In normal adult fibroblasts, transforming growth factor-β (TGFβ) induces the expression of connective tissue growth factor (CTGF). CTGF independently promotes fibroblast proliferation and matrix deposition, and in acute models of fibrosis promotes cell proliferation and collagen deposition acting synergistically with TGFβ. In contrast to normal fibroblasts, fibroblasts cultured from fibrotic tissues express high basal levels of CTGF, even in the absence of added TGFβ. Induction of transcription by TGFβ requires the action of SMAD proteins. In this report we have investigated the role of SMADs in the TGFβ-induction of CTGF in normal fibroblasts and in the elevated levels of CTGF expression found in dermal fibroblasts cultured from lesional areas of patients with scleroderma, a progressive fibrotic disorder that can affect all organs of the body. We have identified a functional SMAD binding site in the CTGF promoter. TGFβ-induction of CTGF is dependent on SMAD3 and SMAD4 but not SMAD2 and is p300-independent. However, mutation of the SMAD binding site does not reduce the high level of CTGF promoter activity observed in dermal fibroblasts cultured from lesional areas of scleroderma patients. Conversely, the previously termed TGFβRE in the CTGF promoter is required for basal CTGF promoter activity in normal fibroblasts and for the elevated level of CTGF promoter activity in scleroderma fibroblasts. Thus, the maintenance of the fibrotic phenotype in scleroderma fibroblasts, as visualized by excess CTGF expression, appears to be independent of SMAD-dependent TGFβ signaling. Furthermore, given CTGF’s activities, the high level of CTGF expression observed in scleroderma lesions may contribute to the excessive scarring observed in this disorder.

Wound healing requires the synthesis and reconstitution of properly organized connective tissue. If activation of collagen gene expression persists, uncontrolled connective tissue deposition results, leading to pathologic scarring and fibrosis (1–3) such as in scleroderma (systemic sclerosis), which is characterized by the progressive scarring of skin and certain internal organs (4). Given the ability of TGFβ to promote fibroblast proliferation and matrix synthesis, attention has been devoted to its potential role in initiating and maintaining the fibrotic phenotype (for reviews, see Refs. 5 and 6), including scleroderma (7). For example, there is a clear correlation between TGFβ action and the initiation of fibrosis; in acute drug- or surgery-induced animal models, anti-TGFβ strategies are effective at blocking the onset of fibrosis (for review, see Ref. 6). However, the fibrosis is clinically a chronic disorder; the involvement of TGFβ in the maintenance of fibrosis and the effectiveness of anti-TGFβ strategies in the reversion of fibrosis is unclear.

In terms of scleroderma, the data supporting the role of TGFβ in the fibrotic phenotype is circumstantial, chiefly depending on the histological distribution of TGFβ mRNA and protein. Unfortunately, the data are often contradictory. For example, mononuclear cells taken from bronchial lavage fluids of scleroderma patients have elevated TGFβ levels (8). However, in the actual lesional areas of skin, TGFβ mRNA is only localized to the leading edge of the scleroderma lesion; i.e. to the region of enhanced inflammatory response that is presumably involved with the initiation of the fibrotic response (9). Furthermore, fibroblasts taken from scleroderma lesions show elevated levels of collagen relative to their normal counterparts, yet show little difference in their ability to produce TGFβ or in their ability to bind TGFβ, nor do they show enhanced sensitivity to TGFβ treatment (10, 11). Thus, although there seems to be circumstantial data to support the role of TGFβ in the onset of the scleroderma fibrotic phenotype, it is unclear as to its precise role in initiating or maintaining the scleroderma phenotype.

In an initial attempt to molecularly characterize the scleroderma phenotype, we recently used differential display analysis to identify genes up-regulated in dermal fibroblasts cultured from patients with scleroderma (12). Perhaps the most interesting gene up-regulated in scleroderma fibroblasts was connective tissue growth factor (CTGF) (12). CTGF is a heparin-binding 38-kDa cysteine-rich peptide that induces proliferation, collagen synthesis, and chemotaxis in mesenchymal cells (13–18) and has been shown to potentiate sustained fibrosis when injected along with TGFβ in an acute animal model (19). Previously, CTGF mRNA and protein were shown to be constitutively expressed in numerous fibrotic disorders both in skin and in internal organs, such as atherosclerosis and pulmonary and renal fibrosis, and that this expression correlated with

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1 The abbreviations used are: TGFβ, transforming growth factor-β; TGFβRE, TGFβ response element; CTGF, connective tissue growth factor; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; PAI-1, human plasminogen activator inhibitor; SEAP, secreted enhanced alkaline phosphatase.
high collagen synthesis (12, 20–24). Hence, in adult tissues, constitutive CTGF expression is considered a faithful, clinical, molecular marker of fibrosis. Furthermore, given its activity, CTGF is considered a mediator of the fibrotic phenotype (12–24).

In contrast to the situation in fibrotic disorders, CTGF is not expressed in normal human dermal or mouse NIH 3T3 fibroblasts unless cells are treated with TGFβ (14–16, 20, 25). Similarly, exogenous addition of TGFβ increases the amount of CTGF protein and promoter activity produced by fibroblasts cultured from scleroderma lesions (12, 20). This induction by TGFβ is cell-type specific, as it occurs in connective tissue cells but not in epithelial cells or lymphocytes (14–16). The regulation of CTGF expression by TGFβ appears to be controlled primarily at the level of transcription (20, 25). Originally, the up-regulation of CTGF by TGFβ was thought to be solely dependent on a relatively small sequence present in the 5′ upstream region of the CTGF promoter (TGFβ response element; TGFßRE) (25). This sequence does not resemble the TGFβ response elements described in other genes, including the SMAD recognition sequence (25). However, recently we have shown that sequences immediately upstream of the previously identified TGFßRE are required for TGFβ to induce CTGF expression (20). Similarly, the high level of CTGF protein observed in scleroderma appears to be due, at least in part, to gene transcription because the CTGF promoter activity is substantially higher in scleroderma dermal fibroblasts relative to normal dermal fibroblasts (12). However, the precise mechanism underlying the control of CTGF gene expression in normal and fibrotic fibroblasts remains unknown.

Activation of TGFβ-dependent gene expression is commonly mediated through SMADs 2, 3, and 4 (for reviews, see Refs. 25 and 26). SMADs 2 and 3 are normally present in the cytosol. Once activated by TGFβ, SMADs 2 and 3 interact transiently with type I TGFβ receptor kinase and become phosphorylated at their carboxyl terminus (27, 28). SMAD2 and SMAD3 then form a heteromeric complex with SMAD4 (29, 30). These complexes then translocate to the nucleus and activate expression of target genes (25), in concert with other nuclear factors the identity of which can vary depending on the promoter and cell type (e.g. Refs. 31–34). Recent studies have recognized a consensus DNA motif, GTCTAGAC that provides the binding site for the SMAD3-SMAD4 complex (35). Homologs of this element have been identified in the promoters of several TGFβ-responsive genes (e.g. Refs. 36–39). More recently, studies in TGFβ-responsive systems have also identified other SMAD family members, such as SMAD6 and 7, which represent a functionally distinct class of SMADs that antagonize SMAD-receptor interactions (40).

In this report, we assess the role of SMADs in CTGF gene expression in normal and scleroderma fibroblasts. Using a combination of Western blot, gel shift, and gene cotransfection/promoter assays, we show that a functional SMAD binding site in the CTGF promoter is necessary for the induction of CTGF by TGFβ in fibroblasts. Conversely, the constitutive CTGF expression observed in scleroderma appears to be independent of SMAD action. Thus, the maintenance of the fibrootic phenotype in scleroderma, as visualized by constitutive CTGF expression, appears to be SMAD-independent. In addition, we show that the previously termed TGFßRE is required for basal CTGF expression in normal fibroblasts and elevated CTGF expression in scleroderma.

MATERIALS AND METHODS

Cell Culture, Reporter Assays, and Transfections—NIH 3T3 fibroblasts (ATCC) were maintained in DMEM, 10% calf serum (Life Technologies). Fetal SMAD3-null mouse fibroblasts, and their wild-type counterparts, were a gift from Anita Roberts (National Institutes of Health). Primary dermal fibroblasts from lesional areas of scleroderma patients and normal individuals were taken from biopsies of patients with diffuse cutaneous scleroderma and age, sex, and anatomically site-matched healthy volunteers, respectively. All patients fulfilled the criteria of the American College of Rheumatology for the diagnosis of scleroderma. Informed consent and ethical approval were obtained for all procedures. Fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mm 1-glutamine (Life Technologies). All cells were used between passages 2 and 5, with reculture at a dilution of 1 in 3 for normal fibroblasts (41). Transfections were performed in 6-well plates using LipofectAMINE plus, as described by the manufacturer (Life Technologies). For reporter assays, 1 μg of a reporter construct was cotransfected with 0.25 μg of CMVβ-galactosidase plasmid, which was used as an internal transfection control (CLONTECH). For assays in which the role of transacting proteins was to be tested, 0.5 μg of reporter, and 1 μg of empty vector or expression vector encoding the protein of interest were used. For transfections involving ski and smoN, amounts of DNA used were as described in the text. SEAP and β-galactosidase kits were used to perform reporter gene assays (Tropix). TGFß2 was from R&D Systems or Celtrix and used at concentrations indicated in the text. The data are presented as mean ± S.E., or representative of experiments performed in at least triplicate in at least 2 separate experiments. Statistical analysis was performed by the Student’s unpaired t test. p values less than 0.05 were considered statistically significant.

DNA Constructs—The full-length CTGF promoter/SEAP reporter construct is described elsewhere (20). Point mutations were introduced using a kit (Stratagene). The mutagenic primer (Sigma Genosys) for the SMAD mutant was 5′-GACCTGGAATTGCGACAGTTTTGGATCGGAGGAATGCTGAGTGTC3′ and for the TGFßRE mutant was 5′-CAGACGAGGAATGCTGAGTTGGAGTACCCAGGATCAAATCC3′. Constructs were fully sequenced in both directions to confirm mutagenesis before use. SMADs 2, 3, 4, 6, and 7 expression vectors were generous gifts from Joan Massague (Sloan-Kettering), and ski and smoN expression vectors were generous gifts from Robert Weinberg (Whitehead Institute). Expression vectors encoding wild-type and dominant-negative p300 were from Upstate Biotechnology. The human plasmid activator inhibitor (PAI-1) luciferase construct was a generous gift from Dan Rifkin (New York University).

Western Blot Analysis—Human dermal fibroblasts were grown in DMEM and 10% fetal bovine serum. Cells were grown to confluence and switched to DMEM supplemented with insulin/transferrin/seminol. Cells were grown for an additional 18 h, and then 10 ng/ml TGFß2 (R&D Systems) was added. Media was removed 24 h later, and 25 μg was electrophoresed through a 12% SDS-polyacrylamide gel (Novex) and blotted to nitrocellulose (Bio-Rad). Filters were blocked overnight at 4 °C in 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween 20. CTGF protein was detected by a 1 h incubation of a 1:1000 dilution of rabbit anti-CTGF antibody in 1% milk, TBS, 0.1% Tween 20. For antibodies, cells were lysed in 2% SDS, and protein concentration was determined (Bio-Rad). Equal amounts of protein were electrophoresed as described above. For experiments involving SMADs, SMAD proteins were detected using a 1:1000 dilution of antibody. SMADs 3, 4, and 7 antibodies were from Santa Cruz Biotechnology. For experiments involving the detection of transfected FLAG-tagged SMADs 3 and 4, 1 μg of a reporter construct encoding the TGFßRE was cotransfected with 0.25 μg of a reporter construct encoding the TGFßRE mutant was 5′-CAGACGAGGAATGCTGAGTTGGAGTACCCAGGATCAAATCC3′. Constructs were fully sequenced in both directions to confirm mutagenesis before use. SMADs 2, 3, 4, 6, and 7 expression vectors were generous gifts from Joan Massague (Sloan-Kettering), and ski and smoN expression vectors were generous gifts from Robert Weinberg (Whitehead Institute). Expression vectors encoding wild-type and dominant-negative p300 were from Upstate Biotechnology. The human plasmid activator inhibitor (PAI-1) luciferase construct was a generous gift from Dan Rifkin (New York University).

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Electrophoretic Mobility Shift Assays—Protein extracts were prepared from ~1 × 10⁶ NIH 3T3 control cells or cells treated with TGFß2 (2 ng/ml) for 60 min following serum starvation for 24 h. Cells were then lysed, and protein nuclear extracts were prepared as previously described (42). Protein concentrations were determined using the Bio-Rad protein assay kit. Oligonucleotide probes as indicated were 32P-labeled gel purified, and incubated with nuclear extracts for 30 min at 30 °C. Gel shift conditions consisted of 1–3 μl of nuclear extract (containing 2–5 μg protein) and 0.5 ng probe (1–5 × 10⁵ cpm) in a final volume of 15 μl of 150 mM NaCl, 10 mM Tris-HCL, pH 7.5 and 50 μg per ml poly(dI-dC) (Roche Molecular Biochemicals). For antibody treatment, nuclear extracts were incubated with 2 μg of the indicated antibodies prior to
addition of probe. SMAD2 antibody was from Zymed Laboratories Inc. and SMAD3 and SMAD4 antibodies were from Santa Cruz Biotechnolog.

For competition experiments, 50- or 100-fold excess cold competitor (25 or 50 ng, respectively) were transfected into NIH 3T3 fibroblasts, and expression was compared with that of the unmutated wild-type full-length construct (FL). Cells were serum-starved for 24 h, followed by an additional 24-h incubation with or without addition of 25 ng/ml TGFβ2 (tgfb or no tgfb, respectively). Assays were performed as described under "Materials and Methods." Relative light units based on SEAP expression (25 μl of conditioned media) normalized to β-galactosidase activity (from a co-transfected CMV-β-galactosidase plasmid) are shown (mean ± S.E., n = 18).

Immunoprecipitations—NIH 3T3 cells were transfected as described above with 2 μg each of expression vector encoding SMAD3-FLAG, SMAD4-FLAG, ski-HA or sno-NHA per 100-mm dish. Forty-eight hours later, cells were lysed in 800 μl of ice-cold freshly prepared 50 mM Hepes, 10% glycerol, 0.5% Nonidet P-40, 50 mM NaCl, 1 mM dithiothreitol, 50 mM sodium fluoride, 25 mM β-glycerol phosphate, 1 mM EDTA, 1 mM sodium pervanadate, 2 mM phenylmethylsulfonyl fluoride, pH 7.9. Cells were lysed on ice, scraped, sonicated, and spun. To 100 μl of lysate, 40 μl of agarose affinity gel conjugated with an anti-FLAG antibody (Sigma) was added. Gel without antibody was used as a control. Gel and protein slurry was rotated on a shaker for 2 h at 4°C. Beads were washed in lysis buffer (1 ml) three times. Protein was eluted with protein sample buffer (100 μl) and stored at −80°C. Samples (25 μl) were then subjected to polyacrylamide gel electrophoresis through a 4–20% gel (Novex), and gels were blotted onto polyvinylidene difluoride membrane (Millipore). After blocking membrane for 2 h at room temperature in phosphate-buffered saline, 5% nonfat dry milk, 0.1% Tween 20, blots were incubated overnight at 4°C with a 1:2000 dilution of anti-HA antibody (Covance). After extensive washing, proteins were detected by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Roche Molecular Biochemicals or Jackson Immunoresearch). Antibodies were diluted in blocking buffer. A chemiluminescent detection kit was used to observe proteins (Pierce).

RESULTS

SMADs Are Necessary for the TGFβ-mediated Induction of CTGF in a p300-independent Fashion—Recently, we identified a segment of the CTGF promoter between nucleotides −244 and −166 that was required for the TGFβ-mediated induction of CTGF (20). Sequence inspection of the region of the CTGF promoter between −244 and −166 resulted in the identification of a putative consensus SMAD binding site (35–38) immediately upstream of −166 (Fig. 1A). Mutating this sequence to a BamHI site in an otherwise wild-type, full-length CTGF promoter/SEAP reporter construct resulted in complete abolition of TGFβ-mediated gene expression when this construct was transfected into NIH 3T3 fibroblasts (Fig. 1B). Thus, a consensus SMAD binding site seems necessary for TGFβ-induction of CTGF. Previously, a sequence immediately downstream of this element was identified (see being necessary for maximal TGFβ induction of CTGF, and hence was called the TGFβRE (25). To compare the relative contribution of the SMAD site and the TGFβRE in terms of the TGFβ induction of CTGF, we mutated the TGFβRE to a BamHI site. In contrast to mutating the SMAD site, mutating the TGFβRE caused a marked decrease in basal CTGF expression, whereas still permitting a response to TGFβ (Fig. 1C). Thus, the factor(s) binding to the TGFβRE are required for basal CTGF transcription and hence for the maximal response of the CTGF promoter to TGFβ (25).

To determine whether SMAD proteins could bind to the putative SMAD binding site, we performed a gel shift assay with NIH 3T3 fibroblast nuclear extract, made from cells that had been treated with TGFβ. A radiolabeled double-stranded oligonucleotide probe containing the putative SMAD binding site was used as probe (see Fig. 1 for sequence). We found that protein complexes could bind the probe (Fig. 2, arrow). This binding was specific as binding could be competed by adding excess unlabeled oligomer (Fig. 2). Binding of one of the complexes could be abolished by preincubating nuclear extract with an anti-SMAD3 or anti-SMAD4 antibody, but not with an anti-
SMAD2 antibody (Fig. 2). Collectively, these data suggest that the putative SMAD site identified as being important for gene expression is a bona fide SMAD binding site. The components of the other complex are under further investigation.

To determine whether SMADs could regulate CTGF gene expression, we cotransfected expression vectors encoding various SMADs with a full-length CTGF promoter/SEAP construct into NIH 3T3 fibroblasts. Cotransfection of SMAD3 and SMAD4 enhanced CTGF expression significantly (Fig. 3A). However, cotransfection of SMAD2 and SMAD4 did not potentiate the TGFβ-induced expression of CTGF (Fig. 3A). Individually, only SMAD3 and SMAD4 mildly increased CTGF promoter activity in the absence of added TGFβ, with SMAD4-induced activity to approximately the same level as SMAD2 and SMAD4 combined (not shown). Transfection of the inhibitory SMAD, SMAD7, markedly attenuated the ability of TGFβ and SMAD3 and 4 to increase CTGF promoter activity, but had little effect on basal expression (Fig. 3A). Conversely, transfecting the inhibitory SMAD, SMAD6, had no effect on the ability of TGFβ to induce CTGF promoter activity (Fig. 3A). SMADs often require p300 as a transcriptional cofactor (32, 43). We found that transfecting either wild-type or dominant-negative p300 had no impact on the TGFβ-induction of CTGF (Fig. 3B). Thus, SMADs modulate CTGF gene expression in a p300-independent fashion.

Our transfection data suggested that SMAD3 is principally involved in the TGFβ-induction of CTGF. To verify this, we obtained fibroblasts cultured from SMAD3-knockout and wild-type fetal mice (45). We treated cells with TGFβ for 24 h and assayed their ability to induce CTGF protein. Both wild-type and knockout embryonic mouse fibroblasts displayed constitutive CTGF expression. This result is not surprising, because CTGF protein is expressed constitutively in several cell types during development (for review, see Ref. 13). We note an elevated basal level of CTGF in the SMAD3-knockout cells, perhaps because of a compensation for a lack of inducibility by TGFβ. However, although wild-type mouse fibroblasts responded to TGFβ by elevating CTGF levels, fibroblasts cultured from SMAD3-knockout animals could not respond to TGFβ (Fig. 4). Thus, the TGFβ-mediated induction of CTGF requires SMAD3.

Regulators of SMADs Modulate TGFβ-induced CTGF Gene Expression, Ski and snoN—To verify that SMADs activate CTGF gene expression, we then examined the ability of several known regulators of SMAD activity to modulate CTGF gene expression. Recently, the oncoproteins ski and snoN have been shown to suppress SMAD signaling in keratinocytes by binding directly to SMADs and thus preventing their participation in functional transcriptional complexes (46–48). We decided to use this fact to further probe the notion that TGFβ-mediated induction of CTGF was SMAD-dependent and to determine whether this silencing mechanism was functional in fibroblasts.

To verify that ski and snoN could interact with SMADs in NIH 3T3 fibroblasts, we cotransfected expression vectors encoding FLAG-tagged SMADs 3 and 4 along with expression vectors encoding either HA-tagged ski or HA-tagged snoN into NIH 3T3 fibroblasts. The presence of these molecular tags allowed easy recognition and manipulation of the transfected proteins. After transfection, cells were serum-starved for 24 h and were then lysed. Because 20 min of TGFβ treatment degrades snoN (47), we also wanted to ensure that transfected snoN existed even in the presence of exogenous TGFβ. Thus, we also prepared lysates from cells that had been treated with TGFβ for 20 min immediately after the serum-starvation treatment. To verify expression of transfected FLAG-tagged SMADs, whole cell lysate was then subjected to SDS-polyacrylamide gel electrophoresis. By Western blot analysis, transfected FLAG-tagged SMADs were readily detected with an
anti-FLAG antibody in lysates from cells that has been cotransfected with expression vectors encoding SMAD3 and SMAD4, which were both FLAG-tagged and either sno or ski, which were both HA-tagged (2 μg of each expression vector per 100-mm dish). Cells were serum-starved for 24 h after transfection, after which cells were immediately lysed or treated for an additional 20 min with 25 ng/ml TGFβ2, as indicated (tgfb). Whole lysates (25 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and Western analysis with an anti-FLAG antibody, to verify expression of SMADs 3 and 4 (LYSATE). Equal amounts of lysate were then either immunoprecipitated with unconjugated agarose gel (IP-BEADS) or FLAG-agarose gel (IP-FLAG). Gels were then subjected to Western analysis with an anti-HA antibody to detect transfected HA-tagged ski and snoN, which were detected only when anti-FLAG conjugated beads were used. B, ski and snoN block the TGFβ- and SMAD-mediated induction of CTGF. Transferring different amounts of ski and snoN expression vectors (as shown) into NIH 3T3 fibroblasts block the ability of TGFβ (25 ng/ml) to induce CTGF promoter activity. The full-length CTGF promoter/SEAP plasmid was used. Empty expression vector (empty) was used as a control at equal amounts to ski or snoN expression vector. Relative light units based on SEAP expression (25 μl of conditioned media) normalized to β-galactosidase activity (from a cotransfected CMV-β-galactosidase plasmid) are shown (mean ± S.E., n = 6).

FIG. 5. ski and snoN block the TGFβ induction of CTGF. A, ski and snoN bind to SMADs in NIH 3T3 fibroblasts. Cells were transfected with expression vectors encoding SMAD3 and SMAD4, which were both FLAG-tagged and either sno or ski, which were both HA-tagged (2 μg of each expression vector per 100-mm dish). Cells were serum-starved for 24 h after transfection, after which cells were immediately lysed or treated for an additional 20 min with 25 ng/ml TGFβ2, as indicated (tgfb). Whole lysates (25 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and Western analysis with an anti-FLAG antibody, to verify expression of SMADs 3 and 4 (LYSATE). Equal amounts of lysate were then either immunoprecipitated with unconjugated agarose gel (IP-BEADS) or FLAG-agarose gel (IP-FLAG). Gels were then subjected to Western analysis with an anti-HA antibody to detect transfected HA-tagged ski and snoN, which were detected only when anti-FLAG conjugated beads were used. B, ski and snoN block the TGFβ- and SMAD-mediated induction of CTGF. Transferring different amounts of ski and snoN expression vectors (as shown) into NIH 3T3 fibroblasts block the ability of TGFβ (25 ng/ml) to induce CTGF promoter activity. The full-length CTGF promoter/SEAP plasmid was used. Empty expression vector (empty) was used as a control at equal amounts to ski or snoN expression vector. Relative light units based on SEAP expression (25 μl of conditioned media) normalized to β-galactosidase activity (from a cotransfected CMV-β-galactosidase plasmid) are shown (mean ± S.E., n = 6).

To determine whether HA-tagged ski and snoN could bind to FLAG-tagged SMADs to inactivate them, we then immunoprecipitated lysates with an anti-FLAG antibody. In extracts from cells that had been cotransfected with HA-tagged ski and FLAG-tagged SMAD3 and SMAD4, we able to immunoprecipitate HA-tagged ski with anti-FLAG-agarose gel, as seen with an anti-HA antibody (Fig. 5A, IP-FLAG). Conversely, no HA-tagged ski was immunoprecipitated when unconjugated beads were used (Fig. 5A, IP-BEADS). Similarly, in extracts from cells transfected with FLAG-tagged SMADs 3 and 4 and HA-tagged snoN, we were able to immunoprecipitate snoN with anti-FLAG-agarose gel (Fig. 5A, IP-BEADS). We found that addition of TGFβ to cells for 20 min before lysis had no detectable effect on anti-FLAG precipitable ski or snoN levels (Fig. 5A; TGFβ-added lanes, IP-FLAG). Thus, transfected ski and snoN bound with SMAD3/4 in NIH 3T3 fibroblasts and thus could be used to test the idea that CTGF gene induction by TGFβ is SMAD-dependent.

We then assessed the effect of transfected ski and snoN constructs on CTGF promoter activity in NIH 3T3 fibroblasts when visualized by readout from a full-length CTGF promoter/SEAP reporter construct. We found that ski and snoN suppressed the induction of CTGF promoter activity by TGFβ (Fig. 5B). There appeared to be a greater effect of ski than snoN on CTGF expression, presumably because TGFβ treatment degrades snoN (47). Given the known abilities of ski and snoN (46–48), these results support the idea that SMADs modulate CTGF gene expression.

CTGF Expression in Scleroderma Fibroblasts Is Independent of SMAD Signaling—Previously, we showed that CTGF protein was constitutively expressed in fibroblasts cultured from lesional areas of scleroderma patients, even in the absence of exogenous TGFβ (12, 20) and that this elevated expression was due at least in part to high levels of CTGF promoter activity (12). To investigate the role of SMAD-dependent signaling in the elevated level of CTGF characteristic of scleroderma, we transfected our full-length CTGF promoter/SEAP reporter construct into normal dermal or scleroderma fibroblasts and compared its expression level to that of our CTGF promoter/SEAP reporter construct with a mutated SMAD binding site. We used dermal fibroblasts cultured from four normal individuals and ten individuals with diffuse scleroderma. Mutation of the...
SMAD binding site had no statistically significant effect on the basal level of promoter activity either in normal or scleroderma fibroblasts (Fig. 6A; Student’s unpaired t test, p > 0.05). Thus, the high level of CTGF promoter activity observed in scleroderma fibroblasts is not dependent on the SMAD recognition sequence.

To further clarify the potential role of SMADs in the scleroderma phenotype, we used Western blot analysis to compare the levels of expression of SMADs 3, 4, and 7 (because of their role in CTGF gene expression) in normal and scleroderma dermal fibroblasts. We used dermal fibroblasts cultured from three normal individuals and affected areas of three individuals with scleroderma with the highest SMAD3 level but also had extremely high SMAD7 (Fig. 6B). That is, our results suggest that the elevated level of CTGF expression observed in scleroderma appears to be independent of SMAD action.

To further clarify if elevated SMAD signaling was responsible for activated expression of genes in scleroderma, we transfected into scleroderma and normal fibroblasts a construct containing the human plasminogen activator inhibitor (PAI-1) promoter linked to firefly luciferase. This construct has been previously shown to be responsive to SMAD-dependent TGFβ signaling (49, 50). No elevation of PAI-1 promoter activity was observed in scleroderma fibroblasts, suggesting that SMAD-dependent TGFβ signaling is not generally activated in scleroderma fibroblasts (Fig. 7).

To determine whether the elevated level of CTGF promoter activity in scleroderma cells reflected elevated level of basal promoter activity, we transfected the TGFβRE-mutant CTGF promoter/SEAP reporter construct into normal and scleroderma fibroblasts. We found that mutating the TGFβRE reduced CTGF promoter levels in both normal and scleroderma cells (Fig. 8). In fact, removing this element reduced CTGF promoter levels observed in scleroderma fibroblasts to that of levels in normal fibroblasts, suggesting that this element is involved with the high level of CTGF expression observed in scleroderma.

**DISCUSSION**

TGFβ is known to activate gene expression through the action of SMAD proteins (25, 26). In the absence of TGFβ, SMADs 2 and 3 are primarily cytosolic. When TGFβ is present, SMADs 2 and 3 are phosphorylated by the TGFβ receptor, bind to SMAD 4, and migrate into the nucleus to activate expression of TGFβ-responsive genes. The inhibitory SMADs, SMAD6 and SMAD7, antagonize this pathway of signaling by TGFβ. The inhibitory SMADs, SMAD6 and SMAD7, antagonize this pathway of signaling by TGFβ. The inhibitory SMADs, SMAD6 and SMAD7, antagonize this pathway of signaling by TGFβ. The inhibitory SMADs, SMAD6 and SMAD7, antagonize this pathway of signaling by TGFβ.
CTGF in normal fibroblasts, we then examined the role of SMAD-dependent TGFβ signaling on the constitutive CTGF expression observed in fibroblasts cultured from the lesional area of scleroderma patients (12, 20). Previously, we have shown that CTGF promoter activity is substantially elevated in scleroderma fibroblasts relative to their normal counterparts (12). Here, we found that the elevated level of CTGF promoter activity observed in scleroderma fibroblasts is not dependent on the SMAD binding site; mutating the SMAD binding site in the context of the CTGF promoter does not decrease CTGF promoter activity in scleroderma fibroblasts. Conversely the previously termed TGFβRE (25) is required for basal CTGF promoter activity in normal fibroblasts and also for the elevated activity in scleroderma fibroblasts. The factor(s) binding this element are currently under investigation, but are not AP-1, CREB, or Sp1; factors known to contribute to TGFβ responses in other contexts, because oligonucleotides containing consensus binding sites for these factors do not compete for protein binding to the TGFβRE in gel shift studies. In any event, the difference in gene expression patterns between normal and scleroderma fibroblasts is not solely caused by activated SMAD-dependent TGFβ signaling because the SMAD-responsive PAI promoter (49, 50) is not elevated in scleroderma fibroblasts.

Collectively, these results suggest that the maintenance of the scleroderma phenotype, as visualized by elevated CTGF levels, is not caused by SMAD-dependent TGFβ signaling. These data do not exclude the possibility that a cryptic SMAD site in the CTGF promoter is used in the scleroderma fibroblasts; however, sequence inspection of the CTGF promoter does not yield another SMAD consensus binding site. In addition, cotransfection of SMAD3 and 4 into dermal fibroblasts does not activate expression of our SMAD mutant CTGF promoter/reporter construct (not shown). Furthermore, TNFα treatment, which suppresses the TGFβ-induction of collagen and CTGF in a manner possibly involving elevation of SMAD7 (20, 53–55), has no effect on basal collagen or CTGF gene expression in scleroderma fibroblasts (20).

These results are consistent with previous studies that have examined the role of TGFβ in acquisition and maintenance of the scleroderma phenotype. For example, recent reports localized TGFβ mRNA to the leading edge of the scleroderma lesion, that is the region of enhanced inflammatory response presumably involved with the initiation of the fibrotic response, but not in the lesional area itself (9, 56, 57). This is in contrast to CTGF whose expression patterns and levels are highly correlated with the severity of fibrosis (44, 58). Similarly, other studies that examined normal and scleroderma fibroblasts failed to see elevated TGFβ levels, enhanced binding of TGFβ to cells, or enhanced responsiveness to TGFβ in scleroderma fibroblasts (10, 11, 12, 52). Combined with our data in this report, these results suggest that although the initial expansion of the sclerotic lesion may require TGFβ action, the maintenance of the sclerotic lesion does not result from TGFβ action per se. Rather, the maintenance of the scleroderma phenotype may result from the failure to suppress downstream responses to TGFβ, such as the induction of CTGF and collagen synthesis. Thus although anti-TGFβ/anti-SMAD strategies may be effective in blocking the onset or progression of fibrosis (see Ref. 6), these strategies may not be effective at reversing fibrosis once it has occurred. Given the known profibrotic activity of CTGF, the high level of constitutive, SMAD-independent, CTGF expression present in the sclerotic lesion may be a cause of the excessive scarring that is observed in scleroderma patients. Thus, an anti-CTGF strategy, or at least identifying why CTGF is overexpressed in fibrosis, might yield a more effective method of reversing the fibrotic process.

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