by TGF- β . Moreover, simultaneous cotransfection of Smad3 and Smad4 activated the TN-C promoter activity in a synergistic manner. Mutation of the Smad-binding sites, the Ets-binding sites, or Sp1/3-binding sites in the TN-C promoter abrogated the TGF- β /Smad-inducible promoter activity. Immunoprecipitation analysis revealed that Smad3, Sp1, and Ets1 form a transcriptionally active complex. Furthermore, the interaction between Smads and CBP/p300 in TGF- β signaling was confirmed. These findings demonstrate the existence of a novel, functional binding element in the proximal region of the TN-C promoter mediating responsiveness to TGF- β involving Smad3/4, Sp1, Ets1, and CBP/p300.

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Introduction

Tenascin-C (TN-C) is a polymorphic high-molecular-mass extracellular matrix glycoprotein composed of six similar subunits joined together at their NH₂ terminus by disulfide bonds (Ekblom and Aufderheide, 1989; Gulcher *et al.*, 1989; Erickson, 1993; Chiquet and Wehrle-Haller, 1994; Gherzi *et al.*, 1995). Its molecular organization is made up of a cysteine-rich N-terminal

domain involved in oligomerization, a series of epidermal growth factor-like repeats, followed by fibronectin type III-like repeats, and a fibrinogen-like globular domain (Erickson, 1993; Chiquet-Ehrismann, 1995; Dandachi *et al.*, 2001).

TN-C displays a developmentally regulated time- and space-dependent tissue distribution. In general, TN-C is transiently expressed during fetal development, and absent or greatly reduced in adult tissues (Dandachi *et al.*, 2001), but is markedly increased during many pathological conditions, including wound healing, inflammation, and oncogenesis (Natali *et al.*, 1991; Erickson, 1993; Crossin, 1996; Mackie, 1997). Although the exact biological functions of TN-C are still unknown, it has been shown that TN-C inhibits cell adhesion to fibronectin and it has been included among the 'antiadhesive molecules', which seem to play some roles in the processes of morphogenesis, wound healing, and cancer progression (Borsi *et al.*, 1994).

Previous studies have identified several cytokines as inducers of TN-C expression in various tissue culture systems. For example, platelet-derived growth factor (PDGF)-BB and angiotensin II induced TN-C mRNA and protein in rat smooth muscle cells (Sharifi *et al.*, 1992). TN-C synthesis is induced by the basic fibroblast growth factor (bFGF) in several cell types, including Swiss 3T3 cells (Tucker *et al.*, 1993). Interleukin (IL)-1 increased TN-C production in human synovial fibroblasts (McCachren and Lightner, 1992). It was also reported that IL-4, PDGF-AB, or bFGF upregulates TN-C synthesis in human dermal fibroblasts (Makhluf *et al.*, 1996). However, very little is known about the signaling pathways involved in these regulations of TN-C.

Transforming growth factor- β (TGF- β) plays a critical role in a wide variety of biological processes, including proliferation, differentiation, extracellular matrix production, and apoptosis (Taylor and Khachigian, 2000). The diverse cellular responses elicited by TGF- β are triggered by the activation of serine/threonine kinase TGF- β receptors, and on the activation by TGF- β or related ligands, signaling from the receptors to the nucleus is mediated by the phosphorylation of cytoplasmic mediators called Smads (Heldin *et al.*, 1997). The receptor-associated Smads, such as Smad1, Smad2, Smad3, and Smad5, interact directly with,

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and are phosphorylated by, activated TGF- β receptor type I (Macias-Silva *et al.*, 1996; Nakao *et al.*, 1997). They are ligand-specific and form, upon phosphorylation, heteromeric complexes with Smad4. The latter functions as a common mediator for all the Smad pathways (Wu *et al.*, 1996; Candia *et al.*, 1997). These complexes are then translocated into the nucleus, where they function as transcription factors, possibly in association with other proteins, such as Sp1 (Czuwara-Ladykowska *et al.*, 2002). The third group of Smad proteins, the inhibitory Smads such as Smad6 or Smad7, prevent phosphorylation and/or nuclear translocation of receptor-associated Smads (Imamura *et al.*, 1997; Hata *et al.*, 1998).

A recent study by Rettig *et al.* (1994) demonstrated that TGF- β has various effects on TN-C expression in fibroblasts and that TGF- β reduces TN-C secretion in newborn fibroblasts. On the other hand, TGF- β increases TN-C synthesis in chick embryo fibroblasts or human renal cells (Chiquet-Ehrismann *et al.*, 1989; Gore-Hyer *et al.*, 2002). Verrecchia *et al.* (2001) demonstrated by cDNA microarray analysis that TN-C is one of the target genes of TGF- β and its expression is upregulated by TGF- β in human dermal fibroblasts. It has already been shown that TGF- β regulates α 2(I) collagen (Chen *et al.*, 1999; Verrecchia *et al.*, 2001) or plasminogen activator inhibitor-1 (PAI-1) (Stroschein *et al.*, 1999) expression via Smad3; however, the regulation of and mechanisms involved in TN-C expression by TGF- β in human dermal fibroblasts remain uncertain.

Therefore, we now report that TGF- β upregulates TN-C transcription in the human dermal fibroblast, and that the CAGA motif of the TN-C promoter is capable of binding Smad2/Smad3. In addition, we confirmed that overexpression of Smad3 or Smad4 mimicked this response, and that Sp1, Ets1, or CREB-binding protein (CBP)/p300 was involved in this signaling pathway. Taken together, these results provide evidence that a Smad-dependent pathway of TGF- β signaling is necessary for potent positive regulation of TN-C gene expression in dermal fibroblasts.

Results

TN-C mRNA expression is upregulated by TGF- β at the transcriptional level in human dermal fibroblasts

First, to determine whether TGF- β can mediate induction of the TN-C mRNA level, human dermal fibroblasts were incubated in the presence or absence of 5 ng/ml TGF- β under the same conditions, and mRNA expression was analysed by the Northern blotting. The TN-C mRNA level was elevated after the stimulation with TGF- β for 3 h, and this increase reached a maximum after 24 h (Figure 1a). Next, we determined whether TGF- β increased the stability of TN-C mRNA. Following the inhibition of transcription, the loss of TGF- β -induced TN-C mRNA was not significantly different from that observed in the TGF- β -untreated cells (Figure 1b). The induction of TN-C mRNA by TGF- β

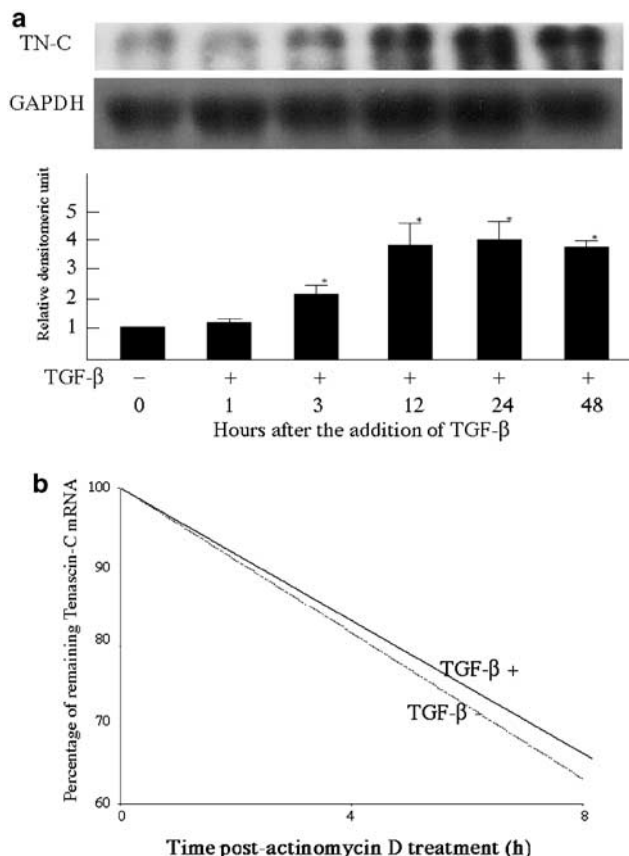


Figure 1 Induction of TN-C mRNA expression in human dermal fibroblasts by TGF- β , and effects of actinomycin D on TGF- β -mediated TN-C upregulation. **(a)** Cultured human fibroblasts were incubated in the presence or absence of 5 ng/ml TGF- β under the same conditions for the indicated time courses, and the Northern blot analysis of TN-C mRNA expression was performed. Levels of GAPDH mRNA are shown as a loading control. One experiment representative of three independent experiments is shown. TN-C mRNA levels quantitated by scanning densitometry and corrected for the levels of GAPDH in the same samples are shown relative to the level in untreated cells (1.0). Data are expressed as the mean \pm s.e. of three independent experiments. * P < 0.05 compared with the value in untreated cells. **(b)** Human dermal fibroblasts were serum-starved for 24 h and incubated in the absence or presence of 5 ng/ml TGF- β for 12 h before the addition of 2.5 μ g/ml actinomycin D. RNA was extracted from the cells at the indicated time after actinomycin D administration. Northern blot analysis of TN-C mRNA expression was performed. The corrected density by GAPDH levels was expressed as a percent of the value at time 0 and plotted on a logarithmic scale. The solid line indicates the TGF- β -treated levels, and the dotted line indicates control (untreated) levels. One experiment representative of three independent experiments is shown

and the failure of TGF- β to increase the half-life of TN-C mRNA suggest that the TGF- β -mediated induction of TN-C expression is regulated at the level of transcription.

Functional analysis of the TN-C promoter upregulation by TGF- β

To identify potential regulatory elements of the human TN-C gene by TGF- β , we performed transient transfection assays using a series of 5'-deletions of the TN-C

promoter linked to the chloramphenicol acetyltransferase reporter gene. The base pair (bp) $-248 \sim +75$ construct responded to TGF- β at the highest level, and the longer constructs with deletion end points at $-516 \sim +75$, $-1239 \sim +75$, and $-2100 \sim +75$ bp and the subsequent deletion $-133 \sim +75$ bp construct showed similar results. However, the $-29 \sim +75$ bp construct decreased reactivity to TGF- β significantly, whereas the $-248 \sim +21$ bp construct did not. These data indicate that the responsive element of the TN-C promoter gene to TGF- β is localized between -133 and -29 bp (Figure 2).

CAGA motifs of the TN-C promoter gene are capable of binding Smad3

Smad3 and Smad4 are able to bind to DNA directly through their N-terminal MH1 domain (Dennler *et al.*, 1998). Although previous studies described two different consensus sequences for Smad binding (GTCTAGAC, called Smad-binding element, and AG(C/A)CAGA CAC, called CAGA box) through a screening of Smad-binding DNA sequences using a random pool of oligonucleotides (Feng *et al.*, 1998), both sequences contain the core motif CAGA. This motif is present in the regulatory regions of several TGF- β target genes, including those for $\alpha 2(I)$ collagen (Chen *et al.*, 1999), PAI-1 (Dennler *et al.*, 1998; Stroschein *et al.*, 1999), type VII collagen (Vindevooghel *et al.*, 1998), Smad7 (von Gersdorff *et al.*, 2000), and PDGF-B-chain (Taylor and Khachigian, 2000).

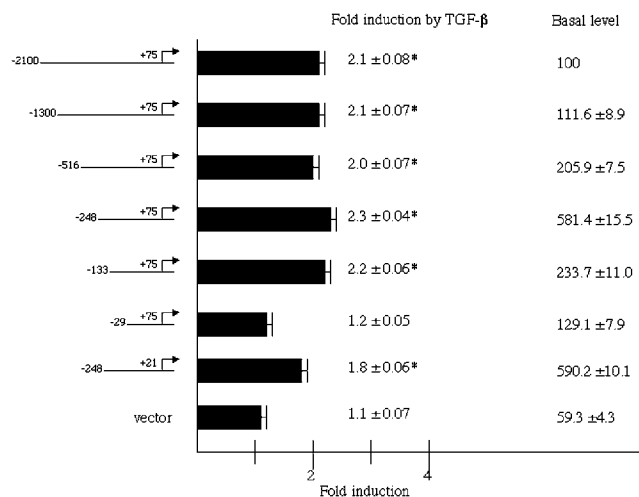


Figure 2 Identification of the TN-C promoter region mediating TGF- β stimulation. The indicated TN-C promoter deletion constructs or the corresponding empty construct were transfected in the absence or presence of 5 ng/ml TGF- β . The bar graph on the right represents fold stimulation of the promoter activity stimulated by TGF- β relative to the promoter activity without TGF- β , which was arbitrarily set at 1. The numbers on the right show the basal levels (i.e. without TGF- β) of each construct relative to the $-2100 \sim +75$ bp promoter, which was arbitrarily set at 100%. Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with the basal promoter activities of each construct ($P < 0.05$, Mann-Whitney U -test)

To determine whether the CAGA motif of the TN-C promoter can bind endogenous Smad2/Smad3 *in vitro*, we performed a DNA affinity precipitation assay. TN-C oligo contains TCTGG and CAGAG sequences of the TN-C promoter (positions $-66 \sim -62$ and $-44 \sim -40$ bp, respectively, Figure 3a). As a positive control, we used the 3 \times CAGA oligo, which is a trimer of the CAGA motif. We also used TN-C-M oligo in which the CAGA motif of TN-C oligo was mutated as follows: TCTGG was changed into TATAG and CAGAG was changed into TATAG. Fibroblasts were serum-starved for 24 h and treated with 5 ng/ml TGF- β for 1 h. The results showed that after TGF- β treatment, Smad2/Smad3 strongly bound to TN-C oligo, which corresponds to positions -78 to -27 bp of the human TN-C promoter. Similar results were obtained for 3 \times CAGA oligo, whereas TN-C-M oligo did not show Smad2/Smad3 binding even after TGF- β stimulation (Figure 3b). Subsequently, to examine the time dependency of the effect of TGF- β on the DNA binding of Smad2/Smad3, cells were incubated in serum-free medium for the indicated time in the presence or absence of 5 ng/ml TGF- β , which was added 1, 3, or 24 h prior to the protein extraction. The Smad3-DNA-binding level was elevated after 1 h compared with the level in untreated cells, and this increase was sustained until 24 h (data not shown). These results suggest that

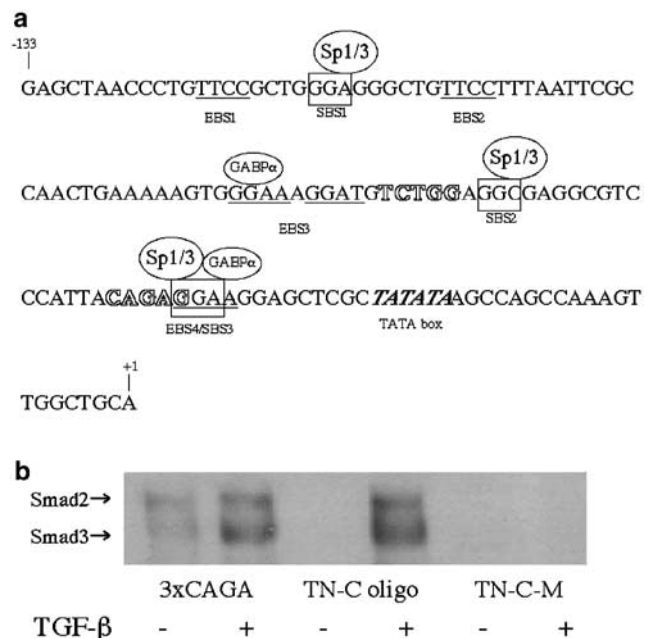


Figure 3 DNA affinity precipitation assay with biotin-labeled oligonucleotides containing the CAGA motifs. (a) Nucleotide sequence of the TN-C promoter region from position -133 to $+1$ bp. The four putative EBS are underlined. The three putative SBS are boxed. The CAGA motifs are outlined. (b) Cell lysates were prepared from dermal fibroblasts and incubated with biotin-labeled oligonucleotides, as described in 'Materials and methods'. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Smad2/3 was detected by immunoblotting analysis

Smad3 bound to this site in an inducible, transient, and specific manner.

Smad3 and Smad4 activate the TN-C promoter

To examine Smad involvement in the upregulation of TN-C transcription by TGF- β , Smads were transiently overexpressed in human fibroblasts cotransfected with the -248~+75 bp TN-C promoter construct. The results showed that ectopic Smad3 was able to upregulate the promoter activity in a dose-dependent manner. The Smad signaling partner Smad4 by itself upregulated the TN-C promoter moderately, whereas Smad2, which shares a high degree of structural similarity to Smad3, failed to upregulate TN-C. This upregulation by the transfection of Smad3 was enhanced significantly by TGF- β stimulation. In addition, we determined whether Smad members collaborate with other Smads to modulate the TN-C promoter activity. Coexpression of both Smad3 and Smad4 resulted in synergistic activation (Figure 4a). These results support that Smads are necessary for TGF- β induction of the TN-C promoter.

Next, the regulation of TN-C protein by Smad3 and Smad4 was examined by immunoblot analysis. At 24 h after the transfection, cells were treated with TGF- β (5 ng/ml) and then 72 h later media were collected. Smads were capable of up-regulating the TN-C protein synthesis in the presence or absence of TGF- β (Figure 4b and c).

The simultaneous transfection of the -133~+75 bp TN-C promoter construct and Smads resulted in a similar outcome, but in contrast, Smad3 or Smad4 was not able to activate the shorter TN-C promoter construct containing -29 to +75 bp of the promoter (Figure 4d). This demonstrates that Smad activation of the TN-C promoter is sequence-specific.

Participation of other transcription factors in TN-C upregulation by TGF- β

It was shown that the TN-C promoter between the -133 and +75 bp construct has four Ets-binding sites (EBS), three Sp1/3-binding sites (SBS), and two GABP-binding sites, and that the TN-C gene is regulated by Ets proteins and Sp1 as potent activators of the gene (Shirasaki *et al.*, 1999). To explore whether Sp1, Sp3, Ets-1, Ets-2, Fli1, GABP α , or GABP β 1 is involved in upregulated TN-C expression by TGF- β , the -248~+75 bp TN-C construct was cotransfected with increasing amounts of

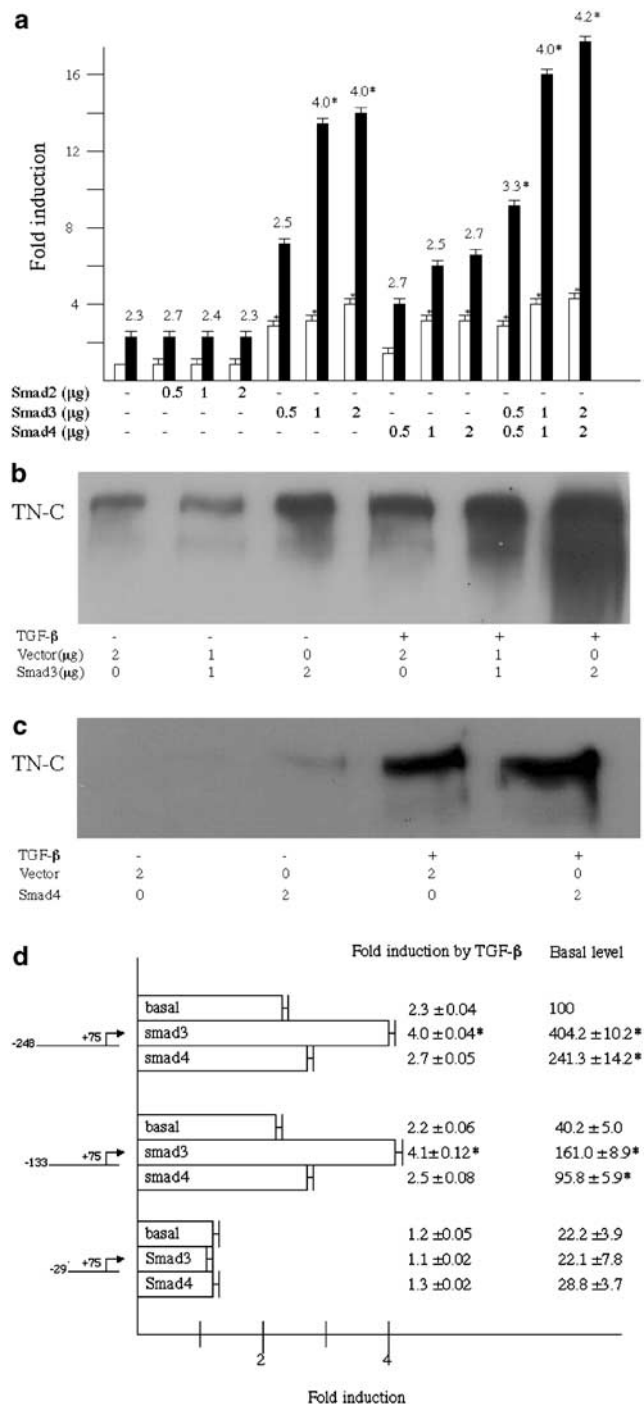


Figure 4 Smad3 and Smad4 are potent transactivators of the TN-C promoter. (a) Dermal fibroblasts were cotransfected with 2 μg of the -248~+75 bp TN-C promoter construct and indicated amounts of the Smad expression vector (or equal amounts of control vector) in the absence (open bars) or presence (closed bars) of 5 ng/ml TGF- β . The total amounts of DNA were kept constant by adding control vector. The graph depicts the TN-C promoter activities, and the basal promoter activity was arbitrarily set at 1. The numbers show the promoter activities stimulated by TGF- β relative to the promoter without TGF- β . Mean \pm s.e. from four independent experiments is presented. *Indicates statistically significant results compared with the -248~+75 bp promoter without Smad expression vectors ($P < 0.05$, Mann-Whitney U -test). (b, c) Overexpression of the Smad3 or Smad4 in human dermal fibroblasts was performed by transient transfection, as described in 'Materials and methods'. The conditioned media were analysed by immunoblot analysis. (d) The indicated TN-C promoter deletion constructs were cotransfected with 2 μg of Smads (or equal amounts of control vector) in the absence or presence of 5 ng/ml TGF- β . The bar graph on the right represents fold stimulation of the promoter activity stimulated by TGF- β relative to the promoter activity without TGF- β . The numbers on the right show the basal promoter activities (i.e. without TGF- β) of each construct relative to the -248~+75 bp promoter, which was arbitrarily set at 100%. Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with the promoter activities of each construct without Smads expression vectors ($P < 0.05$, Mann-Whitney U -test)

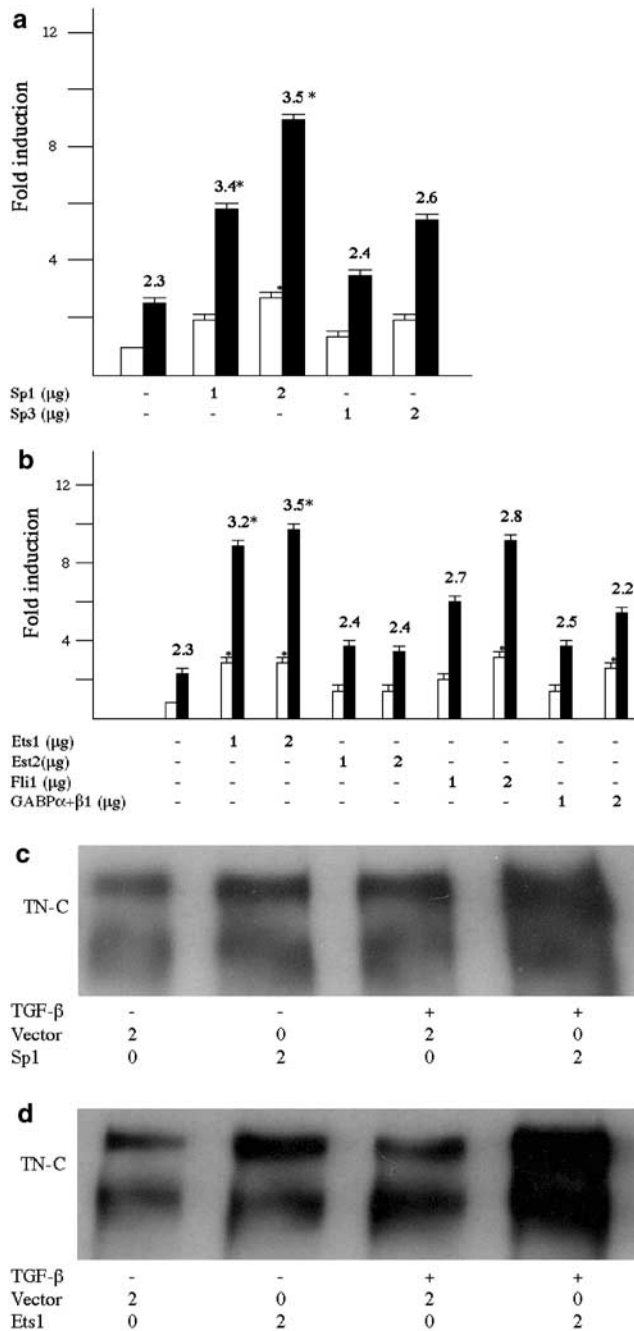


Figure 5 Sp1 and Ets1 are potent transactivators of the TN-C promoter. (a, b) Dermal fibroblasts were cotransfected with 2 μ g of the -248 ~ +75 bp TN-C promoter construct and indicated amounts of the indicated expression vectors (or equal amounts of control vector) in the absence (open bars) or presence (closed bar) of 5 ng/ml TGF- β . The total amounts of DNA were kept constant by adding control vector. The graph depicts the TN-C promoter activities, and the basal promoter activity was arbitrarily set at 1. The numbers show the promoter activities stimulated by TGF- β relative to the promoter activity without TGF- β . Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with the -248 ~ +75 bp promoter without other expression vectors ($P < 0.05$, Mann-Whitney U -test). (c, d) Overexpression of the Sp1 or Ets1 in human dermal fibroblasts was performed by transient transfection, as described in 'Materials and methods'. The conditioned media were analysed by immunoblot analysis

expression vectors of these transcriptional factors. As shown in Figure 5a and b, Sp1 or Ets1 strongly superinduced the TN-C promoter activity by TGF- β in a dose-dependent manner, whereas the others had less stimulatory effects in TGF- β -treated fibroblasts.

In addition, we determined whether the forced overexpression of Sp1 or Ets1 could enhance TGF- β -mediated TN-C protein induction. At 24 h after the transfection, cells were treated with TGF- β (5 ng/ml) and then, 72 h later, media were collected. Immunoblotting revealed that the transient transfection of Sp1 or Ets1 led to superinduction of TGF- β -mediated TN-C expression (Figure 5c and d). These data confirmed that Sp1 or Ets1 is involved in the TGF- β signaling pathway of TN-C.

Effects of mithramycin on the increased expression of the human TN-C promoter activity by TGF- β

Mithramycin is known as a highly specific inhibitor of Sp1 binding (Blume *et al.*, 1991). In previous studies, mithramycin was shown to inhibit the binding of Sp1 specifically, but not to inhibit the binding of other transcriptional factors. Therefore, this reagent was used to determine the role of Sp1 in the elevated expression of the TN-C gene by TGF- β .

Preincubation with 500 nM or 1 μ M mithramycin 1 h prior to stimulation by TGF- β inhibited the TN-C induction by TGF- β in dermal fibroblasts, whereas basal levels of the TN-C promoter activity were not affected by this reagent (Figure 6). These results strongly indicated that Sp1 is involved in the induction of the TN-C gene expression by TGF- β in human dermal fibroblasts.

Substitution mutations inhibit TN-C induction by TGF- β

To determine whether the binding sites of these transcriptional factors contribute to the TGF- β -

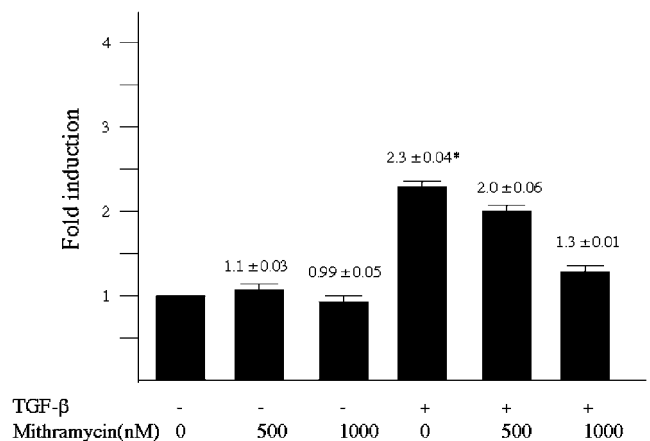


Figure 6 Blocking of increased expression of TN-C gene expression in human dermal fibroblasts by mithramycin. The levels of the TN-C promoter activity were investigated by transient transfection. The TN-C promoter deletion construct -248 ~ +75 bp was transfected in the absence or presence of 5 ng/ml TGF- β . The bar graph represents fold stimulation of the promoter activity, and the basal level was arbitrarily set at 1. Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with untreated cells ($P < 0.05$, Mann-Whitney U -test)

mediated promoter activity, the effects of substitution mutations changing TCTGG to TATAG (CAGA-1) and CAGAG to TATAG (CAGA-2) in the CAGA motif were investigated (Figure 3a). Mutating CAGA-1 resulted in a significant reduction of the promoter activity induced by TGF- β , while mutating CAGA-2 had less effect on the responsiveness (Figure 7a). Mutating both CAGA-1 and -2 showed the most significant reduction of the promoter activity induced by TGF- β . Thus, the CAGA motif is thought to be required for TGF- β -mediated TN-C promoter activation.

Next, the effects of substitution mutations changing GGA to TTA in EBS1-4 were investigated. Mutating either EBS1 or EBS4 resulted in the most significant reduction of the promoter activity induced by TGF- β , while mutating EBS2 or EBS3 had no effect on the responsiveness (Figure 7b). Thus, the four EBSs in the TN-C promoter may have different functions: EBS1 and EBS4 mediate the transactivation of the TN-C gene by TGF- β , whereas EBS2 or EBS3 does not function as a TGF- β response element.

In a similar way, the effects of substitution mutations in SBS were investigated. Mutating SBS1 showed a reactivity similar to the -248 ~ +75 bp TN-C construct, whereas SBS2 or SBS3 decreased the responsiveness to TGF- β significantly (Figure 7c). These results suggest that the integrity of SBS2 or SBS3 is critical for increased promoter activity of TN-C by TGF- β . In addition, mutated SBS2 and SBS3 did not increase the promoter activity by cotransfection with Smad3 or Smad4 (Figure 7c). Taken together, Sp1 binding to SBS2 or SBS3 may cooperate with Smad3.

Interaction between Smad3, Sp1, and Ets1 is associated with TN-C upregulation by TGF- β

First, to examine the amounts of Smad3, Sp1, and Ets1 in cell lysates, immunoblotting was performed using anti-Smad3, Sp1 or Ets1 antibodies. The immunoblotting revealed that the amounts of these proteins were unchanged by TGF- β stimulation (Figure 8a).

The interactions of Smad3/Sp1, Smad3/Ets1, or Ets1/Sp1 have already been reported (Dittmer *et al.*, 1997; Lindemann *et al.*, 2001; Czuwara-Ladykowska *et al.*, 2002). We next examined whether endogenous Smad3/

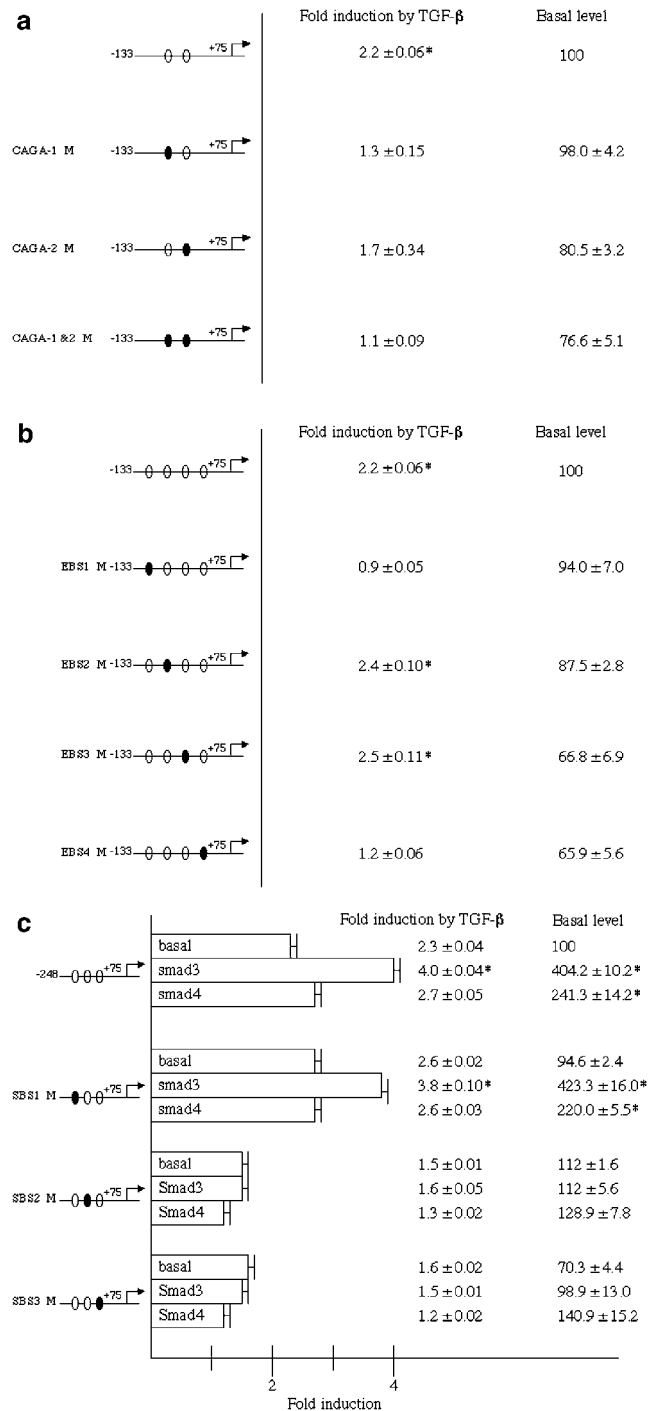


Figure 7 Identification of the functional Sp1/3 or Ets response elements in the human TN-C promoter. **(a)** The diagram on the left indicates the mutant CAGA motif. Mutated plasmids were transfected in the absence or presence of 5 ng/ml TGF- β . The number on the left represents fold stimulation of the promoter activity stimulated by TGF- β relative to the promoter activity without TGF- β , which was arbitrarily set at 1. The numbers on the right show the basal levels (i.e. without TGF- β) of each mutant construct relative to the -133 ~ +75 bp promoter, which was arbitrarily set at 100%. Mean \pm s.e. of four independent experiments is shown. *Indicates significant results compared with the basal promoter activity of each construct ($P < 0.05$, Mann-Whitney U -test). **(b)** The diagram on the left indicates mutant EBS. The mutants were constructed by replacing two guanines in the EBS core sequence with two thymidines, using the -133 ~ +75 bp TN-C promoter construct. **(c)** The diagram on the left indicates mutant SBS. Mutated plasmids were cotransfected with 2 μ g of Smads (or equal amounts of control vector) in the absence or presence of 5 ng/ml TGF- β . The bar graph on the right represents fold stimulation of the promoter activity stimulated by TGF- β relative to the promoter activity without TGF- β . The numbers on the right show the basal levels (i.e. without TGF- β) of each construct relative to the -248 ~ +75 bp promoter, which was arbitrarily set at 100%. Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with the promoter activities of each construct without Smad expression vectors ($P < 0.05$, Mann-Whitney U -test)

Sp1, Smad3/Ets1, and Ets1/Sp1 complexes were affected by TGF- β signaling. Cells were stimulated with TGF- β for 0, 1, 3, and 24 h, and Sp1 or Ets1 was immunoprecipitated using anti-Sp1 or anti-Ets1 antibodies.

The amount of Smad3 or Ets1 associated with the Sp1 complex increased after TGF- β treatment (Figure 8b). Furthermore, after TGF- β treatment for 1 h, the interaction of Smad3/Ets1 or Sp1/Ets1 was also observed (Figure 8c). Interaction of the Smad3/Sp1/

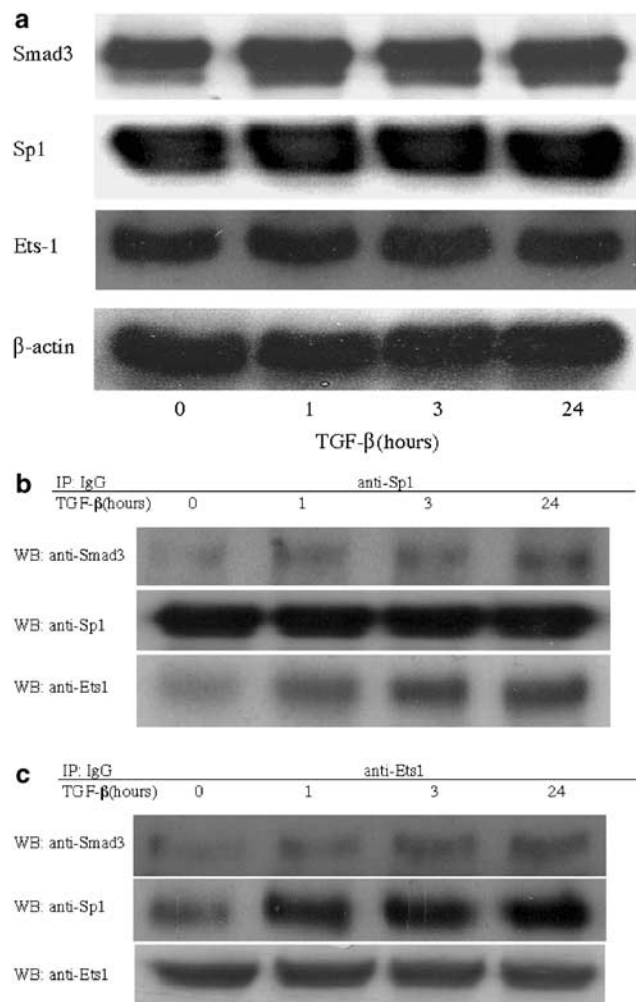


Figure 8 Interaction between Smad3, Sp1, and Ets1. (a) To determine the amounts of Smad3, Sp1 or Ets1 in cell lysates, human dermal fibroblasts were serum-starved for 24 h and treated with 5 ng/ml TGF- β for the indicated time. Immunoblotting was performed using anti-Smad3, Sp1 or Ets1 antibodies. The same membrane was then stripped and reprobed with anti- β -actin antibody to show as a loading control. (b) Human dermal fibroblasts were serum-starved for 24 h and treated with 5 ng/ml TGF- β for the indicated times. Cell lysates (500 μ g of protein/sample) were immunoprecipitated with anti-Sp1 antibody, followed by immunoblotting with anti-Smad3 or Ets1 antibody. The same membrane was then stripped and reprobed with anti-Sp1 antibody to determine the abundance of total Sp1 protein. (c) Human dermal fibroblasts were serum-starved for 24 h and treated with 5 ng/ml TGF- β for the indicated times. Cell lysates (500 μ g of protein/sample) were immunoprecipitated with anti-Ets1 antibody, followed by immunoblotting with anti-Smad3 or Sp1 antibody. The same membrane was then stripped and reprobed with anti-Ets1 antibody to determine the abundance of the total Ets1 protein

Ets1 complex by TGF- β was increased after TGF- β treatment for 1 h, and this increase was sustained until 24 h, which paralleled the induction of TN-C mRNA by TGF- β , as shown in Figure 1a. These results suggest that these three different proteins form a transcriptionally active complex after TGF- β treatment.

Presence of another signaling pathway of TGF- β /Smad in the TN-C promoter activation

Detailed analyses showed the cooperation of CBP/p300 with Smad in the TGF- β response of the α 2(I) collagen gene (Janknecht *et al.*, 1998; Ihn, 2002). We examined whether there is another signaling pathway involved in TN-C induction by TGF- β .

A histone acetyltransferase (HAT)-deficient p300 mutant or full-length p300 was ectopically expressed in dermal fibroblasts. As shown in Figure 9, basal or TGF- β -induced TN-C promoter activity was stimulated less effectively by this mutant form of p300 than by full-length p300. Stimulation of the TN-C promoter activity by a truncated CBP mutant 1891–2175, which solely constitutes the docking region for Smad3 and Smad4, was also reduced, whereas full-length CBP induced the TN-C promoter activity in the absence or presence of TGF- β . These results suggest that TGF- β regulates TN-C expression through CBP/p300, cooperating with Smads, in human dermal fibroblasts.

Discussion

To our knowledge, this is the first report showing that TGF- β induction of the TN-C gene in the human

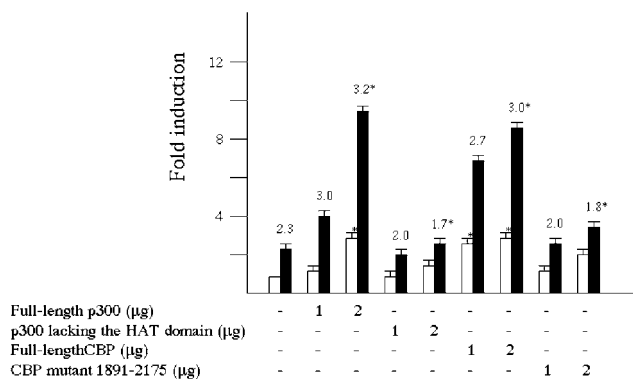


Figure 9 CBP/p300 involvement in TN-C upregulation by TGF- β . Dermal fibroblasts were cotransfected with 2 μ g of the -248 ~ +75 bp TN-C promoter construct and indicated amounts of the various expression vectors (or equal amounts of control vector) in the absence (open bars) or presence (closed bar) of 5 ng/ml TGF- β . The total amounts of DNA were kept constant by adding the control vector. The graph depicts the TN-C promoter activities, and the basal promoter activity was arbitrarily set at 1. The numbers show the promoter activities stimulated by TGF- β relative to the promoter activity without TGF- β . Mean \pm s.e. from four independent experiments is presented. *Indicates significant results compared with the -248 ~ +75 bp promoter without other expression vectors ($P < 0.05$, Mann-Whitney U-test)

dermal fibroblast is mediated by the Smad family of transcriptional regulators. In this paper, we demonstrated that the TGF- β -mediated induction of TN-C expression is regulated at the level of transcription. Serial 5' deletion and the transient transfection analysis defined a region in the TN-C promoter mediating inducible responsiveness to TGF- β . This region contains an atypical nucleotide recognition element for the Smad family that preserves the 5'-CAGA-3' motif. The DNA affinity precipitation assay revealed that the binding of endogenous Smad2/Smad3 to the TN-C promoter by TGF- β appears to be specific and physiologically relevant. This nucleoprotein complex was detected within 1 h after the exposure to TGF- β , consistent with rapid Smad translocation from the cytoplasm to the nucleus and its interaction with the PAI-1 promoter (Dennler *et al.*, 1998). Overexpression of Smad3 or Smad4 activated the TN-C promoter and superinduced the TN-C promoter activity by TGF- β , whereas Smad2 did not affect the TN-C upregulation significantly. These effects were sequence-specific. Simultaneous expression of Smad3 and Smad4 activated the TN-C promoter to a greater degree than an individual transfection of these proteins. Both Smad3 and Smad4 have been shown to bind to DNA directly, whereas Smad2 likely requires a DNA-binding intermediate (Macias-Silva *et al.*, 1996). The major difference between Smad2 and Smad3 resides in the DNA-binding MH1 domain. Possibly, the insertion in this domain of Smad2 alters its characteristics. These findings demonstrate the existence of a novel Smad-binding element in the TN-C promoter.

Once bound to DNA, the mechanism of transcriptional activation by Smad proteins remains elusive, although a complex interaction with multiple elements of the transcriptional machinery appears to be involved. For example, Smad proteins functionally interact with Sp1 in the regulation of the α 2(I) collagen promoter (Poncelet and Schnaper, 2001), NF- κ B in the type VII collagen promoter (Vindevooghel *et al.*, 1998), and AP1 in the c-Jun promoter (Wong *et al.*, 1999). In the context of the TN-C promoter, Sp1 or Ets1 is thought to be the highest superinducer of upregulated expression of TN-C by TGF- β . In addition, the involvement of Sp1 in TGF- β induction of TN-C was confirmed by adding mithramycin. The interactions of Smad3 with Sp1 or Ets1 were found to jointly form a complex in response to TGF- β , suggesting that these three different proteins form a transcriptionally active complex. Therefore, it is possible that Smad regulation of TN-C gene expression may involve cooperative interactions with these other factors including Sp1 or Ets1, because their nucleotide recognition elements lie in relatively close proximity to the Smad-binding site in the TN-C promoter. Synergism between Smad3 and Ets1 in the TGF- β pathway has been shown in the regulation of the parathyroid hormone-related protein (PTHrP) promoter gene in invasive breast cancer cells (Lindemann *et al.*, 2001). In these cells, Ets1 positively regulates TGF- β -induced activation of PTHrP expression and Sp1 regulates the

PTHrP promoter activity in cooperation with Ets1 (Dittmer *et al.*, 1997). In contrast, Ets1 suppressed TGF- β -dependent activation of α 2(I) collagen gene expression in human dermal fibroblasts (Czuwara-Ladykowska *et al.*, 2002). The synergism between Ets1/Smad3 or Sp1/Ets1 observed in the invasive breast cancer cells versus the antagonism observed in dermal fibroblasts may be related to different promoters, as well as to different cellular contexts.

We also examined p300/CBP involvement in TN-C upregulation by TGF- β . Several observations suggest that the CAGA sequence alone is not sufficient to support maximal TGF- β -mediated transcriptional activation of target genes (Poncelet and Schnaper, 2001). Receptor-associated Smads and Smad4 have also been shown to interact with the coactivators CBP/p300 (Wotton *et al.*, 1999; de Caestecker *et al.*, 2000). In our study, transactivation by mutants of p300 lacking the HAT domain or truncated CBP mutant 1891–2175, consisting of the Smad-binding region, was diminished. CBP/p300 do not themselves interact with a specific DNA sequence, but function as transcriptional coactivators or adapter proteins that are recruited to target promoters by sequence-specific DNA-binding proteins (Ghosh *et al.*, 2000). Recruitment of HATs to promoters by sequence-specific DNA-binding factors is likely to play a key role in regulating transcription (Ghosh *et al.*, 2000). Thus, our results suggest that p300 mediates TN-C activation by TGF- β , interacting with the TN-C promoter through its intrinsic HAT. On the other hand, the CBP mutant 1891–2175 competitively inhibits CBP/p300 from coactivating with Smad3, acting as a dominant-negative molecule (Janknecht *et al.*, 1998). Thus, CBP is thought to interact with Smad3 in the TGF- β signaling pathway. Taken together, a hypothetical model of the TGF- β -dependent response of the TN-C promoter gene was provided by our results, as shown in Figure 10.

Smad regulation of TN-C transcription may also occur in fibrotic pathologic settings. For example, it is known that expression of TN-C is increased in the dermis in systemic sclerosis (SSc), an autoimmune disorder characterized by the excessive deposition of extracellular matrix components in the skin and internal organs (Rodnan *et al.*, 1979; Lacour *et al.*, 1992; LeRoy, 1992). TGF- β signaling has been implicated in the primary pathogenesis of fibrosis (Border and Noble, 1994). Previously, we reported that although SSc fibroblasts secreted amounts of TGF- β similar to those secreted by control fibroblasts, the blockade of TGF- β signaling with anti-TGF- β antibodies or a TGF- β 1 antisense oligonucleotide abolished the increased mRNA expression, as well as the upregulated transcriptional activity of the human α 2(I) collagen gene in SSc fibroblasts (Ihn *et al.*, 2001). In addition, we have reported the overexpression of TGF- β receptor type I and type II in SSc fibroblasts compared to normal human dermal fibroblasts (Kawakami *et al.*, 1998). Also, increasing the TGF- β receptor level induced collagen promoter activity (Kawakami *et al.*, 1998).

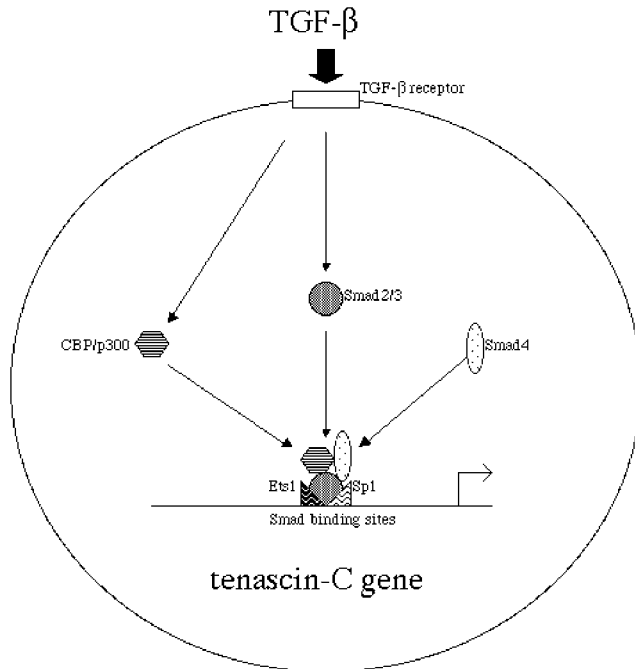


Figure 10 Schematic representation of the TGF- β signaling cascade of the TN-C promoter. TGF- β recruits receptor-associated Smads, such as Smad2 and Smad3, leading to the association with Smad4 and the translocation of the formed heterocomplex into the nucleus. The translocated heterocomplex binds to the target gene with other transcription factors, such as Sp1 or Ets1, and regulates the gene expression cooperating with p300/CBP

Furthermore, in SSc fibroblasts, increased phosphorylation and nuclear accumulation of endogenous Smad2/Smad3 were observed in the absence of exogenously added TGF- β (Chin *et al.*, 2001; Varga, 2002). Thus, overexpression of $\alpha 2(I)$ collagen may result from the activation of autocrine TGF- β signaling. Apart from $\alpha 2(I)$ collagen, the following Smad-regulated genes involved in fibrosis have been reported: elastin (Kucich *et al.*, 2002), tissue inhibitor of metalloproteinases (Gore-Hyer *et al.*, 2002), PAI-1 (Denkler *et al.*, 1998), oncogenes (c-fos, c-jun, JunB, others) (Jonk *et al.*, 1998; Lopez-Rovira *et al.*, 2000), PDGF, connective tissue growth factor (CTGF) (Ashcroft *et al.*, 1999; Taylor and Khachigian, 2000; Holmes *et al.*, 2001; Piek *et al.*, 2001), collagenase-1 (Yuan and Varga, 2001), $\beta 5$ integrin (Lai *et al.*, 2000), α -smooth muscle actin (You and Kruse, 2002), and apoptosis genes (Jang *et al.*, 2002). These findings suggest that TN-C is also upregulated in SSc due to autocrine TGF- β signaling, and that blockade of the TGF- β /Smad signaling pathway may also have therapeutic value by reducing the amount of TN-C as well as $\alpha 2(I)$ collagen in fibrotic diseases.

Materials and methods

Reagents

Recombinant human TGF- $\beta 1$ was obtained from R & D systems (Minneapolis, MN, USA). Actinomycin D, mithramycin A, and antibody for β -actin were purchased from Sigma

(St Louis, MO, USA). Anti-Smad2/3 antibody was from Transduction Laboratories (Lexington, KY, USA). Anti-Smad3, -Sp1, and -Ets1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell cultures

Human dermal fibroblasts WS-1 (derived from the midscapular skin of a 12-week female embryo, CRL-1502) were obtained from ATCC (Manassas, VA, USA). Primary explant cultures were established in 25-cm² culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 50 μ g/ml gentamycin, as described previously (Ihn *et al.*, 1996, 1997). Monolayer cultures were maintained at 37°C in 5% CO₂ in air. Fibroblasts between the third and sixth subpassages were used for experiments.

Immunoblotting

Human dermal fibroblasts were cultured, and then the medium was collected. Aliquots of conditioned medium (35 μ l; 3.5% of the medium from one well) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 1% dry fat milk for 1 h and incubated overnight at 4°C with an anti-human TN-C monoclonal antibody (NEC1b) (kindly provided by Dr Wolfgang Rettig) (Rettig and Garin-Chesa, 1989). The membranes were washed in Tris-buffered saline (TBS) and 0.1% Tween 20, incubated with anti-mouse antibody conjugated to horseradish peroxidase (1:1000 dilution), and washed again. The TN-C bands were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).

To examine the amounts of immunoreactive Smad3, Sp1, or Ets1 in cell lysates, dishes of confluent normal cells were washed twice with cold phosphate-buffered saline (PBS); subsequently, the monolayers were lysed in 1 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin. Aliquots containing 20 μ g of lysate were electrophoresed on 10% polyacrylamide gels at 100 V for 1 h. Following electrophoresis, the blots were transferred to a membrane, blocked for 1 h with 3% powdered milk dissolved in TBS and 0.1% Tween 20, and then incubated overnight at 4°C with polyclonal IgG anti-Smad3 antibody, anti-Sp1 antibody or anti-Ets1 antibody (1:500). As a loading control, immunoblotting was also performed using antibodies against β -actin (1:1000).

RNA preparation and Northern blot analysis

Total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method, and analysed by Northern blotting as described previously (Ihn *et al.*, 1996, 1997). RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels and blotted onto nylon filters (Roche Diagnostics, Indianapolis, IN, USA). The filters were UV crosslinked, prehybridized, and sequentially hybridized with cDNA probes. The following cDNA probes were used: human TN-C 1431-bp *EcoRI* fragment (kindly provided by Dr Mario Bourdon, La Jolla Institute for Experimental Medicine, La Jolla, CA, USA) and GAPDH 437-bp *BamHI-EcoRI* fragment. The membranes were then washed and exposed to X-ray film.

Immunoprecipitation

To prepare the extracts of total cellular proteins, fibroblasts were washed with PBS at 4°C and solubilized in a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin. Total cellular protein (500 μ g) was incubated with antibodies to the Sp1 or Ets1 at 4°C overnight, followed by 2 h incubation with protein G sepharose at 4°C. After three washes in lysis buffer, the immunocomplexes were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and incubated with anti-Smad3, -Sp1, or -Ets1 antibody (1:500). The membrane was washed and then incubated with a secondary antibody against goat (for Smad2/3) or rabbit (for Sp1 or Ets1) IgG (1:500) for 1 h.

DNA affinity precipitation assay

Three oligonucleotides containing biotin on the 5'-nucleotide of the sense strand were used in the assays. The sequences of these oligonucleotides are as follows: (1) 3 \times CAGA oligo, 5'-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACTCGAG, which is a trimer of the CAGA motif; (2) TN-C oligo, 5'-TGGGAAAGGATGTCTGGAGGCGAGGCGTCCCATTACAGAGGAAGGAGCTCGC, which corresponds to positions -78 to -27 bp of the human TN-C promoter; (3) TN-C-M oligo, 5'-TGGGAAAGGATGTATA GAGGCGAGGCGTCCCATTATATAGGAAGGAGCTCGC, which has a mutated CAGA motif of TN-C oligo. These oligonucleotides were annealed to their respective complementary oligonucleotides, and double-stranded oligonucleotides were gel-purified and used. Cell lysate was extracted using a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin. Poly(dI-dC) (5 μ g) competitor was incubated with 500 μ g of cell lysate for 30 min at 4°C, followed by 1 h incubation with 500 pmol of each double-stranded oligonucleotide. After the incubation, 65 μ l of streptavidin–agarose (Sigma) was added to the reaction and incubated at 4°C overnight. The protein–DNA–streptavidin–agarose complex was washed three times with lysis buffer and loaded onto a sodium dodecyl sulfate–polyacrylamide gel (Yagi *et al.*, 2001). Detection of Smad3 was performed with the anti-Smad2/Smad3 antibody (1:500).

Plasmids

The expression vector for Smad3 was kindly provided by Dr Harvey F Lodish (Xiao *et al.*, 2000). The expression vector for Smad4 was kindly provided by Dr Rik Derynck (Wu *et al.*, 1997). Constructs containing N-terminal FLAG-tagged full-length p300 and N-terminal FLAG-tagged p300 lacking the HAT domain (amino acids 1472–1522) were kindly provided

by Dr Joan Boyes (Boyes *et al.*, 1997). Full-length CBP and truncated CBP mutant 1891–2175 were obtained from Dr Ralf Janknecht (Janknecht *et al.*, 1998).

PTN-I-CAT construct was kindly provided by Dr Roberto Gherzi (Gherzi *et al.*, 1995). The deletion constructs were generated by PCR using pTN-I-CAT as a template, and substitution mutations were generated using a Quick Change site-directed mutagenesis kit (Stratagene, LaJolla, CA, USA) and confirmed by sequencing (Shirasaki *et al.*, 1999). The expression vectors for Sp1, Sp3, Ets1, Ets2, Fli1, GABP α , and GABP β 1 were described previously (Shirasaki *et al.*, 1999). Plasmids used in the transient transfection assays were purified twice on cesium chloride (CsCl) gradients, as described previously (Ihn *et al.*, 1997). At least two different plasmid preparations were used for each experiment.

Transient transfection

Fibroblasts were grown to 50% confluence in 100-mm dishes in MEM with 10% FCS. The medium was replaced with serum-free medium, and fibroblasts were transfected with the TN-C promoter constructs, expression vectors, or corresponding empty constructs, employing FuGENE6 (Roche) as described previously (Ihn *et al.*, 2002). In order to control for minor variations in the transfection efficiency, 1 μ g pSV- β -galactosidase vector (Promega, Madison, WI, USA) was included in all transfections. After 24 h incubation, cells were incubated for 24 h additionally in the absence or presence of 5 ng/ml TGF- β . Subsequently, cells were harvested in 0.25 M Tris-HCl (pH 8) and fractured by freeze-thawing. Extracts, normalized for protein content as measured by the Bio-Rad reagent, were incubated with butyl-CoA and [¹⁴C]chloramphenicol for 90 min at 37°C. Butylated chloramphenicol was extracted using an organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting. Each experiment was performed in duplicate.

Statistical analysis

Data presented as bar graphs are the mean \pm s.e. of at least three independent experiments. Statistical analysis was performed using the Mann–Whitney *U*-test ($P < 0.05$ was considered significant).

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