Stimulation of Type I Collagen Transcription in Human Skin Fibroblasts by TGF-β: Involvement of Smad 3

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Transforming growth factor-β (TGF-β) stimulates the transcription of the α2(I) procollagen gene (COL1A2). The intracellular mediators involved in this response remain poorly understood. In this study, we demonstrate that primary human skin fibroblasts express Smads, a novel family of signaling molecules, *in vitro* in the absence of TGF-β. The levels of Smad 7 mRNA was rapidly and transiently increased by TGF-β. Transient overexpression of Smad 3 and Smad 4, but not Smad 1 or Smad 2, caused *trans*-activation of a CAT reporter gene driven by a 772 bp segment of the human COL1A2 promoter containing putative TGF-β response elements. Smad stimulation of promoter activity was ligand independent, but was further enhanced by TGF-β. Overexpression of a phosphorylation-deficient Smad 3 mutant or wild-type Smad 7, which lacks the carboxy-terminal phosphorylation motif, specifically inhibited TGF-β-induced activation of COL1A2 promoter. A CAGACA sequence shown to be a functional Smad-binding element in the plasminogen activator inhibitor-1 gene promoter was found within the TGF-β-response region of the proximal COL1A2 promoter. Gel mobility shift assays showed protein phosphorylation-dependent binding activity in fibroblast nuclear extracts specific for this sequence; TGF-β treatment strongly stimulated the formation of this DNA-protein complex. Smad was identified as a component of the CAGACA-binding transcription complex in TGF-β-treated fibroblasts by antibody supershifting. These results demonstrate that (i) Smad 3 transmits TGF-β signals from the receptor to the COL1A2 promoter in human fibroblasts, and is likely to play an important role in stimulation of COL1A2 promoter activity elicited by TGF-β; (ii) in fibroblasts, Smads appear to function as inducible DNA-binding transcription factors; and (iii) Smad 7 may be involved in autocrine negative feedback in the regulation of COL1A2 promoter activity by TGF-β. *Key word: Smad signaling. J Invest Dermatol 112:49–57, 1999*

Type I collagen is the major structural component of the extracellular matrix. Transforming growth factor-β (TGF-β) stimulates type I collagen transcription (Penttinen *et al*, 1988; Inagaki *et al*, 1994; Jimenez *et al*, 1994; Chung *et al*, 1996). Transient transfection studies with α2(I) procollagen gene (COL1A2) promoter constructs have localized a complex TGF-β response element called TbRE to a region between –330 bp and –255 bp (Inagaki *et al*, 1994). The functional TbRE consists of a GC-rich 5′ portion and Box B. In contrast to the detailed structural analysis of TGF-β-responsive elements in the COL1A2 promoter, little is currently known about the intracellular signaling mechanisms that enable TGF-β to stimulate collagen transcription *in vivo* and *in vitro*.

TGF-β signaling in mammalian cells is mediated through interactions with type I and type II TGF-β receptors. These transmembrane serine-threonine kinase receptors exist as independent homodimers in the absence of ligand (Massagué, 1996). Binding of TGF-β to the high-affinity type II receptor results in recruitment of the type I receptor into a ternary signaling complex. The type I receptor then becomes phosphorylated, enabling it to propagate the signal to intracellular targets. Genetic screens in *Drosophila*, *Xenopus*, and *C. elegans* have identified a novel class of genes whose products function downstream from receptors for TGF-β-like ligands, and regulate architectural patterns of development (Sekelsky *et al*, 1995; Graff *et al*, 1996; Savage *et al*, 1996). Nine distinct vertebrate homologs of these proteins, called Smads, have been cloned to date (Hoodless *et al*, 1996; Chen *et al*, 1997a; Liu *et al*, 1996; Zhang *et al*, 1996; Topper *et al*, 1997). The identification of Smad mutations in human cancers suggests a role for these genes as tumor suppressors (Hahn *et al*, 1996; Eppert *et al*, 1996). The Smads share conserved amino- and carboxy-terminal domains separated by a more divergent proline-rich linker region. Smad 1 and Smad 5 signal downstream of bone morphogenetic protein, whereas Smad 2 and its close homolog Smad 3 are thought to be activated primarily by TGF-β and activin (Chen *et al*, 1996; Hoodless *et al*, 1996; Liu *et al*, 1996; Macías-Silva *et al*, 1996; Yingling *et al*, 1996; Nakao *et al*, 1997b). It is likely that Smads are also involved in signaling by agonists not related to the TGF-β superfamily (Mucsi and Goldberg, 1997). After stimulation of cells with TGF-β, cytoplasmic Smad 2 and Smad 3 interact transiently with the activated type I TGF-β receptor, become phosphorylated at carboxy-terminal serine residues, and associate with Smad 4 (Lagna *et al*, 1996; Macías-Silva *et al*, 1996; Zhang

Manuscript received July 1, 1998; revised October 7, 1998; accepted for publication October 9, 1998.

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Abbreviations: GST, glutathione-S-transferase; PAI-1, plasminogen activator inhibitor-1; SBE, Smad binding element.

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Table I. Sequences and positions of primers used in RT-PCR assays

mRNA		Primer sequence (5′–3′)[a]	GenBank accession	Position (5′–3′)	Size of amplified product (bp)
Smad 2	S	GGAGCAGAATACCGAAGGCA	AF 68018	1322	128
	AS	CTTGAGCAACGCACTGAAGG		1449	
Smad 6	S	CAAGCCACTGGATCTGTCCGA	AF035528	1800	321
	AS	TTGCTGAGCAGGATGCCGAAG		2120	
Smad 7	S	ATGCTGTGCCTTCCTCCGCT	AF015261	680	494
	AS	CGTCCACGGCTGCTGCATAA		1173	
GAPDH	S	TGACCACAGTCCATGCCATC		584	609
	AS	TACATGGCAACTGTGAGGAGG		1192	

[a]S, sense orientation; AS, antisense orientation.

et al, 1996; Liu *et al*, 1997; Nakao *et al*, 1997; Wu *et al*, 1997). The hetero-oligomeric Smad 2/3–Smad 4 complex translocates into the nucleus, where it regulates the transcription of target genes through as-yet poorly understood mechanisms. Recently, Smad 6, Smad 7, and Smad 9 have been identified in endothelial and epithelial cells. These closely related proteins form a structurally and functionally distinct class of Smads, and act as negative effectors of TGF-β signaling *in vitro* (Topper *et al*, 1997; Imamura *et al*, 1997; Nakao *et al*, 1997).

In this study, we investigated the role of Smad signaling in mediating the effects of TGF-β on the transcription of the type I collagen gene. The results indicate that Smad 3 and Smad 4 are expressed in primary human fibroblasts, *trans*-activate the COL1A2 promoter *in vitro*, and show inducible DNA-binding activity specific for a TGF-β-response element of the COL1A2 promoter. Smad 7, whose expression in fibroblasts is rapidly induced by TGF-β, abrogates ligand-induced stimulation of collagen promoter activity, and may play a role in an intracellular negative feedback loop for TGF-β signaling.

MATERIALS AND METHODS

Fibroblast cultures Primary cell cultures were established from neonatal foreskin biopsies by previously described explant techniques (Varga *et al*, 1987), and COS cells were from ATCC. Media were obtained from Biowhittaker (Walkersville, MD); all other tissue culture reagents were from Gibco BRL (Grand Island, NY). Cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's (COS cells) or modified Eagle's medium supplemented with 10% fetal calf serum, 1% vitamins, 1% penicillin/streptomycin, and 2 mM L-glutamine, and studied between passages 4–8. When the cells reached confluence, fresh medium with varying concentrations of fetal calf serum with or without TGF-β2 (from Celtrix, Santa Clara, CA), or TGF-β1 (from Amgen, Thousand Oaks, CA) was added, and cultures were harvested following a further 24–48 h incubation.

Extraction and analysis of RNA At the end of each experiment, total RNA was isolated from fibroblasts maintained in medium with 1% fetal calf serum using TRIZOL Reagent (Gibco BRL). Relative levels of mRNA were examined by northern analysis with [α-^{32}P]dCTP-labeled cDNA probes. Following washing of the nylon membranes, the cDNA-mRNA hybrids were visualized by autoradiography on Kodak X-AR5 films exposed for 24–72 h with intensifying screens. The following cDNA probes were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.4 kb restriction fragment including the entire coding region of Smad 3, and a 1.6 kb restriction fragment including the entire coding region of Smad 4 (Zhang *et al*, 1996).

Coupled reverse transcription-polymerase chain reaction (RT-PCR) assay To examine the expression of Smad 2, Smad 6, and Smad 7 mRNA in fibroblasts, a coupled RT-PCR assay was employed. At the end of the indicated periods of incubation with or without TGF-β1, total RNA was extracted from confluent fibroblasts, as described for northern analysis. Samples were treated with RNase-free DNase according to the manufacturer's instructions (Promega, Madison, WI) to remove potentially contaminating DNA. One microgram of RNA was then copied into cDNA in 12 μl reaction mixture using the SuperScript Preamplification System for first-strand cDNA synthesis kit (Gibco BRL). Aliquots of the resulting cDNA were then subjected to PCR amplification in the presence of 1 unit of AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT), 15 μM of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 10 Ci [α-^{32}P] dCTP, and 2.5 μl PCR Buffer (Perkin-Elmer) in a total volume of 25 μl. Oligonucleotide primers specific for Smad 2, Smad 6, and Smad 7 cDNA shown in **Table I** were designed using "Primer Design" software (Scientific and Educational Software, Durham, NC). The reaction was incubated at 94°C for 30 s and at 72°C for 30 s, stopped at 72°C for 5 min, and cooled on ice. At the end of the amplification, the size of the PCR products was determined by comparison with standard DNA markers, using 8% polyacrylamide gel electrophoresis and ethidium bromide staining. In other experiments, 9 μl of each reaction was taken at different amplification cycles, and analyzed by electrophoresis. The gels were then dried and exposed to Kodak X-ray film for 5 min. The segments of the gel corresponding to the bands detected by autoradiography were excised, and the amount of radioactivity was determined by liquid scintillation counting. As positive controls, duplicate samples of cDNA were amplified in parallel with GAPDH-specific primers, and analyzed as described above. As negative control, cDNA synthesis without RT was performed to exclude PCR contamination. Experiments were repeated three times.

Transient transfections For transient transfection experiments, the p3TP-lux, plasminogen activator inhibitor-1 (PAI-1)-CAT, COL1A2-CAT, and pSV0CAT constructs were used as reporters. The p3TP construct contains three concatamerized repeats of the AP-1 site of the collagenase promoter, and the TGF-β response element of the PAI-1 promoter; the PAI-1-CAT chimeric construct contains sequences from –1237 to +20 bp of the rat PAI-1 promoter fused to the CAT reporter gene (Bruzdzinski *et al*, 1993); the COL1A2-CAT construct contains sequences from –772 to +58 bp of the human COL1A2 promoter fused to the CAT reporter gene (Ihn *et al*, 1997); and pSV0CAT is a negative control plasmid. Fibroblasts were grown to 70% confluence in 60 mm dishes. The media were changed, and 4 h later cultures were transfected with the reporter plasmids (20 μg), along with the indicated wild-type or mutant expression plasmids or corresponding empty constructs (1 μg), employing the calcium-phosphate/DNA coprecipitation method. Expression vectors for human Smad 1 and Smad 2 (gifts from L. Attisano, University of Toronto, Toronto) contain in-frame amino-terminal FLAG epitope tag (Eppert *et al*, 1996; Hoodless *et al*, 1996). Expression vectors for Smad 4 (from R. Derynck, University of California, San Francisco), and Smad 3 and Smad 3A, which carries three carboxy-terminal serine-to-alanine substitutions (from H. Lodish, Whitehead Institute), contain the FLAG epitope tag at the amino-terminal domain (Zhang *et al*, 1996; Liu *et al*, 1997). Expression vector for Smad 7 (from P. ten Dijke, Ludwig Institute for Cancer Research) was constructed by subcloning a Smad 7 cDNA in the pcDNA3 expression vector (Nakao *et al*, 1997). Expression vector for Smad 6 (from M. Kawabata, Japanese Foundation for Cancer Research) contains a FLAG epitope in the amino terminal domain in pcDNA3 (Imamura *et al*, 1997). Expression vector for Smad 9 (from T. Watanabe, Otsuka Pharmaceuticals) was constructed by subcloning a Smad 9 cDNA in the pT7Blue expression vector (Watanabe *et al*, 1997). A Smad 2 mutant expression vector (from H. Goldberg, Hospital for Sick Children) was constructed by subcloning a Smad 2 cDNA with a premature stop codon in the pcDNA3 expression vector (Mucsi and Goldberg, 1997). In order to control for minor variations in transfection efficiency, 50 ng renilla luciferase DNA (Promega), which expresses luciferase under the control of the cytomegalovirus promoter, was included in all transfections. Fresh media containing 0.1% fetal calf serum and TGF-β1 or TGF-β2 (12.5 ng per ml) were then added, and the cells were harvested 48 h after transfection. The total protein content of the extracts was quantitated by the Bradford Coomassie Blue G binding assay (Bradford, 1976) (Bio-Rad Laboratories, Richmond, CA). Identical amounts of protein from each cell extract (30 μg per assay) were used for parallel determination of CAT activity. CAT activity was determined by an organic solvent extraction assay using [^{14}C] chloramphenicol and

butyryl-CoA at assay conditions predetermined to be within the linear range for CAT activities of the samples. Radioactive acetylated chloramphenicol was quantitated by liquid scintillation counting. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Preparation of nuclear extracts and electrophoretic gel mobility shift assay Fresh media with or without TGF-β1 (12.5 ng per ml) were added to confluent fibroblasts. At the end of the indicated incubation periods, nuclear extracts were prepared as described (Andrews and Faller, 1991), and protein concentrations were determined by the Bio-Rad assay. Glutathione-S-transferase (GST)-Smad 3 MH1-domain fusion protein vectors (Zawel *et al*, 1998) were expressed in *Escherichia coli* and partially purified by chromatography on glutathione-Sepharose 4B columns (Pharmacia, Piscataway, NJ). Double-stranded oligonucleotides corresponding to a 24 bp sequence spanning –272 to –249 bp of the COL1A2 promoter (COL1A2-CAGA, 5′-GGAGGTATGCAGACAACGAGTCAG-3′) harboring the CAGACA motif, a 24 bp sequence spanning –386 to –363 bp of the COL1A2 promoter (COL1A2–386/–363, 5′-CTAGCGGCCTCTAGACGTTTAAGA-3′) harboring a CAGA motif, or a 26 bp sequence [Smad binding element (SBE), 5′-GGAGTATGTCTAGACTGA-CAATGTAC-3′] harboring a consensus palindromic Smad-binding element (Zawel *et al*, 1998) were generated. Additional probes were prepared by introducing subsitution mutations (underlined) in the –272 to –249 COL1A2 sequence: COL1A2-CAGA-m2, 5′-GGAGGTATG<u>ACT</u>ACAACGAGTCAG-3′; COL1A2-CAGA-m3, 5′-GGAGGTAT-GCAG<u>CAC</u>ACGAGTCAG-3′; COL1A2-CAGA-m4, 5′-GGAGGTATGCAGACA<u>CAT</u>-AGTCAG-3′; and COL1A2-CAGA-m5, 5′-GGAGGTATG<u>ACTCAC</u>ACGAGTCAG-3′. The probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, or with [α-^{32}P]dATP using Klenow fragment of DNA polymerase I for the phosphatase studies. Electrophoretic gel mobility shift assays were performed in binding reactions (total volume 20 μl) containing 2 μg of double-stranded poly[d(I-C)], 3–10 μg nuclear extract or 1 μg of recombinant Smad 3, and 100,000 cpm of radiolabeled probes (≈0.2–0.5 ng). In order to establish the specificity of the protein-DNA complexes, unlabeled oligonucleotide competitors in 2–50-fold molar excess were added in the binding reactions 5 min before the labeled probes. Polyclonal SED antibodies (1–10 μl, as indicated) to Smad 2/3 (Nakao *et al*, 1997), or pan-FOS antibodies, which react with all members of the FOS family (Santa Cruz Biotechnology, SC-235X), or preimmune serum were added to the binding reactions 15 min prior to the probes. Following incubation of the reaction mixtures on ice for 30 min, protein-DNA complexes were resolved from free probes in nondenaturing 5.5% polyacrylamide gels using low ionic strength buffers. The gels were then dried under vacuum and exposed to X-ray film at –70°C. To examine the effect of *in vitro* dephosphorylation on DNA-binding activity, nuclear extracts were incubated with 0.8 units per g protein calf intestinal alkaline phosphatase (MBI Fermentas, Amherst, NY) at room temperature for 30 min, followed by the phophatase inhibitor sodium orthovanadate (Sigma, St. Louis, MO), and then examined by gel shift assays with Klenow-labeled DNA probes, as described above.

Immunoblotting and western analysis In order to detect endogenous Smad proteins, nuclear extracts from confluent fibroblasts incubated with TGF-β1 or left untreated for 48 h were prepared, and boiled at 90°C with sample buffer for 5 min. Extracts (15–30 μg) were subjected to electrophoresis in 7% SDS polyacrylamide gels in a minigel apparatus for 1 h. The proteins were then trans-blotted onto nitrocellulose membranes for 30 min at 15 V. After blocking with 5% milk, the membranes were incubated with primary antibodies or preimmune serum for 1 h, followed by biotinylated goat anti-rabbit IgG secondary antibodies for 1 h, and

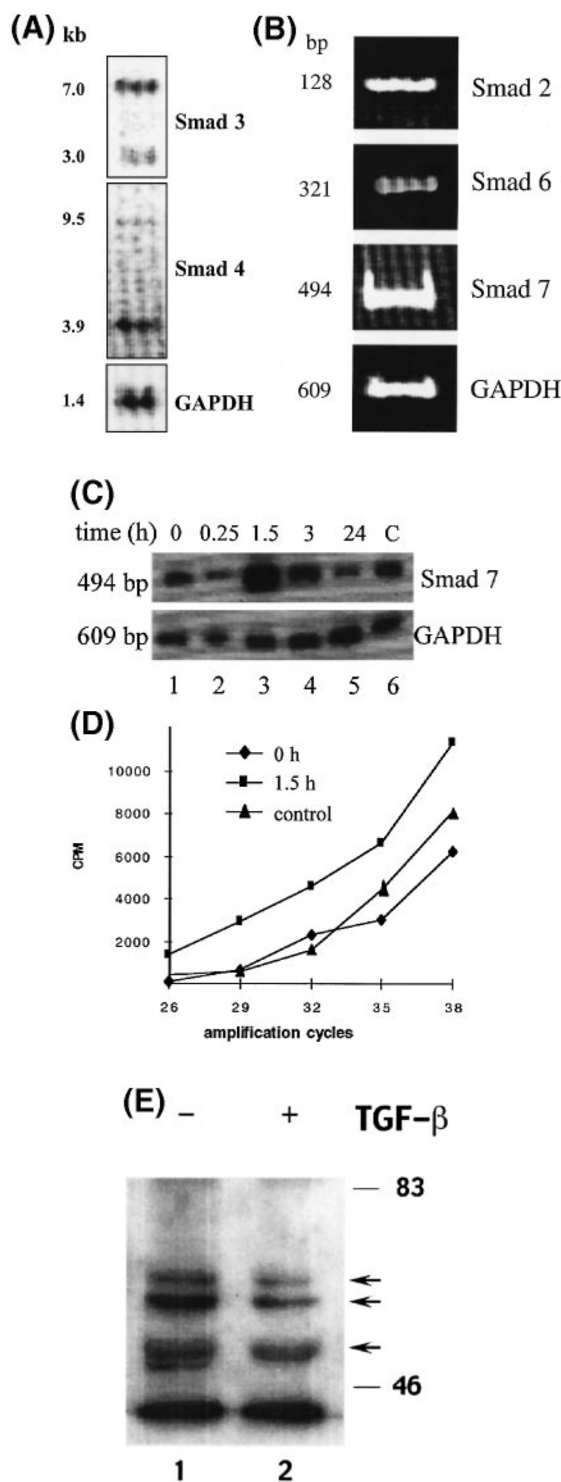

Figure 1. Expression of Smad mRNA and protein in human dermal fibroblasts. (*A*) Northern analysis. Total RNA was isolated from confluent fibroblasts and analyzed by northern hybridization with a Smad 3 cDNA. The membranes were then stripped, and re-hybridized to Smad 4 and GAPDH cDNA. A representative autoradiogram is shown. (*B*) Demonstration of Smad expression in fibroblasts by coupled RT-PCR. Primers specific for Smad 2, Smad 6, Smad 7, or GAPDH, described in **Table I**, were used to amplify cDNA from confluent fibroblasts by PCR. Amplification products in the linear range for Smads and GAPDH were electrophoresed and visualized under UV light. Representative results are shown. (*C, D*) Rapid induction of Smad 7 mRNA expression by TGF-β. RNA was prepared from confluent fibroblasts, and Smad 7 and GAPDH mRNA levels were determined by coupled RT-PCR in the presence of [α-^{32}P]dCTP. (*C*) Fibroblasts were treated with TGF-β1 for varying periods up to 24 h as indicated. C, fibroblasts incubated for 24 h in medium only. A representative autoradiogram is shown. (*D*) At the indicated number of PCR cycles, equal aliquots from the control (0), 90 min TGF-β-treated, and 24 h untreated samples were taken for electrophoresis, and the amplification products were visualized by autoradiography. The cpm incorporated in each band was determined by scintillation counting. The values shown are normalized for coamplified GAPDH. (*E*) Western immunoblot analysis of Smad 2/Smad 3. Nuclear extracts from untreated fibroblasts (*lane 1*) or fibroblasts treated with TGF-β1 (*lane 2*) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis, as described in *Materials and Methods*. A representative chemiluminogram is shown. *Arrows* indicate the relative migration of Smad 2 and Smad 3. Molecular mass markers in kDa are shown.

washed with Tris-saline buffer. The SED anti-Smad antibody recognizes Smad 2 and Smad 3. Secondary antibodies were detected by incubation with avidin-biotinylated horseradish peroxidase macromolecular complex (Vector Laboratories, Burlingame, CA) for 1 h, and detected by incubation with super-signal chemiluminescent HRP substrate (Pierces, Rockford, IL) for 3 min, followed by exposure to X-ray film for 1 s to 3 min. Molecular standards (Bio-Rad Laboratories, Hercules, CA) were also electrophoresed. In order to detect protein expression from transiently transfected Smads, COS-7 cells were transfected with Smad expression vectors, and harvested following a 48 h incubation. Proteins from the transfected cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis, and probed with the anti-FLAG M2 antibody (Kodak, New Haven, CT), and detected by ECL system (Amersham, Arlington Heights, IL).

RESULTS

Expression of endogenous Smad in primary human fibroblasts The pathway-restricted Smad 2 and Smad 3, and their shared signaling partner Smad 4, have been shown to be broadly expressed in mammalian tissues, and in established epithelial and osteoblastic cell lines (Nakao *et al*, 1997a, b; Yamamoto *et al*, 1997; Yang *et al*, 1998). We were interested in determining if primary fibroblasts, which are less likely to contain mutations in TGF-β signaling proteins than established cell lines, showed expression of these Smads. Total RNA was isolated from low-passage confluent human skin fibroblasts and examined by northern analysis. Two distinct Smad 3 mRNA transcripts, with approximate sizes of 3.0 and 7.0 kb, and two Smad 4 mRNA transcripts (4.0 and 9.5 kb) were detected (**Fig 1*A***). In agreement with previous findings in mouse embryos, the 4.0 kb Smad 4 transcript was the major form (Yang *et al*, 1998). These results were consistent in four independent experiments with fibroblasts from different individuals, and in media with 1% fetal calf serum or no serum. In order to detect low abundance Smad mRNA expression, a coupled RT-PCR reaction was used. Amplification products of the predicted 128 bp, 321 bp, or 494 bp size, corresponding to Smad 2, Smad 6, and Smad 7 mRNA, could be detected in unstimulated fibroblasts (**Fig 1*B***). The relative amount of the Smad 7 mRNA was increased rapidly and transiently in fibroblasts treated with TGF-β1 (12.5 ng per ml), whereas mRNA levels for Smad 2, Smad 6, and GAPDH remained unchanged (**Fig 1*C***, ***D***, and data not shown).

Immunoblotting with the cross-reactive SED anti-Smad antibody indicated that Smad 2/Smad 3 protein could be detected in fibroblast nuclear extracts in the absence of added TGF-β. The levels of Smad 2/3 were not consistently altered by treatment of the fibroblasts with TGF-β (**Fig 1*E***).

Overexpression of wild-type or mutant Smads In order to examine the regulation of TGF-β-responsive genes in primary fibroblasts, transient transfections were employed. First, the regulation of the 3TP promoter, a well-characterized artificial construct that is a sensitive indicator of TGF-β-dependent transcriptional responses in mammalian cells (Keeton *et al*, 1991), was examined. In agreement with results from earlier studies with immortalized Mv1Lu and SW480.7 cells, the activity of the 3TP promoter was substantially induced in the fibroblasts cotransfected with Smad 3 (**Fig 2*A***, *left panel*). This activation was further enhanced in the presence of TGF-β (data not shown). Smad 4 caused only a modest (50%) stimulation of 3TP promoter activity. Similar effects were obtained with PAI-1-CAT as the reporter (**Fig 2*A***, *right panel*), but

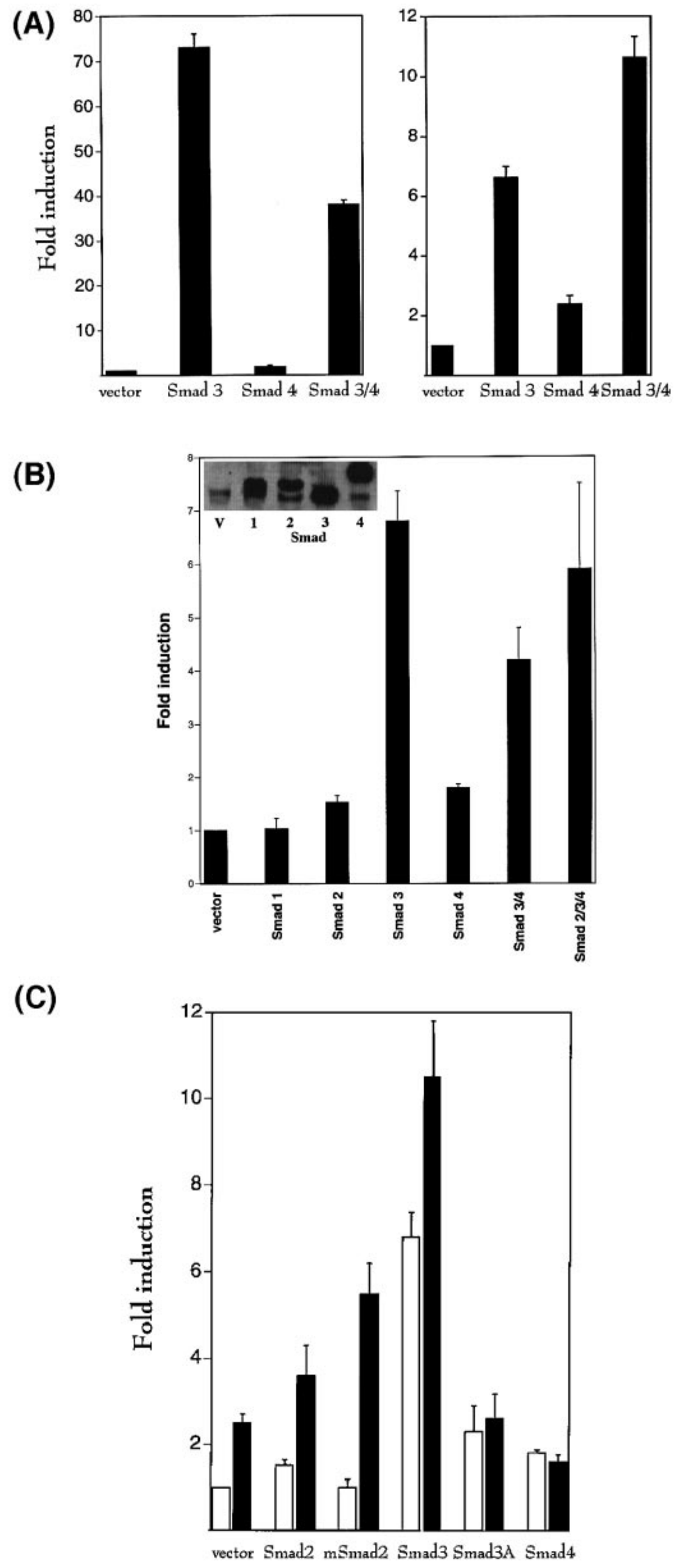

Figure 2. Overexpression of Smad 3 or Smad 4 trans-activates COL1A2, whereas mutant Smad 3 abrogates stimulation. (*A*) Ligand-independent activation of reporter constructs. p3TP-lux (3 μg) and PAI-1-CAT (20 μg) (*left* and *right panels*) were transfected into fibroblasts along with the indicated Smads, as described in *Materials and Methods*. CAT and luciferase activities were determined following a 48 h incubation, and corrected for minor variations in transfection efficiencies by renilla luciferase. The results, expressed as -fold increase in CAT or luciferase activity induced by Smad compared with the activity of the reporter in the presence of the empty vector (set to 1), represent the mean ± SEM of four determinations. Note the difference in scales between the *left* and *right panels*. (*B*) Stimulation of COL1A2-CAT by Smads. The COL1A2-CAT, used as the reporter, was cotransfected with the indicated Smads alone, or in combination. *Inset*, western blot of COS-7 cells transfected with the indicated Smad-FLAG expression constructs, and probed with anti-FLAG antibodies. V, vector only. (*C*) Inhibition of Smad signaling by mutant Smad 3. Fibroblasts were transfected with the indicated wild-type Smads, phosphorylation-deficient Smad 3 (Smad 3A), or Smad 2 (mSmad 2) mutants, or the empty vector along with COL1A2-CAT. CAT activity was determined following treatment of the fibroblasts with TGF-β1 for 24 h. The results shown represent the mean of at least two independent experiments in duplicates. □, Untreated fibroblasts; ■, TGF-β-treated fibroblasts.

the response of the p3TP construct to Smad was substantially greater than that of PAI-1. This is consistent with previous reports, and probably reflects the presence of additional enhancer sequences in the 3TP promoter (de Winter *et al*, 1997). CAT activity driven by the negative control plasmid pSV0CAT was not stimulated by Smads or by TGF-β.

Next, equal amounts of Smad 1, Smad 2, Smad 3, or Smad 4, all under CMV promoter regulation, were transiently transfected into confluent fibroblasts along with the COL1A2-CAT construct, which contains 772 bp of the promoter including the putative TGF-β response elements. Smad 3 by itself caused a 6–9-fold increase in COL1A2 promoter activity compared with fibroblasts transfected with empty vector (**Fig 2*B***). Smad 4 caused a similar though less marked stimulation. In contrast, neither bone morphogenetic protein-specific Smad 1, nor TGF-β/activin-specific Smad 2 had an effect on promoter activity. Furthermore, in contrast to previous studies (Liu *et al*, 1997; Nakao *et al*, 1997), combinations of Smad 2, Smad 3, and Smad 4 did not consistently result in an additive effect. The expression of each Smad protein in the transfected COS cells was comparable (**Fig 2*B***, *upper panel*). Next, the effect on TGF-β on Smad-regulated COL1A2 activity was examined. The results indicated that addition of TGF-β to the cultures caused a further increase in stimulation of COL1A2 promoter activity induced by Smad 3 (**Fig 2*C***). These results were identical whether TGF-β1 or TGF-β2 was used (data not shown).

To investigate the functional role of individual Smads in mediating the effects of TGF-β on COL1A2 promoter activity in fibroblasts, loss-of-function experiments were performed. In Smad 3, replacement of three carboxy-terminal serine residues corresponding to amino acids 422, 423, and 425 with alanine interferes with phosphorylation of the protein by the activated TGF-β type I receptor (Liu *et al*, 1997). As shown in **Fig 2(*C*)**, this phosphorylation-deficient Smad 3 mutant almost fully blocked the stimulation of COL1A2 activity by TGF-β. In contrast, a truncated form of Smad 2 lacking the 44 carboxy-terminal amino acids did not prevent stimulation of the promoter, indicating the specificity of the inhibitory effect, and suggesting that in fibroblasts, Smad 3, but not Smad 2, was involved in transducing the signal from TGF-β receptors to the COL1A2 promoter.

Smad 6, Smad 7, and Smad 9 are distinct members of the Smad family. Transient overexpression in the fibroblasts of Smad 7, but not Smad 6 or Smad 9, reduced by 45% the basal activity of COL1A2 in the presence of the empty vector, and significantly abrogated its induction by TGF-β (**Fig 3**). Because the expression of Smad 7 mRNA is induced by TGF-β (**Fig 1**), it is likely that Smad 7 functions as an autocrine negative regulator of TGF-β signaling in fibroblasts.

Smad DNA-binding activity TGF-β has been shown to stimulate the translocation of Smad 2 and Smad 3 from the cytoplasm into the nucleus, but the precise nuclear function of these Smads has not been established. In order to examine if endogenous fibroblast Smads could modulate transcription directly by binding to DNA sequences in target genes, gel mobility shift assays were performed. Nuclear extracts were prepared from confluent fibroblasts left untreated, or incubated with TGF-β1. Synthetic double-stranded oligonucleotides spanning from –272 to –249 bp of the COL1A2 promoter were prepared and used as probes. This region of the COL1A2 promoter contains a CAGACA sequence that was shown to be a functional Smad-binding element in the human PAI-1 promoter (Dennler *et al*, 1998) Furthermore, the CAGACA sequence lies within Box B of a previously identified complex TGF-β response element of COL1A2 (Inagaki *et al*, 1994). The results showed that nuclear extracts from untreated fibroblasts generated a DNA-protein complex with the –272/–249 COL1A2-CAGA probe, or with a consensus SBE probe (**Fig 4*A***). Identical pattern of band shift was observed with nuclear extracts prepared from different fibroblast lines in three independent experiments (data not shown). Treatment of the fibroblasts with TGF-β1 caused a rapid and transient increase in the formation of the

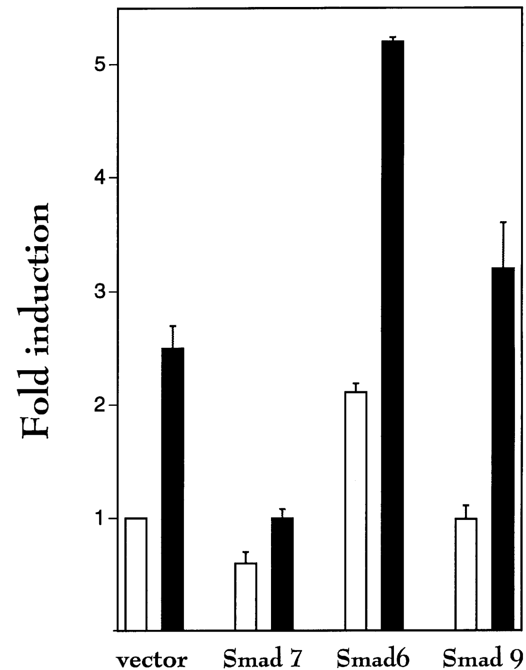

Figure 3. Smad 7 abrogates stimulation of COL1A2 by TGF-β. Smad 6, Smad 7, Smad 9, or empty vector (1 μg) were transfected into fibroblasts along with COL1A2-CAT (20 μg), as described in *Materials and Methods*. TGF-β1 was added to the cultures, and CAT and luciferase activities were determined following a 24 h incubation, and corrected for minor variations in transfection efficiencies by renilla luciferase. The results, expressed as -fold increase in CAT or luciferase activity induced by Smad compared with activity of the reporter in the presence of the control vector (set to 1), represent the mean ± SEM from three independent experiments. □, Untreated fibroblasts; ■, TGF-β-treated fibroblasts.

DNA-protein complex (**Fig 4*A***). Competition with molar excesses of homologous COL1A2-CAGA, SBE, or COL1A2–386/–363 probes, or unrelated AP-1-binding oligonucleotides, established the specificity of this DNA-protein complex (**Fig 4*B***). Specificity was further supported by the reduction of endogenous transcription factor binding to mutated COL1A2-CAGA probes (**Fig 4*C***).

In order to identify the *trans*-acting factors participating in the transcription complex assembled on the CAGACA sequence of COL1A2, supershift assays with antibodies were performed. The polyclonal SED antibody, which cross-reacts with Smad 2 and Smad 3 (Nakao *et al*, 1997), caused a supershift, confirming that the DNA-protein complex generated with nuclear extracts from TGF-β-treated fibroblasts included a pathway-restricted Smad (**Fig 4*D***). In the presence of excess antibody (10 μl), the DNA-protein complex was completely supershifted, and the residual binding was fully eliminated (data not shown). Pan-FOS antibodies or preimmune serum failed to cause a supershift. Taken together, these results indicate that a transcription complex including Smad or related proteins in nuclear extracts from TGF-β-treated fibroblasts binds directly and specifically to COL1A2 promoter sequences homologous to a Smad recognition site in the PAI-1 promoter, and previously implicated in TGF-β responsiveness (Inagaki *et al*, 1994). To determine if DNA binding of the endogenous Smad-containing transcription complex was phosphorylation dependent, nuclear extracts were subjected to *in vitro* dephosphorylation. As shown in **Fig 4(*E*)**, *in vitro* calf intestinal alkaline phosphatase treatment of nuclear extracts prepared from TGF-β-treated fibroblasts resulted in a loss of binding to the CAGACA Smad-binding sequence. In order to define the core sequence required for optimal Smad DNA binding, mutated CAGA probes were used in gel shift assays with bacterially expressed GST-Smad 3 MH1 domain fusion proteins. The results, shown in **Fig 5**, indicated that the integrity of the CAGACA core sequence was essential for efficient binding of recombinant Smad 3.

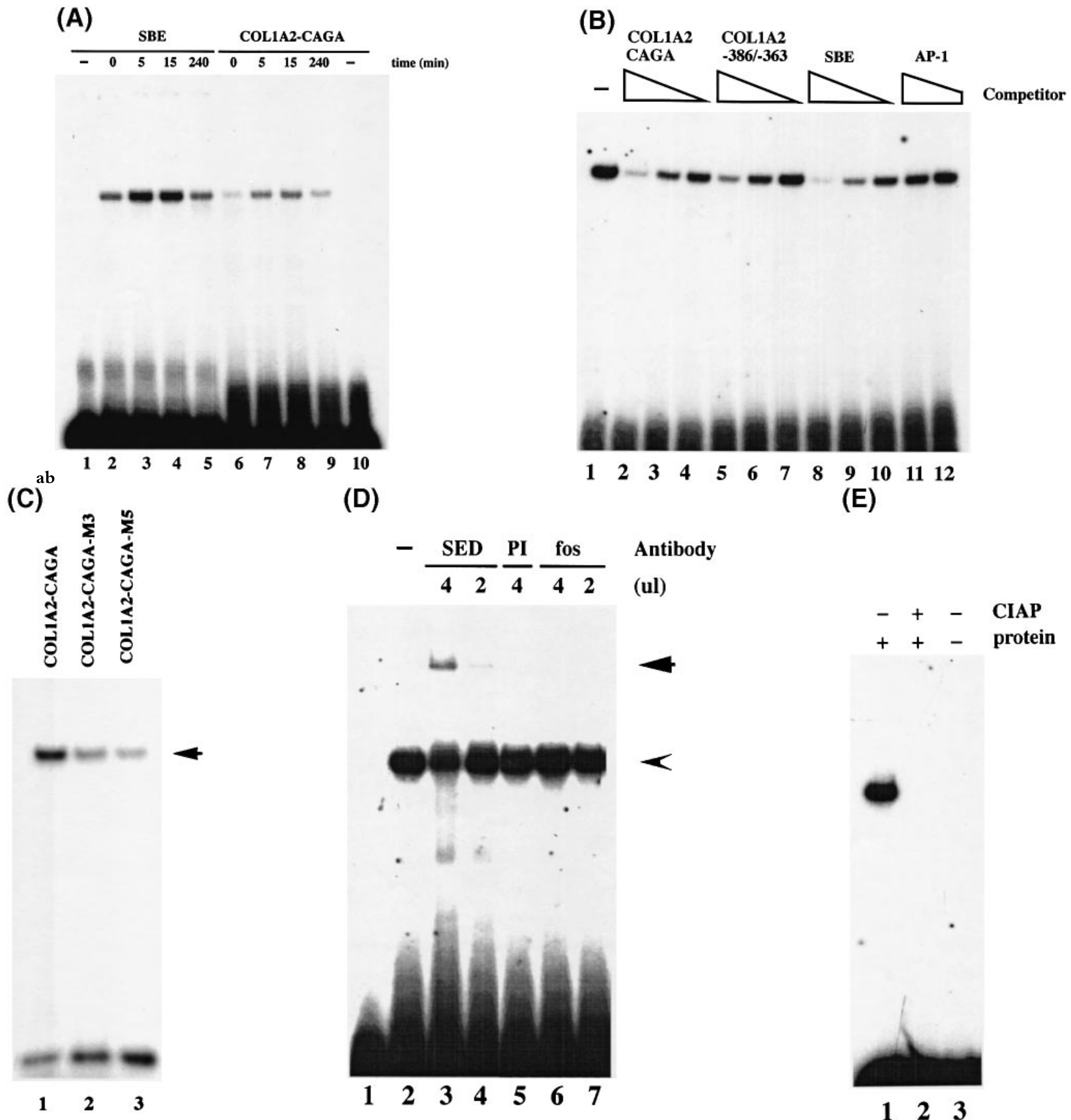

Figure 4. Endogenous Smads display sequence-specific inducible DNA-binding activity. A 24 bp oligonucleotide corresponding to the human COL1A2 promoter sequences between –272 and –249 bp (COL1A2-CAGA), a 26 bp oligonuclotide containing a consensus SBE, and a 24 bp oligonucleotide corresponding to the human COL1A2 promoter sequences between –386 and –363 bp (COL1A2–386/–363) were synthesized. Radiolabeled oligonucleotides were then used as probes in gel mobility shift assays, as described in the *Materials and Methods*. Unlabeled oligonucleotides were used as competitors. (*A*) Inducible DNA-binding activity. The SBE (*lanes 1–5*) and the COL1A2-CAGA (*lanes 6–10*) oligonucleotides were used as probes. The nuclear extracts were as follows: *lanes 2*, *6*, untreated fibroblasts; *lanes 3–5* and *7–9*, fibroblasts treated with TGF-β1 for 5–240 min, as indicated on top; *lanes 1*, *10*, no protein. (*B*) Competition assay. Nuclear extracts were prepared from fibroblasts treated with TGF-β for 15 min, and incubated with the radiolabeled COL1A2-CAGA probe. *Lane 1*, no competitor DNA; *lanes 2–4*, 100-, 20-, or 4-fold molar excesses of unlabeled homologous COL1A2-CAGA oligonucleotide; *lanes 5–7*, 100-, 20-, or 4-fold molar excesses of unlabeled COL1A2–386/–363 oligonucleotide; *lanes 8–10*, 100-, 20-, or 4-fold molar excesses of unlabeled consensus SBE oligonucleotide; *lanes 11*, *12*, 100- or 20-fold molar excesses of unlabeled AP-1 consensus binding site oligonucleotide. (*C*) Binding to nuclear extract prepared from fibroblasts treated with TGF-β1 for 15 min to the indicated mutated COL1A2-CAGA probes (described in *Materials and Methods*). (*D*) Antibody supershifting. Nuclear extracts from fibroblasts treated with TGF-β1 for 15 min were preincubated without antibodies (*lane 2*), SED anti-Smad 2/Smad 3 antibodies (2 or 4 μl as indicated, *lanes 3*, *4*), or 2 or 4 μl of pan-FOS antibodies (*lanes 6*, *7*) or preimmune serum (*lane 5*) for 30 min prior to binding reaction. The COL1A2-CAGA oligonucleotide probe was Klenow-labeled and incubated with 10 μg nuclear extract, and free probe was separated from DNA-protein complexes by electrophoresis. The shifted (*arrowhead*) and supershifted (*arrow*) bands are indicated on the right side of the autoradiogram. *Lane 1*, free probe. (*E*) Modulation of nuclear extract DNA-binding activity by *in vitro* dephosphorylation. Nuclear extract prepared from fibroblasts treated with TGF-β for 60 min were pretreated with calf intestinal alkaline phosphatase for 30 min, followed by sodium orthovanadate (1 mM). *Lane 1*, no calf intestinal alkaline phosphatase; *lane 2*, calf intestinal alkaline phosphatase; *lane 3*, free probe.

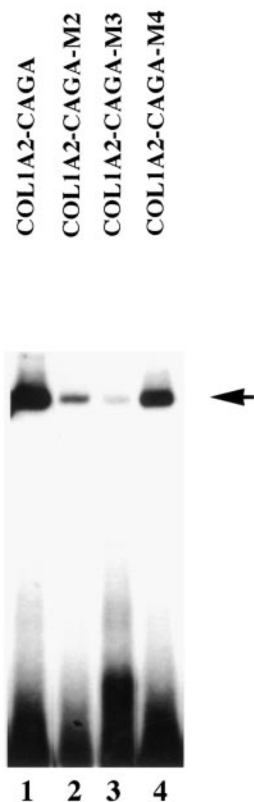

Figure 5. Recombinant Smad 3 binding requires the CAGACA sequence. COL1A2-CAGA oligonucleotides carrying mutations (described in *Materials and Methods*) were used as probes in gel shift assays with bacterially expressed GST-Smad 3 MH1 domain fusion proteins. A representative autoradiogram is shown.

DISCUSSION

TGF-β stimulates the transcription of collagen and other genes coding for extracellular matrix proteins *in vitro* and *in vivo*, and plays a central role in tissue repair and pathologic fibrosis (Varga and Jimenez, 1995). Although the *cis*-acting DNA elements mediating the activation of target gene expression in response to TGF-β have been extensively investigated, the intracellular signaling molecules that couple the activated TGF-β receptor to the nucleus have remained largely unknown. The recent identification of the Smad protein family has provided novel insights regarding TGF-β signaling. In this study, we explored whether Smad proteins play a role in activation of collagen transcription in human fibroblasts. The expression of specific Smad moieties appears to widely distributed in different tissues, but so far little is known about their cell lineage-specific expression. These results indicate that Smad 2, Smad 3, and Smad 4, as well as Smad 6 and Smad 7 mRNA, are detectable in cultured fibroblasts in the absence of TGF-β. Northern analysis indicated the presence of two distinct mRNA transcripts for Smad 3 as well as for Smad 4. The size of the transcripts is consistent with previous reports from cancer cell lines and embryonic tissues (Nakao *et al*, 1997; Zhou *et al*, 1998). Whether the existence of multiple Smad mRNA transcripts indicates alternative splicing or several polyadenylation sites remains to be determined. Smad 2/ Smad 3 protein was detected in the nucleus of unstimulated fibroblasts, and its levels were not appreciably changed by exposure of the fibroblasts to TGF-β. Constitutive ligand-independent nuclear localization of the pathway-restricted Smads has been previously described (Moustakas and Kardassis, 1998).

We show that transient overexpression in fibroblasts of Smad 3, and to a lesser degree Smad 4, stimulated activity of a proximal COL1A2 promoter segment that contains putative TGF-β response elements of the gene (Inagaki *et al*, 1994; Chung *et al*, 1996), in the absence TGF-β. *Trans*-activation of COL1A2 was specific for Smad 3 and Smad 4, because overexpression of Smad 1, which is implicated in bone morphogenetic protein signaling, failed to stimulate transcription. Surprisingly, the TGF-β/activin-restricted Smad 2 was also unable to *trans*-activate the promoter. Smad 2 has a 92% amino acid sequence similarity to Smad 3 (Zhang *et al*, 1996), differing only by the presence of two stretches of amino acids in the MH1 domain, and has been considered to be redundant with Smad 3 in TGF-β signaling (Hu *et al*, 1998). Significant functional differences between Smad 2 and Smad 3, however, were recently demonstrated on the transcription of the PAI-1, p21, and goosecoid genes (Dennler *et al*, 1998; Labbe *et al*, 1998; Moustakas and Kardassis, 1998), and are likely to be due to their differential ability to bind to DNA (Zawel *et al*, 1998). Taken together with these findings, these results suggest that Smad 2 and Smad 3 may have different subsets of target genes and different functional roles in transcriptional regulation; however, we cannot exclude the possibility that Smad 2 also plays a role in mediating the effect of TGF-β on COL1A2 activity. In contrast to previous reports showing that Smad 3 synergizes with Smad 2 and Smad 4 to stimulate the transcription of TGF-β responsive genes (Nakao *et al*, 1997), we were not able to demonstrate synergistic interactions among the Smads in fibroblasts. Further studies will be necessary to delineate the functional relationship between Smad 2 and Smad 3 in mediating TGF-β signals to the COL1A2 promoter. The ligand-independent activation of target gene transcription by Smad 3 and Smad 4 suggests that they may function as intracellular mediators of TGF-β signaling in fibroblasts.

The highly conserved carboxy-terminal domain has been shown to play a critical role in the signaling function of the pathway-restricted Smads (Lagna *et al*, 1996; Liu *et al*, 1997; Wu *et al*, 1997). Because phosphorylation and multimeric complex formation are considered to be important for nuclear translocation and transcriptional activation by Smads, mutations or loss of the serine residues results in interruption of Smad signaling. In these experiments, a Smad 3A mutant harboring substitutions that replace critical serine residues with alanine (Liu *et al*, 1997) abrogated the stimulation of COL1A2 promoter activity by TGF-β. In contrast, a phosphorylation-deficient mutant of Smad 2 (Mucsi and Goldberg, 1997) failed to inhibit TGF-β signaling. These findings further implicate Smad 3 as an important component of Smad signaling, and point to hitherto unappreciated functional differences between Smad 2 and Smad 3.

Transient overexpression of Smad 7, but not Smad 6 or Smad 9, efficiently blocked the stimulation of COL1A2 promoter activity by TGF-β in the fibroblasts. The "anti-Smads" lack the amino-terminal domain and carboxy-terminal SSXS phosphorylation sequences that are conserved among the pathway-restricted Smads. The interaction of Smad 7 with the activated TGF-β type I receptor is thought to result in the formation of a stable complex, which in turn blocks the phosphorylation of Smad 2 and Smad 3 by the receptor, thus interrupting ligand-induced signaling via the pathway-restricted Smads and downregulating the sensitivity of the cell to TGF-β (Hayashi *et al*, 1997; Nakao *et al*, 1997; Ishisaki *et al*, 1998). Whether Smad 7 abrogates TGF-β stimulation of COL1A2 promoter activity by interfering with activation of Smad 3, or via competetive inhibition of its association with Smad 4 or coactivators, remains to be determined (Hu *et al*, 1998). Smad 6 blocks bone morphogenetic protein-induced Smad signaling by forming a complex with Smad 1, but not with the TGF-β-specific Smads, thus acting as a "decoy" via competition for the obligate signaling partner Smad 4 (Hata *et al*, 1998). In these studies, overexpression of Smad 6 did not block the stimulation of COL1A2 promoter activity by TGF-β, indicating ligand-specific differences in the functional roles of the anti-Smads.

The expression of Smad 7 mRNA in the fibroblasts was rapidly and transiently induced by TGF-β. These results indicate that Smad 7 is a novel and relatively early response gene for TGF-β. Smad 7 may function as an autocrine negative regulator of COL1A2 transactivation by TGF-β in a manner reminescent of other

cytokine-inducible autoregulatory switch-off signals that are likely to have important physiologic roles in limiting the duration or intensity of the cytokine-induced response. A failure of the Smad 7-mediated endogenous inhibitory feedback loop could lead to unchecked activation of the intracellular TGF-β signaling cascade, resulting in constitutive activation of COL1A2 transcription following a transient exposure of the fibroblasts to TGF-β.

The mechanisms of target gene activation by Smads are not well understood. Recent evidence indicates that Smad proteins can interact with DNA. In *Xenopus*, Smad forms a heterodimer with the winged-helix DNA-binding protein forkhead activin signal transducer FAST-1 (Chen *et al*, 1997b). In contrast, in *Drosophila*, the amino-terminal domain of Mad, though not the full-length protein, can bind directly to decapentaplegic-responsive elements within the *vestigial* gene promoter (Kim *et al*, 1997). Full-length human Smad 4 was shown to bind directly to DNA in the artificial 3TP promoter; however, the putative Smad-binding sequence was dispensable for TGF-β-dependent activation of this promoter, raising doubts about the functional significance of this binding (Yingling *et al*, 1997). Recently, a palindromic sequence (SBE) binding to bacterially expressed GST-Smad 3 MH1 domain and Smad 4 full-length fusion proteins, was identified using a PCR selection strategy (Zawel *et al*, 1998). The sequence contains the CAGA motif, which is also found in the Smad-binding element of the PAI-1 promoter (Dennler *et al*, 1998). In these studies, we demonstrate that endogenous transcription factors in nuclear extracts from both untreated and TGF-β-treated fibroblasts, as well as bacterially expressed GST-Smad 3 MH1 domain fusion protein, bound specifically to a COL1A2 promoter region containing the CAGACA motif and displaying strong homology to the PAI-1 Smad-binding element. Remarkably, this newly identified Smad-binding element lies within a region of the COL1A2 promoter previously implicated in TGF-β responsiveness of the gene (Inagaki *et al*, 1994). Although these observations strongly suggest that Smad binding to the −272 to −249 bp region mediates activation of the COL1A2 promoter in response to TGF-β, the functional role of this region must be confirmed with additional studies. Furthermore, it remains to be established whether *in vivo*, additional nuclear factors might also be required for the formation of the transcriptionally active complex. The close proximity of several Sp1 binding sites to the Smad-binding CAGACA sequence in the COL1A2 promoter is significant, raising the possibility that stimulation of COL1A2 transcription by TGF-β may involve synergistic interactions among Smad and Sp1, as has been shown in the case of p21 promoter regulation (Moustakas and Kardassis, 1998).

The transcription complex assembled on the COL1A2-CAGA probe included Smad 2/3, as demonstrated by antibody supershifting. Furthermore, *in vitro* dephosphorylation of nuclear proteins inhibited their binding to the probe, suggesting that phosphorylation of one or more amino acid residues of Smad was important for DNA-binding activity. These findings are consistent with a model in which ligand-stimulated Smad phosphorylation plays a role in activation of the intracellular TGF-β signaling cascade. A relatively low level of CAGA-specific DNA-binding activity was detected in untreated fibroblasts, and was rapidly induced by TGF-β. This observation, taken together with the results of Smad mRNA expression, and western immunoblotting studies, suggests that in primary fibroblasts in culture, Smad genes are expressed, and their products translocate into the nucleus and bind to target DNA sequences independent of ligand. This may be the result of low-level consitutive activation of the fibroblast TGF-β receptor caused by endogenous TGF-β (Kawakami *et al*, 1998), or by TGF-β in the media, and may account for the basal activity of the COL1A2 promoter. Stimulation of fibroblasts by TGF-β causes Smad phosphorylation and increased Smad nuclear translocation, or the recruitment of additional *trans*-acting factors such as Sp1, into the Smad-containing DNA-binding complex, resulting in enhanced transcription of target genes. The functional role of the putative Smad-binding elements of COL1A2 in mediating the TGF-β response of the gene, and the precise composition of the binding complex, remain to be further delineated.

We thank Rik Derynck, Harvey Lodish, Howard Goldberg, Peter ten Dijke, Masa Kawabata, Masao Takase, Takeshi Watanabe, Liliana Attisano, and Carolyn Bruzdzinski for their generous gifts of reagents used in this study. The work was supported by the National Institutes of Health (AR-42309).

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