Opposite Smad and Chicken Ovalbumin Upstream Promoter Transcription Factor Inputs in the Regulation of the Collagen VII Gene Promoter by Transforming Growth Factor-β*

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A critical component of the epidermal basement membrane, collagen type VII, is produced by keratinocytes and fibroblasts, and its production is stimulated by the cytokine transforming growth factor-β (TGF-β). The gene, COL7A1, is activated by TGF-β via Smad transcription factors in cooperation with API. Here we report a previously unsuspected level of complexity in this regulatory process. We provide evidence that TGF-β may activate the COL7A1 promoter by two distinct inputs operating through a common region of the promoter. One input is provided by TGF-β-induced Smad complexes via two Smad binding elements that function redundantly depending on the cell type. The second input is provided by relieving the COL7A1 promoter from chicken ovalbumin upstream promoter transcription factor (COP-TF)-mediated transcriptional repression. We identified COP-TFI and -TFII as factors that bind to the TGF-β-responsive region of the COL7A1 promoter in an expression library screening. COP-TFs bind to a site between the two Smad binding elements independently of Smad or API and repress the basal and TGF-β-stimulated activities of this promoter. We provide evidence that endogenous COP-TF activity represses the COL7A1 promoter. Furthermore, we show that TGF-β addition causes a rapid and profound down-regulation of COP-TF expression in keratinocytes and fibroblasts. The results suggest that TGF-β signaling may exert tight control over COL7A1 by offsetting the balance between opposing Smad and COP-TFs.

Type VII collagen belongs to an extensive family of closely related proteins involved in cell anchoring to extracellular matrix and cartilage formation. Although some collagens have a widespread distribution, type VII collagen is found exclusively in the basement membrane of stratified squamous epithelia (1, 2). Its subunit, the type VII collagen α-chain (COL7A1), is expressed in both dermal fibroblasts and epidermal keratinocytes (3–5). COL7A1 forms homotrimers that are assembled into fibrils (6). These fibrils are thought to anchor the epidermal basement membrane to the underlying dermal extracellular matrix (7). Mutations that cause structural alterations or defective expression of COL7A1 lead to dystrophic epidermolysis bullosa, a group of inherited skin disorders in which blisters form between the basement membrane and the papillary dermis (8).

Transforming growth factor-β (TGF-β)1 in particular is a potent inducer of COL7A1 expression in fibroblast and keratinocytes (9–11). A multifunctional cytokine, TGF-β critically regulates cell adhesion and extracellular matrix production among other cellular functions (12, 13). In addition to activating the production of collagen VII and other types of interstitial collagens, TGF-β controls the expression of fibronectin, extracellular matrix proteoglycans, integrin cell adhesion receptors, pericellular proteases, and protease inhibitors. The effects of TGF-β on genes encoding the cell adhesion apparatus, along with its effects on cell proliferation and differentiation, exert a profound influence on tissue development and homeostasis. TGF-β activates COL7A1 expression at the transcriptional level (9–11). Although a general signal transduction pathway for transcriptional regulation by TGF-β has been established, little is known about the specific mechanisms involved in the COL7A1 gene response. Activated TGF-β receptors directly phosphorylate Smad2 and Smad3, inducing their accumulation in the nucleus to regulate the expression of a large set of genes (14). Receptor-phosphorylated Smads associate with Smad4, which in most instances is indispensable for transcriptional regulation. Smad proteins recognize the sequence CAGAC, commonly referred to as the Smad binding element (SBE). To regulate specific target genes, however, activated Smad complexes must additionally interact or functionally cooperate with other transcription factors. In the case of COL7A1 activation by TGF-β, previous studies have shown the involvement of Smad and API transcription factors (9–11). The TGF-β-responsive region in the COL7A1 gene contains an canonical SBE. Mutation of this element inhibits the activation of the COL7A1 promoter by TGF-β in fibroblasts (9). Paradoxically, this SBE is not required for this response in keratinocytes (11) suggesting that the cellular context and other complexities play an important role in the regulation of COL7A1 expression by TGF-β.

To address these questions, we investigated the role of various elements present in the TGF-β-responsive region of the COL7A1 promoter in keratinocytes and fibroblasts. We report the existence of a second Smad binding site in the COL7A1 promoter that provides cell type-dependent redundancy, clarifying previous controversies. Our results also suggest that

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1 The abbreviations used are: TGF-β, transforming growth factor-β; COP-TF, chicken ovalbumin upstream promoter transcription factor; FBS, fetal bovine serum; SBE, Smad binding element.
COL7A1 activation in response to TGF-β involves not only activation by a Smad complex but also the relief of inhibition by COUP-TF transcriptional repressors.

EXPERIMENTAL PROCEDURES

Cell Lines—HaCaT, NIH 3T3, and COS1 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% FBS (Invitrogen).

Plasmids—The human COL7A1 promoter fragment spanning from -496 to +92 was generated by PCR, using human DNA as template and oligonucleotides primers with flanking SalI/HindIII sites. This fragment was cloned into the XhoI/HindIII sites of the low basal activity luciferase reporter plasmid pGL2-basic (Promega). Mutant forms of the wild type promoter were obtained by site-directed mutagenesis using oligonucleotides carrying the indicated mutations. FLAG-tagged COUP-Dim was generated by oligonucleotides primers with flanking SalI/HindIII sites. This fragment was cloned into the pCMV5 vector. FLAG-tagged COUP-TFI or -TFII or COUP-Dim using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Transcriptional Assays—HaCaT cells were transfected by using the DEAE-dextran method as described previously (15). NIH 3T3 cells were transfected using LipofectAMINE or the calcium-phosphate precipitation method. Briefly, 3 μg of plasmid DNA were diluted in 250 μl of 2× HEPES-buffered saline (50 mM HEPES, 280 mM NaCl, 1.5 mM Na2HPO4, to final pH 7.1). 80-μl aliquots of this precipitate were overlaid on wells of a 12-well dish containing 1 ml of freshly added Dulbecco’s modified Eagle’s medium plus 10% FBS. After transfection cells were incubated in medium containing 10% FBS for 6–8 h. The medium was changed to 0.2% FBS, and cells were incubated with 100 pt TGF-β for 20–24 h. Cell lysates were then subjected to luciferase assays (Promega) in a Berthold luminometer (Nashua, NH). A cytokomegalovirus promoter Renilla luciferase plasmid (Promega) was used as a control to normalize the transfection efficiency and was assayed as described previously (16).

Oligonucleotide Precipitation Assays—Cells were treated with TGF-β for 1 h under normal culture conditions and then lysed by sonication in buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 1% glycerol, 1 mM dithiothreitol, 0.5% Nonidet P-40) with phosphatase and protease inhibitors. Cell debris was removed by 5-min centrifugation at 10,000 × g at 4°C. Cell extracts were incubated for 16 h with 1 μg of biotinylated double-strand oligonucleotide corresponding to the wild type or mutant forms of the COL7A1 -495/-431 promoter region. DNA-bound proteins were collected with streptavidin-agarose beads for 1 h, washed with lysis buffer, separated on a SDS-polyacrylamide gel, and identified by Western blotting.

RNA Assays—Exponentially growing cells were incubated with 100 pt TGF-β for the indicated time. Cells were harvested, and total RNA was extracted by using Qiagen (Chatsworth, CA) RNaseasy minikit. 100 μg of total RNA was then used to obtain poly(A) RNA using a Clontech kit. The poly(A) RNA obtained was run in parallel denaturing gel and subjected to Northern analysis. Blots were probed with probes corresponding to human or mouse COL7A1, actin, COUP-TFI, or COUP-TFII.

Yeast One-hybrid Screening—A NIH 3T3 cDNA library in the pGAD10 fusion vector (Clontech), which provides an N-terminal GAL4 transcriptional activation domain, was transformed into a yeast strain bearing four consecutive copies of the -495/-431 region of the COL7A1 promoter upstream of both HIS3 and a LacZ reporter gene. cDNA clones that allowed growth in -His plates and gave a strong β-galactosidase activity in a colony lift assay were identified as positive.

RESULTS

Role of Two Smad Binding Elements in the COL7A1 Promoter—To investigate the transcriptional activation of COL7A1 by TGF-β, we generated luciferase reporter constructs driven by the -496/+92 region of the human COL7A1 (9) (Fig. 1A). Versions of this promoter were generated containing mutations that target the previously described SBE site (9), which we refer to as 5’-SBE, and an adjacent AP1 site (10) as well as other sites in this region that are conserved in the human and mouse genes (Fig. 1A). TGF-β addition strongly stimulated the expression of the wild type COL7A1 promoter in mouse NIH 3T3 fibroblasts, and this response was diminished by mutations targeting the 5’-SBE (Fig. 1B). These results are in full agreement with those reported in human dermal fibroblasts (9). TGF-β also stimulated the expression of the wild type COL7A1 promoter in HaCaT human skin keratinocytes. Surprisingly, however, this response was not diminished by mutations targeting the 5’-SBE (Fig. 1B).

Several lines of evidence suggested that this tolerance for a mutant 5’-SBE in HaCaT keratinocytes reflected a genuine difference between fibroblasts and keratinocytes and not an anomaly of the HaCaT cell line. HaCaT cells are well charac-
terized in terms of their responsiveness to TGF-β (17). Further, we verified that TGF-β stimulates the expression of the endogenous COL7A1 gene at the mRNA level (Fig. 1C). Moreover, the lack of an effect of mutations in this SBE has been reported recently in mouse keratinocytes as well (11).

Searching for a possible basis for this difference between keratinocytes and fibroblasts, we noticed a perfect inverted SBE (3′-SBE) −50 base pairs downstream of the 5′-SBE (Fig. 1A). This site was not investigated in previous studies (9–11). We tested the effect of mutating this site alone or in combination with the 5′-SBE. Mutation of the 3′-SBE affected the response of the COL7A1 promoter to TGF-β in a manner similar to mutation of the 5′-SBE site, as the 3′-SBE mutations diminished the TGF-β response in fibroblasts but not in keratinocytes (Fig. 1B). Remarkably, the simultaneous mutation of the 5′-SBE and 3′-SBE sites completely eliminated the TGF-β response not only in fibroblasts but also in keratinocytes (Fig. 1B). These results suggest that the 5′-SBE and 3′-SBE in the COL7A1 promoter are important for the TGF-β response in both cell types, acting redundantly in keratinocytes but not in fibroblasts.

Cell Type-dependent Cooperation with AP1—AP1 sites and the Fos-Jun complexes that recognize these sites have been shown to play a role in certain TGF-β responses (10). To identify factors that may regulate COL7A1 from the −495/−431 promoter region, we screened a cDNA expression library for gene products that bind to this region. To this end, we generated a yeast strain expressing HIS3 and LacZ under the control of four copies of this promoter region and then used these cells to screen a mouse fibroblast cDNA library fused to the GAL4 transcriptional activation domain. In this approach, cDNAs encoding GAL4 fusion proteins that bind to the −495/−431 promoter region would confer HIS3 phenotype and activate LacZ expression. Only four cDNA clones were isolated that fulfilled these criteria. One of these cDNAs encoded the full-length COUP-TFII (also known as ARP-1) (Fig. 3B).

COUP-TFII and COUP-TFII are closely related orphan members of the nuclear/steroid receptor family (18, 19). They consist of an N-terminal DNA binding domain containing two zinc finger motifs and a C-terminal transcription regulatory domain containing a dimerization region (Fig. 3B). COUP-TFI and COUP-TFII bind to DNA as dimers that recognize two direct TGACC(C/T) repeats separated by a single base pair spacer (20). COUP-TFI and -TFII are thought to act primarily as transcriptional repressors (20–25).

Examination of the COL7A1 −495/−431 region revealed that the sequence of the −450 conserved box is similar to the COUP-TF consensus binding sequence (Fig. 3C). As mutations of the −450 conserved box augmented the activity of the COL7A1 promoter, we hypothesized that this effect might be due to a loss of endogenous COUP-TF binding to this region. To determine whether this region can bind COUP-TF proteins, we carried out DNA precipitation assays using wild type and mutant biotinylated COL7A1 −495/−431 oligonucleotide probes (Fig. 3C). A FLAG-tagged COUP-TFII construct expressed in COS cells bound to the wild type probe but not to two different probes that contain mutations in the −450 conserved box (Fig. 3D). COUP-TFII binding to the wild type promoter was not
affected by cell treatment with TGF-β and was not disrupted by SBE or AP1 site mutations that block the binding of Smad or Jun, respectively (Fig. 3E). We looked for, but could not find, evidence of Smad3 or Smad4 binding to COUP-TFI or COUP-TFII. In extracts from cells overexpressing Smads and COUP-TFs, these proteins neither enhanced nor interfered with the binding of each other to the COL7A1 −495/−431 probe (data not shown). These results suggest that the −450 conserved box is a COUP-TF site that binds COUP-TF proteins independently of Smad and AP1.

**Down-regulation of COUP-TF Expression by TGF-β**—Interestingly, GeneChip transcriptomic profiling data in HaCaT cells revealed that TGF-β treatment decreases the level of COUP-TFI transcripts (17). We verified by Northern analysis that TGF-β addition causes a rapid and profound decrease in the level of COUP-TFI mRNA in HaCaT and NIH 3T3 cells (Fig. 4). COUP-TFI expression, which was present in NIH 3T3 cells but barely detectable in HaCaT cells, was also inhibited by TGF-β (Fig. 4). The down-regulation of COUP-TFI by TGF-β in HaCaT cells was rapid (t1/2 ≈ 2 h; Fig. 4) and preceded the up-regulation of COL7A1 expression (t1/2 ≈ 4 h; refer to Fig. 1C). Thus, TGF-β action down-regulates the expression of a putative COL7A1 repressor.

**Repression of COL7A1 Promoter by COUP-TF**—We investigated whether COUP-TFs can act as repressors of the COL7A1 promoter in transfected HaCaT cells. Indeed, both COUP-TFI and COUP-TFII markedly inhibited the basal activity of the wild type COL7A1 promoter as well as its activation by TGF-β (Fig. 5A). The promoter construct containing mutations in the −450 conserved box (the COUP-TF binding element) was not only hyperactive under basal conditions but also completely resistant to inhibition by exogenous COUP-TFI or COUP-TFII (Fig. 5A).

As no anti-COUP-TF antibodies could be obtained for these studies, we resorted to alternative approaches to determine whether the COL7A1 promoter is sensitive to endogenous COUP-TFs. First, we mutated the COUP-TF binding region to disrupt the COUP-TF site (mt-COUP1 and 2) or bring the sequence of this site closer to the consensus COUP-TFI binding sequence (COUP+). The mouse sequence as well as the consensus COUP-TFI binding site is shown. D, COS1 cells were transfected with a vector encoding FLAG-tagged COUP-TFII. Cell lysates were precipitated with biotinylated probes corresponding to the wild type or the indicated mutant versions of the COL7A1 −495/−431 promoter region. Protein-DNA complexes were subjected to Western immunoblotting with anti-FLAG antibodies. E, cells were transfected with mouse FLAG-tagged COUP-TFII and then treated with 100 pm TGF-β for 1 h or not treated. Cell lysates were precipitated with biotinylated probes corresponding to the wild type or the indicated mutant versions of the COL7A1 −495/−431 promoter region. Protein-DNA complexes were subjected to Western immunoblotting using anti-FLAG, anti-Smad4, or anti-pan-Jun antibodies.
and the other by the relief of COUP-TF-mediated transcriptional repression of the COL7A1 promoter. This complex regulatory process has several features that distinguish it from previously characterized TGF-β-regulated promoters.

We provide evidence that regulation of the COL7A1 promoter by TGF-β-activated Smad factors occurs via two SBEs in the TGF-β-responsive region of this promoter. The SBE, or CAGAC sequence, is the best characterized of the various sequences implicated in DNA binding by signal-activated Smad factors (14, 26, 27). In many TGF-β target promoters characterized to date, the responsive region contains only one SBE. The COL7A1 promoter was previously thought to be in this class, with only one identified functional SBE (referred to here as the 5′-SBE) (9–11). Our evidence, however, indicates that a second SBE, located ~50 bp downstream of the 5′-SBE in the human and mouse promoters, is also involved. In promoter construct assays at least, these two SBEs are functionally equivalent and act redundantly in keratinocytes but not in fibroblasts. This partial redundancy explains the previously encountered paradox that COL7A1 promoter constructs devoid of the only identified SBE lack TGF-β responsiveness in fibroblasts (9) but remain responsive in keratinocytes (11).

The results of our oligonucleotide binding experiments show that an endogenous, TGF-β-dependent Smad complex can specifically bind to the TGF-β-responsive region of the COL7A1 promoter. This binding requires the integrity of both SBEs but not the integrity of other conserved elements in this region, including AP1 and COUP-TF sites, whose mutation prevents the binding of their cognate factors. While we cannot exclude the possibility that the mutations in the AP1 and COUP-TF sites may still allow other, as yet unknown factors to cooperate with Smads in binding to this promoter, this possibility seems unlikely given the density of our mutational analysis. This is of interest because the affinity of an isolated Smad-SBE interaction is too low to support effective binding and gene regulation in vivo on its own (27). In promoters that contain one single SBE, receptor-phosphorylated Smads must associate with Smad4 and at least one other DNA binding factor to achieve high affinity binding. Examples include the activation of Xenopus Mix2 by a Smad2-FoxH1 complex (28) and goosecoid by a Smad2-Mixer complex (29) in response to nodal/activin signals and the activation of an Igα promoter by a Smad3-E2F4/5 complex (30–32). This also applies to promoters regulated by bone morphogenetic proteins via Smad1, as in the activation of Xenopus Vent2 by a Smad1-OAZ complex (34, 35) and mouse Tlx2 by a Smad1-Escit complex (36) and the repression of c-MYC by a Smad3-E2F4/5 complex in response to TGF-β in human epithelial cells (33), with Smad4 included in these complexes in all cases.

Although the requirement of a DNA binding cofactor for Smad binding to target promoters containing one single SBE is well established, recent work has suggested that another class of Smad target promoters may achieve high affinity binding by cooperative interactions of multiple SBEs with the multiple DNA binding domains present in the phosphoSmad-Smad4 heteromer. Previous examples of this paradigm include Smad7 (37, 38) and Id1 (39, 40), which are common targets of TGF-β and BMP, and CDKN1A (p21Cip1), which is a target of TGF-β (41). A ligand-activated Smad complex may bind to these promoters independently of other cofactors, although it may have to recruit additional transcription factors for regulation of the gene. Thus, a TGF-β-activated Smad3-Smad4 complex recruits FoxO to the CDKN1A promoter for activation (41) and recruits ATF3 to the Id1 promoter for repression (17).

The present results suggest that COL7A1 belongs to this class of TGF-β target genes. COL7A1 contains two functional
TGF-β is proposed to activate the COL7A1 promoter via two inputs. TGF-β activates a Smad complex that binds to the COL7A1 promoter and activates transcription cooperatively with a TGF-β-independent AP1 complex. In parallel, TGF-β action facilitates the activation of this promoter by relieving it from COUP-TF-mediated repression. TGF-β rapidly and profoundly down-regulates the expression of COUP-TFI and -TFII, which act as COL7A1 promoter repressors. The involvement of Smads in the COUP-TF down-regulation response remains to be determined.

SBEs that are recognized by a Smad complex apparently without a strict dependence on additional factors, and it contains an AP1 site that in fibroblasts at least is required for strong TGF-β-dependent transactivation (10). COLIA2 (42), PAI1 (43, 44), and c-Jun (45) have also been found to respond to TGF-β through a cooperation between SBE and AP1 sites. Although Smad and AP1 factors may interact in the cell and have been proposed to bind to the PAI1 promoter as a complex (43), our results agree with reports that Smad and AP1 bind to their respective elements independently of each other (10, 45, 46).

A second set of findings in the present studies led to evidence that TGF-β down-regulates the expression of COUP-TF, and this effect may contribute to relieving COL7A1 from COUP-TF-mediated repression in keratinocytes and fibroblasts. The finding that COUP-TFs can negatively regulate the COL7A1 promoter was unexpected. Interestingly, the only four positive clone identifiers in our cDNA expression library screening for COL7A1 wild type, and proteins-DNA complexes were subjected to Western blotting and probed with anti-FLAG antibody. Western blotting was performed with whole extracts to verify the expression of the two constructs. B, NIH 3T3 cells were transfected with increasing amounts of FLAG-COUP-Dim expression plasmid together with a COL7A1 promoter reporter construct. Cells were untreated or treated with TGF-β for 20 h, and luciferase activity was determined.

We provide several lines of evidence suggesting that COUP-TFs may function as repressors of the COL7A1 promoter. First, COUP-TFI and -TFII strongly decrease the activity of the COL7A1 promoter in transcriptional assays. Second, mutations that disrupt the COUP-TF site increase the basal activity of the COL7A1 promoter, whereas mutations that increase the affinity of this site decrease the activity of the promoter. Third, expression of a dominant-negative construct that inhibits COUP-TF binding to the COL7A1 promoter strongly increased the basal activity of this promoter and its stimulation by TGF-β in keratinocytes.

We found that TGF-β addition to keratinocytes or fibroblasts causes a rapid and profound decrease in the expression of COUP-TFI and -TFII. This decrease preceded the increase in COL7A1 mRNA levels. These observations suggest that in addition to inducing the formation of a COL7A1-activating Smad complex, TGF-β action may facilitate the activation of this gene by relieving it from COUP-TF-mediated repression. This dual input is similar to the recently delineated mechanism of activation of CDKN1A (p21Cip1) and CDKN2B (p15Ink4b) by TGF-β in epithelial cells. TGF-β elevates the expression of these two cyclin-dependent kinase inhibitors through the induction of activator Smad complexes and the relief of c-MYC-mediated repression (41, 50, 51). As in the case of COUP-TF, c-MYC expression is rapidly down-regulated by TGF-β, allowing the depletion of c-MYC from the CDKN1A and CDKN2B promoters. A dual activation switch on the COL7A1 promoter would likewise afford a tight control over its expression and control by TGF-β, perhaps reflecting the critical importance of COL7A1 regulation in epidermal homeostasis.

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