Smad-dependent Transcriptional Activation of Human Type VII Collagen Gene (COL7A1) Promoter by Transforming Growth Factor-β*

We have previously shown that transforming growth factor-β (TGF-β) increases type VII collagen gene (COL7A1) expression in human dermal fibroblasts in culture (Mauviel, A., Lapierre, J.-C., Halchin, C., Evans, C. H., and Uitto, J. (1994) J. Biol. Chem. 269, 25–28). To gain insight into the molecular mechanisms underlying the up-regulation of COL7A1 by this growth factor, we performed transient cell transfections with a series of 5′-deletion promoter/chloramphenicol acetyltransferase reporter gene constructs. We identified a 68-base pair region between nucleotides −524 and −456, relative to the transcription start site, as critical for TGF-β response. Using electrophoresis mobility shift assays (EMSA) with an oligonucleotide spanning the region from −524 to −444, we discovered that a TGF-β-specific protein-DNA complex was formed as early as 11 min after TGF-β stimulation and persisted for 1 h after addition of the growth factor. Deletion analysis of the TGF-β-responsive region of the COL7A1 promoter by EMSA identified segment −496/−444 as the minimal fragment capable of binding the TGF-β-induced complex. Furthermore, two distinct segments, −496/−490 and −453/−444, appeared to be necessary for TGF-β-induced DNA binding activity, suggesting a bipartite element. Supershift experiments with a pan-Smad antibody unambiguously identified the TGF-β-induced complex as containing a Smad member. This is the first direct identification of binding of endogenous Smad proteins to regulatory sequences of a human gene.

The collagens comprise a superfamily of proteins that play a critical role in the maintenance of extracellular matrix integrity. Type VII collagen is found primarily in the basement membrane zone of specialized squamous epithelia, such as in the skin, various mucous membranes, and the cornea of the eye (1, 2). It is the predominant, if not the exclusive, component of anchoring fibrils, attachment structures that play a critical role in ensuring stability to the association of the epithelial basement membrane zone to the underlying papillary dermis (3, 4). Synthesis of functional anchoring fibrils is of critical importance in providing integrity to the cutaneous basement membrane zone, and abnormalities in these adhesion structures clinically manifest as dystrophic forms of epidermolysis bullosa, a group of heritable bullous diseases characterized by cutaneous fragility and the tendency to sub-basal lamina densa blister formation (5). In fact, our laboratory recently demonstrated that mutations within the COL7A1 gene are associated with different forms of dystrophic epidermolysis bullosa (6, 7).

Analysis of the 5′-end sequences of the human COL7A1 gene has revealed a promoter devoid of a canonical TATA or CAAT box (GenBank/EMBL accession no. L23982). Its expression requires the integrity of an Sp1-binding site located between residues −512 and −505, relative to the transcription start site (8). Despite these structural features usually associated with promoters of so-called “housekeeping” genes, type VII collagen expression has been shown to be transcriptionally regulated by several cytokines, including transforming growth factor-β (TGF-β)3 (9–11), and by other biological response modifiers, such as ultraviolet irradiation (12). Interestingly, we have previously shown that cytokine-mediated regulation of COLT1A1 gene expression is strikingly different from that of type I collagen, as evidenced by synergistic activation of COL7A1 gene expression by TGF-β and tumor necrosis factor-α (11). Thus far, however, little is known about the transcriptional mechanisms underlying the above mentioned regulation of COL7A1 gene expression.

In this study, we have investigated the molecular mechanisms by which TGF-β up-regulates the activity of human COL7A1 in dermal fibroblasts. We report, for the first time, evidence for Smad-mediated, immediate-early activation of a human gene by TGF-β through direct interaction of a Smad-containing transcription complex with the TGF-β-responsive region of the COL7A1 promoter.

MATERIALS AND METHODS

Cell Cultures—Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskins, were utilized in passages 3–6. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G, and 0.25 μg/ml Fungizone™). Human recombinant TGF-β2 was a kind gift from Dr. David Olsen, Celsity Co., Palo Alto, CA. It is referred to as TGF-β throughout the text.

* This work was supported in part by National Institutes of Health Grants 29-AR43751 (to A. M.) and RO1-AR41439 and T32-AR07651 (to J. U.) and by a Research Career Development Award from the Dermatology Foundation (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org

1 The abbreviations used are: TGF-β, transforming growth factor-β; ARE, activin-responsive element; CAT, chloramphenicol acetyltransferase; EMSA, electrophoresis mobility shift assay; TBSB, TGF-β-specific band; TBRs, TGF-β-responsive sequence; bp, base pair(s).
Plasmid Constructs—To study the transcriptional regulation of human type VII collagen gene (COL7A1) expression, transient transfection experiments were performed with various COL7A1 promoter 5′-deletion fragments cloned into promoterless pBSOCAT vector (13), as described previously (8).

Transient Cell Transfections and CAT Assays—Transient cell transfections of human dermal fibroblasts were performed with a calcium phosphate/DNA co-precipitation procedure (14). Briefly, cultured cells were transfected with 10 μg of plasmid DNA and 2 μg of the pRSV-β-galactosidase plasmid DNA to monitor the transfection efficiencies (15). After glycerol shock, the cells were placed in Dulbecco's modified Eagle's medium containing 1% fetal calf serum, and TGF-β2-galactosidase plasmid DNA to monitor the transfection efficiencies (15).

After glycerol shock, the cells were placed in Dulbecco's modified Eagle's medium containing 1% fetal calf serum, and TGF-β was added 3 h later. After 40 h of incubation, the cells were rinsed once with phosphate-buffered saline, harvested by scraping, and lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI). The β-galactosidase activities were measured according to a standard protocol (15). Unless stated otherwise, aliquots corresponding to identical β-galactosidase activity were used for each CAT assay with [14C]chloramphenicol as substrate (16), using thin layer chromatography. Following autoradiography, the plates were cut and counted by liquid scintillation to quantify the acetylated [14C]chloramphenicol.

Electrophoresis Mobility Shift Assays—Several fragments spanning the region between nucleotides −524 and −444 of the COL7A1 promoter responsive to TGF-β were generated by polymerase chain reaction amplification using the plasmid −722COL7A1 as template and purified by electroelution after electrophoresis in a 2% agarose gel. Each oligonucleotide was used either as a probe or as a competitor in electrophoresis mobility shift assay (EMSA) experiments. Nuclear extracts were isolated from human dermal fibroblasts using a small scale preparation (17), aliquoted in small fractions to avoid repetitive freezing, and stored at −80 °C until use. The protein concentration in the extracts was determined using a commercial assay kit (Bio-Rad). Nuclear extracts (5 μg) were incubated for 20 min on ice in binding reaction buffer (10 mM HEPES-KOH, pH 7.9, at 4 °C, 4% glycerol, 0.4 mM KCl, 0.4 mM EDTA, and 0.4 mM dithiothreitol) in the presence of 1 μg of poly(dI-dC), prior to the addition of [32P]5′-end-labeled oligomers (0.05–0.1 pmol, 2–6 × 10⁶ cpm) for another 20 min of incubation at 4 °C. For competition experiments, a 1–60-fold molar excess of unlabeled DNA was added to the binding reaction. For supershift experiments, nuclear extracts were incubated overnight with antisera prior to the binding reaction. Sp1 and c-Jun/AP-1 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The pan-Smad antibody 367 was described previously (21). Samples were then separated by electrophoresis on a 4% polyacrylamide gel in 0.5 Tris borate-EDTA buffer (TBE) at 4 °C, fixed for 1 h in 30% methanol, 10% acetic acid, vacuum-dried, and autoradiographed.

RESULTS

TGF-β Up-regulates Human COL7A1 Expression at a Transcriptional Level—We and others have previously demonstrated that TGF-β is a potent inducer of type VII collagen gene expression in dermal fibroblasts, as determined at both protein and mRNA levels (10, 11). To investigate whether TGF-β up-regulates COL7A1 gene expression at the transcriptional level by activation of the promoter, human dermal fibroblast cultures were transiently transfected with several 5′-end deletion/CAT reporter gene constructs spanning the COL7A1 promoter region from positions −722 to +92, relative to the transcription start site, +1 (8). Cells were subsequently treated with TGF-β (10 ng/ml) for 40 h, at which point CAT activity (representing COL7A1 promoter activity) was determined. As shown in Fig. 1A, a stimulatory effect of TGF-β was observed with constructs −722COL7A1/CAT (−4.4-fold induction) and −524COL7A1/CAT (−5.3-fold induction), indicating that the up-regulation of type VII collagen gene expression by TGF-β occurs, at least in part, at the transcriptional level through activation of the promoter. Subsequent 5′-deletion to position −456 abolished the COL7A1 promoter responsiveness to TGF-β, which was similarly lost when further 5′-deletions extended to positions −396 and −230 (Fig. 1A). These data indicate that the DNA sequences between residues −524 and −456 of the COL7A1 promoter are essential in providing TGF-β responsiveness in fibroblasts.

We have recently demonstrated that a GT box, −512/−505, binds the transcription factor Sp1 and is crucial for high expression of COL7A1 (8). Sp1 has been shown to play a role in TGF-β-mediated up-regulation of COL1A2 (18), although other transcription factors, such as AP-1, are also involved (18–20). We, therefore, tested the effect of a functional mutation in this GT box on the TGF-β responsiveness of COL7A1 promoter. For this purpose, the TGF-β responsiveness of mutant −524mCOL7A1/CAT construct, harboring the GT box mutation, was compared with that of −524COL7A1/CAT in transient cell transfection experiments. As anticipated from our previous studies (8), the mutation drastically reduced the basal activity of the promoter. It did not, however, alter its up-regulation by TGF-β. On the other hand, 5′-deletion up to position −456 not only reduced basal promoter activity by −90%, but also totally eliminated TGF-β responsiveness, confirming the data presented in Fig. 1A. These data indicate that the −512/−505 GT box is not involved in the TGF-β response.

TGF-β Induces the Rapid Formation of a Nuclear Protein-DNA Complex Binding to the −524/−444 Fragment of COL7A1 Promoter—To determine whether the region of COL7A1 promoter shown above to confer TGF-β responsive-
Accurate determination of the earliest time point at which the TBSB could be observed was performed in a third set of experiments. For this purpose, fibroblast nuclear extracts were prepared every 2 min, from 5 to 15 min, after addition of TGF-β to the fibroblast cultures and tested in EMSAs, using the COL7A1 promoter fragment −524/−444 as a probe. The results indicate that binding of the TGF-β-specific complex appears with maximal intensity as early as 11 min poststimulation (not shown).

To address the specificity of the TBSB and to attempt to identify the protein(s) that it may contain, competition experiments were carried out with several oligonucleotides spanning the −524/−444 region of COL7A1 promoter, as well as oligonucleotides containing consensus sequences for known transcription factors, Sp1, AP-2, and AP-1. As shown in Fig. 2B, a 30-fold molar excess of unlabeled −524/−444 fragment efficiently competed all binding associated with the probe (lane 3). Fragment −524/−491, containing the GT box described above, efficiently competed Sp1-related binding (lane 4), confirming our previous observations (8), whereas fragments −490/−457 (lane 5) and −500/−475 (lane 6) failed to compete either Sp1 or TBSB binding. Fragment −504/−444 (lane 7) competed TBSB binding and most of the Sp1 binding, suggesting that the TBRS is, indeed, comprised within this fragment. As expected from our previous observations (8), an Sp1 oligonucleotide (60-fold molar excess) abolished shifts 1 and 2 but did not alter the TBSB. Also, an AP-2 oligonucleotide significantly displaced both shifts 1 and 2 but without altering TBSB formation (lane 9). Competition of Sp1 binding is likely due to the sequence similarity between Sp1 and AP-2 consensus sequences. Finally, an AP-1 oligonucleotide (lane 10) did not displace either Sp1 binding or the TBSB.

**Formation of the TBSB Requires Two Distinct Sites on the −524/−444 Region of COL7A1 Promoter**—The next set of experiments was designed to further refine the cis-acting element(s) responsible for the appearance of the TBSB. Toward this end, a series of oligonucleotides was generated, representing a stepwise deletion from either the 5′- or 3′-ends of the −504/−444 promoter fragment. Their sequences and relative positions are depicted in Fig. 3A. First, EMSA experiments were performed with nuclear extracts from fibroblast cultures treated for 30 min with TGF-β, using each of the various stepwise deletion oligonucleotides as probes. As expected from the competition experiments presented above, the −504/−444 fragment efficiently bound the TBSB (Fig. 3B, lane 2). 5′-End deletion from nucleotide −504 to nucleotide −496 did not affect TBSB formation with oligonucleotides extending to position −444 in 3′ (lanes 4 versus lane 2). However, further 5′-end deletion to residue −490 or 3′-end deletion to residue −453 resulted in complete loss of TGF-β-induced binding activity (lanes 6 and 8, respectively). Three conclusions could be drawn from these experiments. First, the minimal COL7A1 promoter fragment capable of binding the TBSB is −496/−444. Second, it is likely that two distinct sites, one located within the sequences surrounding nucleotides −496/−490 at the 5′-end of the TBRS and the other between nucleotides −453/−444 at the 3′-end of the TBRS, are simultaneously required for providing the TGF-β-induced binding activity. Third, deletion of the Sp1 binding GT box between −512 and −505 did not influence the formation of the TBSB, further indicating that the GT box is not involved in TGF-β response. Finally, despite the removal of the GT box, residual Sp1 binding activity was still observed, suggesting the presence of a secondary Sp1 binding sequence within the −490/−453 DNA fragment of COL7A1 promoter. The latter observation was confirmed in two subsequent experiments. First, recombinant Sp1 protein was capable of binding

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**Fig. 2. Binding of fibroblast nuclear proteins to the TGF-β-responsive region of human COL7A1 promoter.** Gel mobility shift assays were performed with a labeled oligonucleotide spanning the region from −524 to −444 of the COL7A1 promoter. A, nuclear proteins were prepared from fibroblast cultures treated with TGF-β (10 ng/ml) for various lengths of time, as indicated, and binding reactions were carried out as described under “Materials and Methods.” DNA-protein complexes were separated from unbound oligonucleotides by nondenaturing 4% acrylamide gel electrophoresis. Note the appearance of a new oligonucleotide resulted from 5′/3′-footprint digestion (TBSB). Also, an AP-2 oligonucleotide significantly displaced both shifts 1 and 2 but without altering TBSB formation (lane 9). Competition of Sp1 binding is likely due to the sequence similarity between Sp1 and AP-2 consensus sequences. Finally, an AP-1 oligonucleotide (lane 10) did not displace either Sp1 binding or the TBSB.

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this fragment, as determined in gel mobility shift assays, and second, an antibody specifically directed against Sp1 supershifted the weak protein-DNA complexes 1 and 2 generated with the −504/-444 COL7A1 probe (not shown).

Confirmation of the data presented above was provided in a series of competition experiments in which the deletion fragments described in Fig. 3A were utilized to compete the TGF-β-induced protein-DNA complex formation with the longer −524/-444 COL7A1 fragment. Only the fragments containing both discrete regions −496/−490 and −453/−444 were able to compete binding of the TBSB to the −504/-444 probe, whereas shorter fragments lacking either one or both of these elements were unable to compete (not shown).

Identification of Smad as Part of the TGF-β-induced DNA-Protein Complex—We next attempted to delineate the signaling pathway by which TGF-β induces the formation of the TBSB. For this purpose, fibroblast cultures were first incubated with several inhibitors of different signaling pathways, prior to TGF-β stimulation (30 min) and subsequent nuclear protein preparation. All inhibitors were tested at three concentrations, which have been shown in previous publications to effectively block their target pathways. Protein kinase C inhibitors (GP1992X, staurosporine, calphostin, H7), phospholipase C inhibitors (D609, U73122), tyrosine kinase inhibitors (genistein, tyrphostin 51), the tyrosine/threonine phosphatase inhibitor sodium orthovanadate, mitogen-activated protein kinase pathway inhibitors (SB203580, PD 98059), calcium ionophore, pertussis and cholera toxins, and okadaic acid all failed to block TBSB formation (not shown). This lack of effect from all inhibitors tested suggests a rapid mechanism that may be triggered directly through the TGF-β receptors, without a complex cytoplasmic cascade of events, involving the several pathways listed above. In this context, Smad proteins were recently identified as immediate-early response factors, which are translocated into the nucleus following their phosphorylation by TGF-β receptor type I (reviewed in Refs. 22–25).

To identify the transcription factor(s) participating in the TGF-β-induced complex, we performed supershift assays with antibodies specific for Sp1, c-Jun, and a recently developed pan-Smad antibody, 367 (Ref. 21). As shown in Fig. 4, an Sp1 antibody recognized and supershifted both shifts 1 and 2 without altering the TGF-β-specific band (lane 4). These results were anticipated from our previous demonstration of the essential role played by Sp1 in high basal activity of the COL7A1 promoter (8). Anti-c-Jun had no effect on any of the three DNA-protein complexes (lane 5). Interestingly, the anti-Smad polyclonal antibody 367 was able to supershift part of, and to reduce the total amount of, the TBSB (lane 3). Collectively, these data indicate that a member of the Smad family is rapidly complexing to the TGF-β-responsive region of the COL7A1 promoter following treatment of fibroblasts with TGF-β.

DISCUSSION

Our studies of the human COL7A1 promoter reported here demonstrate that this gene is an immediate-early target of TGF-β transcriptional activation in dermal fibroblasts, with evidence of formation of a TGF-β-specific transcriptional complex within 11 min after TGF-β addition in a ligand-dependent manner. Smad proteins have recently been identified as critical intracellular mediators of TGF-β family-induced signals (reviewed in Refs. 22–25). Upon ligand binding to TGF-β type I and II receptors, two Smad isoforms, Smad2 and Smad3, are recruited to the type I receptor where they undergo phosphorylation on conserved serine residues in the C terminus. Following activation, these proteins associate with the tumor suppressor Smad4/DPC4 and are translocated to the nucleus where they presumably act in transcriptional complexes (reviewed in Refs. 22–25). In our experiments, supershift assays using a pan-Smad antibody (21) show that the protein-DNA complex, TBSB, contains one or more Smad proteins. Based on signaling specificity, this complex likely contains Smad2 or Smad3 along with Smad4.

2 R. J. Lechleider and A. B. Roberts, unpublished data.
The method of transcriptional activation by Smads has recently begun to be elucidated. Chen et al. (26) identified a DNA-binding protein of the forkhead family (FAST-1) that specifically and inducibly interacts with an activin-responsive element (ARE) in the promoter of the Xenopus homeobox gene Mix.2 (27). FAST-1 also interacts with Smad2 and Smad4/DPC4 in the ARE binding complex (28). Reconstitution of this system in mammalian cells demonstrates that efficient DNA binding and transcriptional activation require FAST-1, Smad2, and Smad4 in the same complex (29). Our data represent the first example of a mammalian promoter directly regulated by interactions with endogenous Smad proteins and as such make COL7A1 the first example of an immediate-early gene regulated by TGF-β in a Smad-dependent fashion.

Although the cis-elements necessary for interaction with Smad transcriptional complexes are not known, some architectural constraints may be deduced from comparison of the TBRs in the COL7A1 promoter with the ARE from the Mix.2 gene. Chen et al. (26) identified a direct 6-bp AAATGT repeat separated by 11 bp, and this sequence was used to clone the DNA binding protein FAST-1. Mutation of either element of this repeat disrupts DNA binding by FAST-1 (26). Similarly, the COL7A1 element contains two 5-bp ATGGC repeats, two adjacent CAGA repeats in the 5′end and two pairs of 4-bp repeats, CCCA and ACAG. Deletion studies suggest that two elements are necessary for Smad/DNA interaction, as both the COL7A1 –490/–444 and –496/–453 probes failed to bind the TGF-β-inducible complex, whereas the –496/–444 fragment was the minimum required to generate a gel-shifted band. This suggests a mechanism similar to that observed with the Mix.2 ARE, with two separated elements required for full activity (26). The nature of the exact cis-elements required for DNA-Smad interaction is currently under investigation. Whether a Smad protein binds the COL7A1 promoter directly cannot be ascertained from our data. The Drosophila Smad1 homologue, the mad gene product, can bind elements in the vestigial promoter directly, and a consensus Mad binding sequence (GCCnCGc) that can bind recombinant Mad protein has been identified (30). Similarly, the tumor suppressor Smad4 can bind DNA directly (31), but this has not been convincingly demonstrated in an endogenous complex. In the COL7A1 promoter, an accessory DNA binding protein may be required, as in the Mix.2 ARE, or a Smad or Smads may bind the TGF-β-responsive elements without additional binding proteins. Experiments are under way to explore these possibilities.

Acknowledgment—The expert technical assistance of Ying-Jee Song is gratefully acknowledged.

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doi: 10.1074/jbc.273.21.13053

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