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Antagonistic Regulation of Type I Collagen Gene Expression by Interferon-γ and Transforming Growth Factor-β

INTEGRATION AT THE LEVEL OF p300/CBP TRANSCRIPTIONAL COACTIVATORS*

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Asish K. Ghosh, Weihua Yuan, Yasuji Mori, Shu-jen Chen, and John Varga‡

From the Section of Rheumatology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60607

Among the extracellular signals that modulate the synthesis of collagen, transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ) are preeminent. These two cytokines exert antagonistic effects on fibroblasts, and play important roles in the physiologic regulation of extracellular matrix turnover. We have shown previously that in normal skin fibroblasts, TGF-β positively regulates α2(I) procollagen gene (*COL1A2*) promoter activity through the cellular Smad signal transduction pathway. In contrast, IFN-γ activates Stat1α, down-regulates *COL1A2* transcription, and abrogates its stimulation induced by TGF-β. The level of integration of the two pathways mediating antagonistic collagen regulation is unknown. We now report that IFN-γ abrogates TGF-β-stimulated *COL1A2* transcription in fibroblasts by inhibiting Smad activities. IFN-γ appears to induce competition between activated Stat1α and Smad3 for interaction with limiting amounts of cellular p300/CBP. Overexpression of p300 restored *COL1A2* stimulation by TGF-β in the presence of IFN-γ, and potentiated IFN-γ-dependent positive transcriptional responses. In contrast to fibroblasts, in U4A cells lacking Jak1 and consequently unable to activate Stat1α-mediated responses, IFN-γ failed to repress TGF-β-induced transcription. These results indicate that as essential coactivators for both Smad3 and Stat1α, nuclear p300/CBP integrate signals that positively or negatively regulate *COL1A2* transcription. The findings implicate a novel mechanism to account for antagonistic interaction of Smad and Jak-Stat pathways in regulation of target genes. In fibroblasts responding to cytokines with opposing effects on collagen transcription, the relative levels of cellular coactivators, and their interaction with regulated transcription factors, may govern the net effect.

Transforming growth factor-β regulates cellular responses through modulation of transcription of genes encoding cell cycle regulators, extracellular matrix proteins, adhesion molecules, cytokines, and transcription factors (1). One of the most potent effects of TGF-β[1] is connective tissue accumulation, achieved in part by stimulation of the transcription of type I collagen genes (2–5). Thus, TGF-β plays crucial roles in embryonic development and organogenesis, and physiologic connective tissue remodeling during wound healing and tissue repair. On the other hand, excessive TGF-β activity is implicated in the development of pathological fibrosis, the "dark horse of tissue repair" (6).

TGF-β initiates cellular signals through two distinct transmembrane serine-threonine kinase receptors. Upon ligand binding, the activated TGF-β receptor complex transiently interacts with receptor-activated Smads which propagate TGF-β signals (7). Smad2 and Smad3 are direct substrates of the TGF-β receptor kinase, and interact with the common partner Smad4 (8–10). Smad4-containing heteromeric Smad complexes then translocate from the cytoplasm into the nucleus where they function as transcriptional regulators. Smads2–4 share highly conserved DNA-binding MH1 and transactivating MH2 domains; the latter also mediates Smad interactions with other proteins (11, 12). Smad7 contains a characteristic MH2 domain, but lacks the conserved SS*X*S phosphorylation motif, and its MH1 domain shows marked divergence from that of Smad3 (13). Furthermore, in contrast to receptor-activated Smads, Smad7 stably binds to TGF-β receptors and interferes with ligand-induced phosphorylation of Smad2 and Smad3 (14). Because its expression is markedly induced by the ligand, Smad7 appears to serve an autoregulatory negative feedback function in cellular TGF-β signaling.

We have shown previously that overexpression of Smad3 in primary skin fibroblasts mimicked the action of TGF-β, markedly inducing *COL1A2* promoter activity *in vitro* (15). Transactivation was blocked by phosphorylation-defective dominant negative mutants of Smad3, establishing the critical role of endogenous Smads in transducing information from the activated TGF-β receptor in these cells. The *COL1A2* promoter contains Smad3/Smad4-binding consensus "CAGA boxes" (16), also found in the promoters of *PAI-1*, *jun*B, and other TGF-β inducible genes (17, 18), that are necessary and sufficient to mediate transcriptional responses induced by TGF-β (19). However, as *CAGA* boxes are widely distributed in the promoters of mammalian genes, and the affinity and specificity of SMAD binding to these is relatively low, other nuclear factors are likely to contribute to the specific and tight Smad-DNA interactions that are required for transcriptional regulation (20). Cooperation with FAST-1 (21), AP-1 (22, 23), TFE3 (24), Sp1 (25), and vitamin D receptor (26) are implicated in Smad-mediated transcription of TGF-β-responsive genes.

CREB-binding protein (CBP) and adenovirus E1A-associated protein p300 are structurally conserved large proteins

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‡ To whom correspondence should be addressed: Section of Rheumatology (M/C733), University of Illinois Chicago College of Medicine, 1158 Molecular Biology Research Bldg., 900 S. Ashland Ave., Chicago, IL 60607. Tel.: 312-413-9310; Fax: 312-413-9271; E-mail: jvarga@uic.edu.

[1] The abbreviations used are: TGF-β, transforming growth factor-β; CBP, CREB-binding protein; IFN, interferon; GAS, γ-activated sites; bp, base pair(s); CAT, chloramphenicol acetyltransferase; SBE, Smad-binding element.

that function as essential coactivators in several signal transduction pathways. p300/CBP enhance transcription by bridging DNA-bound factors and the basal transcriptional machinery, thereby stabilizing the pre-initiation complex. Furthermore, by nature of their intrinsic histone acetyltransferase enzyme activity, these coactivators acetylate amino-terminal lysine residues of nucleosomal histones (27, 28). Local histone hyperacetylation causes nucleosomal relaxation, thus promoting access of transcription factors to target DNA sequences. The transcriptional activity of genes is strongly correlated with their acetylation state (29). The presence of conserved protein-binding domains enables p300/CBP to interact with distinct classes of regulated transcription factors. Through binding to CREB, the p65 component of NF-κB, the c-Jun, and c-Fos components of AP-1, and Stats, p300/CBP integrate converging cellular signaling pathways (30–35). Receptor-activated Smads interact directly with p300/CBP via the Smad MH2 domain and the p300 COOH-terminal region overlapping the E1A-binding site (36–41). We previously demonstrated that p300 markedly enhanced TGF-β-stimulated *COL1A2* transcription in fibroblasts (42). By competing with activated Smad3 for limiting amounts of cellular p300/CBP, the adenoviral oncoprotein E1A abrogated TGF-β transactivation, indicating the critical role of p300/CBP in collagen gene transcription in fibroblasts.

Interferon-γ (IFN-γ), a pleiotropic cytokine produced by T cells and NK cells, plays fundamental roles in both innate and acquired immune responses (43). Transcriptional responses induced by IFN-γ in most cells are mediated through the Jak-Stat pathway (44). Upon stimulation by IFN-γ, tyrosine-phosphorylated cytoplasmic Stat1α forms homodimeric complexes that can translocate into the nucleus, and bind directly to palindromic γ-activated sites (*GAS*) of IFN-γ-responsive target gene promoters. Stat1α thus serves as an essential mediator of IFN-γ-induced transcriptional responses. Stat1α physically associates with p300/CBP near its amino-terminal domain; this interaction plays an important functional role in positive regulation of IFN-γ-induced transcriptional responses (45, 46). In addition to transcriptional stimulation, IFN-γ can also negatively regulate the transcription of selected genes, but no common IFN-γ-specific inhibitory elements have been identified. We and others have shown previously that IFN-γ inhibits the transcription of collagen in fibroblasts independent of Stat1-promoter interactions, and abrogates its stimulation induced by TGF-β (47–51). Thus, TGF-β and IFN-γ exert opposite effects on collagen synthesis. Because these two cytokines are secreted by inflammatory cells at sites of tissue injury, their antagonistic interactions regulating collagen synthesis are likely to be of great importance in the maintenance of connective tissue homeostasis.

As well as suppressing the stimulation of collagen transcription, IFN-γ abrogates other TGFβ responses, including collagenase-3 expression in epithelial cells (52), perlecan expression in colon carcinoma cells (53), and fibronectin and laminin receptor expression in monocytic cells (54). The basis underlying antagonistic modulation of TGF-β signaling by IFN-γ is incompletely understood. In the present report, we characterized the repression of TGF-β-stimulated collagen transcription by IFN-γ in normal skin fibroblasts. The results indicate that the stimulatory effects of TGF-β on Type I collagen gene (*COL1A2*) transcription were abrogated by IFN-γ through a minimal Smad-binding element of the *COL1A2* promoter. Inhibition of TGF-β signaling in fibroblasts was not mediated through antagonistic Smad7. The stimulatory effect of TGF-β on *COL1A2* transcription could be rescued in the presence of IFN-γ by overexpression of p300. These findings indicate that p300/CBP integrate IFN-γ/TGF-β-induced signals that positively or negatively regulate collagen gene transcription in fibroblasts, and suggest that an increase in activated Stat1α in IFN-γ-treated fibroblasts suppressed Smad-mediated transactivation by titrating away the coactivators. The findings provide novel understanding of the physiologically important antagonistic regulation of collagen gene transcription by cytokines.

MATERIALS AND METHODS

Reagents and Cell Culture—All tissue culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY). Recombinant human IFN-γ was from Genentech Inc. (South San Francisco, CA), and TGF-β from Amgen (Thousand Oaks, CA). Primary cell cultures were established from neonatal foreskin by previously described explant techniques (15), and studied between passages 4 and 8. Cells were grown at 37 °C in a 5% CO_2 atmosphere in modified Eagle's medium supplemented with 1% or 10% fetal calf serum, 1% vitamins, and 2 mM L-glutamine. The U4A *Jak1*-deficient cell line, which does not support IFN-γ-induced gene expression (55), and U4A/*Jak1* were a kind gift of O. Colamonici. Transforming growth factor-β and IFN-γ were added simultaneously, and cultures were harvested following 24–48 h incubation. In previous studies under similar conditions, we found that neither TGF-β nor IFN-γ significantly effected cell number or viability in confluent fibroblast cultures (15). Total RNA was isolated with TRIZOL Reagent (Life Technologies, Inc.) following the indicated periods of incubation, and relative levels of mRNA were examined by Northern analysis using radiolabeled *Smad3, Smad7*, and *glyceraldehyde-3-phosphate dehydrogenase* cDNA probes. The cDNA-mRNA hybrids were visualized by autoradiography on Kodak X-AR5 films exposed for 24–48 h with intensifying screens.

Immunoprecipitation and Western Immunoblotting—At the end of the indicated period of incubation, cells were washed with ice-cold phosphate-buffered saline, and whole cell lysates prepared. Lysates were either directly resolved by electrophoresis in polyacrylamide gels, or first immunoprecipitated for 1 h using anti-p300 antibody (Santa Cruz Biotechnology, Santa Cruz CA). Gels were blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and subjected to immunoblotting with primary antibodies (human anti-p300, anti-Smad7, and anti-actin (C-2) from Santa Cruz; anti-Smad3 from Zymed Laboratories Inc., San Francisco, CA; anti-Type I collagen from Southern Biotech (Birmingham, AL); anti-Stat1 from Transduction Labs (Lexington, KY); and anti-phosphotyrosine Stat1 (Y701) from Upstate Biotechnology (Lake Placid, NY) for 2 h at room temperature. The blots were then washed, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, and visualized by chemiluminescence.

Cellular Immunofluorescence Imaging—The expression and intracellular localization of endogenous SMADs and Stat1α in the presence or absence of IFN-γ or TGF-β was studied by indirect immunofluorescence. For this purpose, fibroblasts were seeded into chamber glass slides and incubated in media with 0.1% fetal calf serum and IFN-γ added 30 min before TGF-β. After 2 h incubation, cells were then fixed with methanol, incubated with primary antibodies (anti-Smad3 and anti-Smad4 from Santa Cruz; anti-Stat1α from Transduction Labs) for 1 h, followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). After three washes, the slides were stained, and the intracellular distribution of Smads and Stat1 was examined by fluorescence or confocal microscopy. Quantitation was performed in a blinded fashion by scoring 100 fibroblasts in different fields as showing predominantly nuclear or cytoplasmic immunofluorescence.

Transient Transfection—To measure transcriptional responses to IFN-γ or TGF-β, *772COL1A2/CAT* consisting of the −772 to +58 bp segment of human *COL1A2* promoter linked to the *CAT* gene (56), *CAGA-COL1A2/luc* containing six copies of the −266/−258 bp sequence of *COL1A2* (17), or *SBE4-luc* containing four tandem repeats of an 8-bp palindromic consensus Smad-binding consensus sequence (16) were used. Expression vectors for wild type and ΔHAT-mutant *p300* (57), *Smad3* (58), and antisense *Smad7* cDNA (59), and appropriate control vectors were transfected. As controls, Δ-*152DR-CAT* (from J. Ting, University of North Carolina, Chapel Hill, NC) containing an IFN-γ-responsive 152-bp segment of the *HLA-DR* promoter ligated to the *CAT* gene (60), and *GAS-tk-CAT* (from G. Sen, Cleveland Clinic Foundation Research Institute) containing an IFN-γ-responsive 24-bp segment of the IRF-1 promoter ligated to the *CAT* gene, were used. Transient transfections were performed by calcium phosphate/DNA co-precipitation or using Superfect reagent (Qiagen, Valencia, CA), as described previously (15). The total amount of DNA in each transfection was kept constant by addition of appropriate empty vectors, as re-

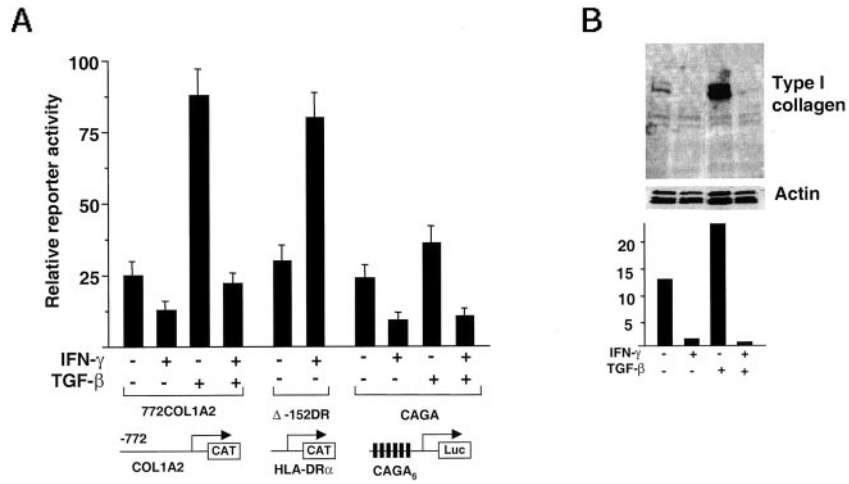

FIG. 1. **Stimulation of collagen transcription is abrogated by IFN-γ.** *A,* fibroblasts were transfected with *772COL1A2/CAT* (*left panel*), the IFN-γ responsive minimal promoter Δ*-DR152-CAT* (*middle panel*), or *CAGA-COL1A2/luc*, which contains tandem copies of the COL1A2 Smad-binding consensus CAGA sequence (*right panel*). Following 48 h incubation with IFN-γ (500 units/ml) and/or TGF-β (500 pM), cultures were harvested and CAT and luc activities were determined. The results for *772COL1A2/CAT* and *CAGA-COL1A2/luc*, shown as relative reporter gene activity, indicate the means of corrected duplicates from three independent experiments. Schematic representation for the constructs used is shown below. *B,* levels of Type I collagen in fibroblasts treated with TGF-β and/or IFN-γ for 48 h was determined by Western blot of whole cell lysates. A representative autoradiogram is shown on *top*. The intensities of the bands quantiated by densitometry and corrected for actin are shown at the *bottom*.

quired. Following incubation with the indicated cytokines, CAT, and luciferase activities were determined in duplicate and normalized with protein concentration.

Preparation of Nuclear Extracts and Electrophoretic Gel Mobility Shift Assay—Fresh media with 0.2% fetal calf serum and IFN-γ alone or with TGF-β were added to confluent fibroblasts. At the end of the indicated incubation periods, nuclear extracts were prepared according to the method of Andrews and Faller (61), and protein concentrations were determined using the Bio-Rad assay. Double-stranded oligonucleotide probes corresponding to a consensus Smad-binding element (*SBE*). The probes were end-labeled using T4 polynucleotide kinase (Promega, Madison WI). Electrophoretic gel mobility shift assays were performed as described previously (15).

Statistical Analysis—Statistical differences between experimental groups were determined by analysis of variance, and values of $p < 0.05$ by Fisher's test were considered significant.

RESULTS

Antagonistic Regulation of Collagen Transcription by TGF-β and IFN-γ, Convergence at the Level of Smad3—To examine the regulation of collagen transcription, normal skin fibroblasts were transiently transfected with *COL1A2* promoter-*CAT* reporter constructs. TGF-β caused marked stimulation of CAT activity driven by 772 bp of the *COL1A2* promoter. This induction was completely abrogated by IFN-γ, indicating that cis-elements necessary for negative regulation of *COL1A2* expression were contained within this region of the promoter (Fig. 1*A, left panel*). The activity of Δ*152DR-CAT* was induced by IFN-γ, demonstrating that the inhibitory effect of IFN-γ was selective for the *COL1A2* promoter (Fig. 1*A, middle panel*). Changes in the intracellular pool of collagen in response to the cytokines paralleled those in promoter activity (Fig. 1*B*). Inhibition of collagen synthesis did not require pretreatment with IFN-γ prior to stimulation. IFN-γ induced the rapid nuclear translocation of Stat1, an essential mediator of IFN-γ transcriptional responses (Fig. 2). To further examine Stat1 activation, the consensus Stat1α-binding *GAS* oligonucleotide was used as probe in gel shifts. As expected, IFN-γ treatment of fibroblasts for 15 min resulted in the formation of a *GAS*-specific DNA-protein complex (Ref. 62; and data not shown). A careful analysis of the proximal *COL1A2* promoter failed to identify the presence of consensus Stat1α-binding GAS elements (TTN_5AA). Furthermore, in contrast to classical Stat1-mediated transcriptional responses, the inhibitory effect of IFN-γ on

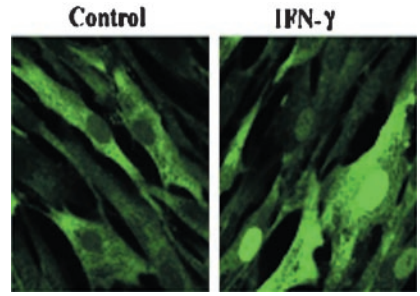

FIG. 2. **IFN-γ induces Stat1 activation in fibroblasts.** Fibroblasts were treated with IFN-γ for 2 h, or left untreated. At the end of the incubation period, cells were processed for immunocytochemistry, and STAT1-specific immunofluorescence was detected by confocal microscopy, as described under "Materials and Methods."

COL1A2 promoter activity was delayed (>8 h) (51). Therefore, these observations suggested that Stat1α activation could not be fully responsible for suppression of *COL1A2* transcription by IFN-γ .

Because we have previously established a fundamental role for Smad3 in TGF-β stimulation of *COL1A2* transcription in fibroblasts (15), the possibility that the Smad signal transduction pathway was a target for the inhibitory activities of IFN-γ was considered. To directly examine the involvement of Smad3, the heterologous minimal construct *CAGA-COL1A2/luc*, which contains six tandem copies of the *COL1A2* Smad-binding *CAGA* element shown to be sufficient to mediate TGF-β responses (19) was used. The TGF-β-induced increase in activity of this promoter was suppressed in IFN-γ-treated fibroblasts (Fig. 1*A, right panel*). Identical results were obtained with *SBE4-luc*, containing a consensus *SBE* (16). IFN-γ had no effect on endogenous Smad3 or Smad4 mRNA or protein expression (data not shown). Together, these findings indicated that IFN-γ suppressed TGF-β stimulation of *COL1A2* transcription mediated by multimerized Smad3-recognition sites, while inducing endogenous Stat1α activation. Inhibition did not result from decreased expression or DNA binding of cellular Smads, suggesting instead that IFN-γ targeted their transcriptional activities.

Smad7-independent Inhibition of COL1A2 Transcription in Fibroblasts—Intracellular cross-talk among cytokines with op-

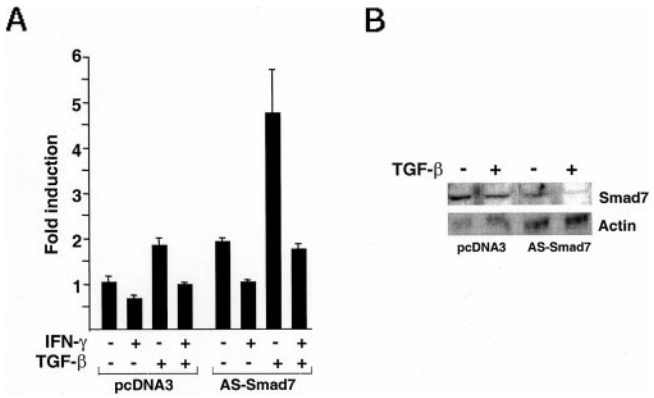

FIG. 3. **Antisense *Smad7* does not prevent inhibition of TGF-β-induced *COL1A2* transcription by IFN-γ.** *A,* fibroblasts were co-transfected with *772COL1A2/CAT* (10 μg) along with antisense *Smad7* expression constructs (5 μg) or empty vector. Six h later, TGF-β (500 pM) was added to the cultures. Following further 24 h incubation in media with 0.1% fetal calf serum without or with IFN-γ and/or TGF-β, cultures were harvested and CAT activities were determined. The results, shown as -fold change in CAT activity, are expressed as the means of duplicates from three independent experiments. *B,* whole cell lysates from fibroblasts transfected with *pcDNA3* or *antisense Smad7* (*AS-Smad7*) were subjected to immunoblotting with antibodies against Smad7 or actin.

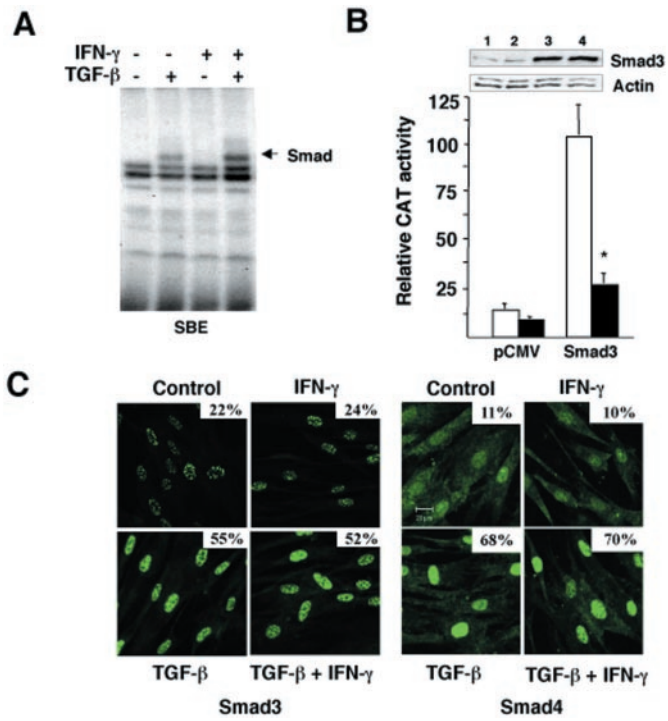

FIG. 4. **Antagonistic regulation of Smad-mediated transcriptional responses by IFN-γ.** *A,* confluent fibroblasts were incubated with TGF-β and/or IFN-γ for 60 min. Nuclear extracts were analyzed in gel mobility shift assays with a radiolabeled oligonucleotide probe containing the *SBE* core sequence. The shifted band identified as Smad is indicated by an *arrow. B,* fibroblasts were co-transfected with a plasmid expressing Smad3 along with *772COL1A2/CAT*. Following 48 h incubation of the cultures in media without (*open bars*) or with IFN-γ (500 units/ml, *closed bars*), CAT activities were determined. The results shown represent the mean of three independent determinations; *, $p < 0.05$. *Inset*, expression of recombinant Smad3 unaffected in fibroblasts treated with IFN-γ. Equal amounts of whole cell lysates from untreated (*lanes 1* and *3*) or IFN-γ-treated (*lanes 2* and *4*) fibroblasts transiently transfected with plasmid expressing *Smad3* (*lanes 3* and *4*) or empty vector (*lanes 1* and *2*) were separated by electrophoresis and probed with antibodies to Smad3 (*upper panel*) or actin (*lower panel*). *C,* fibroblasts were treated with TGF-β and/or IFN-γ for 2 h. At the end of the incubation period, cells were fixed and processed for immunocytochemistry, and Smad3 (*left panel*) or Smad4 (*right panel*)-specific immunofluorescence was detected by confocal microscopy, as described under "Materials and Methods." The percentage of fibroblasts showing predominantly nuclear Smad localization, shown in the *upper right panels*, was determined by counting 100 cells. Representative photomicrographs are shown. Results were essentially identical when incubation with IFN-γ/TGF-β was continued for 4 h.

posing effects can be mediated through induction by one cytokine of autocrine mediators that block signaling triggered by the another cytokine. For instance, IFN-γ abrogates interleukin-4-induced transcriptional responses via endogenous suppressor of cytokine signaling SOCS-1, which prevented STAT6 activation in these cells (63, 64). In a similar vein, TGF-β stimulation of *3TP-lux* transcription is prevented by IFN-γ or TNF-α via induction of antagonistic cellular Smad7, which blocks ligand-induced Smad3 phosphorylation and its attendant events through stable interaction with the TGF-β receptors (65, 66). These observations suggested a possible mechanism to account for the antagonistic effect of IFN-γ on TGF-β-stimulated *COL1A2* transcription: induction of an endogenous inhibitor of TGF-β signaling by IFN-γ. In contrast to SOCS-1, which has not been shown to suppress TGF-β signaling, Smad7 specifically blocked Smad3-mediated responses in fibroblasts (15). Therefore, we sought to examine whether endogenous Smad7 could be implicated in IFN-γ suppression of TGF-β-stimulated *COL1A2* transcription. The results showed that, whereas it consistently induced rapid and transient increase in Smad7 mRNA expression in HepG2 epithelial cells, IFN-γ had no detectable effect on the levels of endogenous Smad7 determined by immunocytochemistry and Western immunoblotting, or on Smad7 mRNA expression determined by Northern analysis (data not shown). Our failure to demonstrate induction of Smad7 expression by IFN-γ in fibroblasts, in contrast to cells of epithelial origin, is consistent with recent report (66).

We next examined the functional consequences of down-regulating endogenous Smad7 using an antisense expression plasmid (59). Fibroblasts were transfected with 10 μg of *772COL1A2/CAT* along with 5 μg of antisense *Smad7* cDNA, or empty vector. As expected, transactivation of *COL1A2* by TGF-β was greater in fibroblasts expressing antisense *Smad7* than in control fibroblasts transfected with empty vector, indicating that the level of endogenous Smad7 determined the magnitude of the TGF-β-induced response (Fig. 3*A*). *Smad7* antisense cDNA markedly decreased the amount of cellular Smad7 (Fig. 3*B*). The inhibitory effect of antisense on cellular Smad7 was unaffected by IFN-γ (data not shown). Down-regulation of endogenous Smad7 did not abrogate the inhibitory effect of IFN-γ on TGF-β-induced *COL1A2* promoter in these fibroblasts (Fig. 3*A*). Similar results were obtained when antisense oligonucleotides were used to down-regulate endogenous Smad7 (data not shown). Taken together, these results indicate that endogenous Smad7 is not responsible for abrogation of TGF-β-stimulated *COL1A2* transcription by IFN-γ in primary fibroblasts.

IFN-γ Abrogates COL1A2 Stimulation Downstream from Smad3 Activation—The antagonistic effect of IFN-γ on Smad-mediated *COL1A2* transactivation could result from interference with TGF-β-induced Smad3 activation. Three complementary approaches were undertaken to examine this possibility. First, we sought to determine whether inhibition of Smad-mediated transactivation by IFN-γ altered the DNA binding activity of nuclear proteins. Electrophoretic mobility shift assays with fibroblast nuclear extracts showed that incubation with IFN-γ slightly enhanced TGF-β-induced binding of endogenous Smad3 to the consensus *SBE* (Fig. 4*A*). To assess the effects of IFN-γ on the transactivation function of Smad3, fibroblasts overexpressing recombinant Smad3 were treated

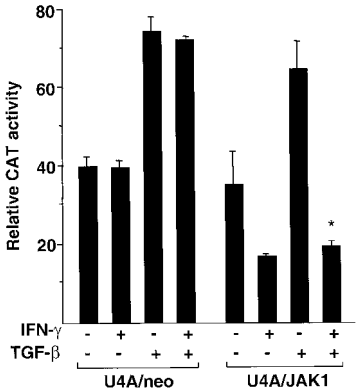

FIG. 5. **IFN-γ repression of TGF-β-induced transcription requires endogenous activation of Stat1α.** *Jak1*-deficient U4A cells and U4A/*Jak1* cells were transfected with *772COL1A2/CAT*. Following 48 h incubation in the presence of TGF-β with or without IFN-γ, cells were harvested and CAT activities were determined. The results shown represent the mean of two determinants; *, $p < 0.05$.

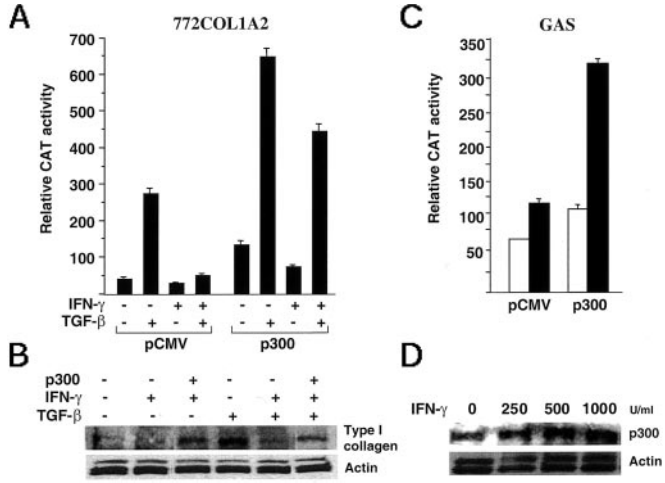

FIG. 6. **Overexpression of p300 relieves the antagonistic effect of IFN-γ on *COL1A2* stimulation.** *A,* fibroblasts were co-transfected with the plasmid expressing *p300*, along with *772COL1A2/CAT*. Following 48 h incubation with or without TGF-β and/or IFN-γ, CAT activities were determined. Results shown represent the mean of several independent experiments. *B,* levels of Type I collagen in fibroblasts transfected with *p300* expression vector. Whole cell lysates were prepared following a 48-h incubation with IFN-γ and/or TGF-β, and following gel electrophoresis, proteins were subjected to Western blotting using antibodies to Type I collagen and actin. *C,* fibroblasts were co-transfected with the plasmid expressing *p300*, along with a minimal construct containing the consensus *GAS* linked to the *CAT* reporter gene. Following 6 h incubation with (*closed bars*) or without (*open bars*) IFN-γ (10 units/ml), CAT activities were determined. *D,* modulation of cellular p300 levels by IFN-γ. Whole cell lysates were prepared following a 48-h incubation of confluent fibroblasts with the indicated concentrations of IFN-γ, and examined by immunoblot using antibodies to p300 and actin.

with IFN-γ. As shown in Fig. 4*B*, the striking elevation in *COL1A2* promoter activity in *Smad3*-transfected fibroblasts was substantially abrogated, indicating that in these cells IFN-γ disrupted signaling downstream of the activated TGF-β receptor. IFN-γ did not alter the level of recombinant Smad3 expression (Fig. 4*B, inset*; and data not shown). Next, the effect of IFN-γ on the subcellular distribution of endogenous Smad3 was examined. Nuclear import of Smad3, essential for regulating target gene transcription, is highly dependent on its ligand-induced phosphorylation (11). Treatment of the fibroblasts with TGF-β caused rapid (<2 h) accumulation of endogenous Smad3 and Smad4 within the nucleus (Fig. 4*C*). IFN-γ by itself had no effect on Smad cellular localization, and pretreatment of the cells failed to prevent TGF-β-induced nuclear translocation at early or late time points. The inability of IFN-γ to disrupt ligand-induced nuclear import of activated Smads further indicates that mechanisms distinct from blocking ligand-induced Smad activation underlie the antagonistic effects of IFN-γ on TGF-β stimulation of *COL1A2* transcription.

IFN-γ treatment of fibroblasts caused rapid Stat1 nuclear accumulation (Fig. 2); and increase in its DNA binding activity (62). To directly determine the role of Stat1α in mediating repression of TGF-β-induced *COL1A2* transcription by IFN-γ, the response of Jak1-deficient U4A cells was examined. These cells are a genetically defined system commonly used to delineate IFN-γ signaling (55). Treatment of U4A cells with IFN-γ had little effect on *COL1A2* promoter activity in the presence of TGF-β (Fig. 5). In contrast, U4A cells rescued with stable expression of exogenous *Jak1* (U4A/*Jak1*) demonstrated normal Stat1α-mediated responses, and IFN-γ caused >70% decrease in TGF-β-stimulated CAT activity. As expected, the activity of the GAS-driven reporter was markedly up-regulated by IFN-γ in the parental, but not in the signaling defective, cells (data not shown). We therefore conclude that Stat1α activity is required for IFN-γ abrogation of TGF-β-induced, Smad3-mediated *COL1A2* transactivation. Because Stat1 activation is rapid and precedes repression of *COL1A2*, it is likely to represent an early step in a series of events culminating in transcriptional repression.

TGF-β and IFN-γ Signals Are Integrated at the Level of p300/CBP Coactivators—Certain responses mediated by IFN-γ/Stat1α and TGF-β/Smad3 pathways are critically dependent on p300/CBP coactivators (9, 36–42, 45). We reasoned, therefore, that the antagonistic effect of IFN-γ on Smad-mediated TGF-β transactivation of *COL1A2* could result from competition between Smad3 and IFN-γ-activated signal transducers such as Stat1α for interaction with a limiting cellular pool of p300/CBP. Consistent with this possibility, we found that overexpression of p300 in fibroblasts rescued TGF-β stimulation of *COL1A2* promoter activity (Fig. 6*A*) and endogenous collagen accumulation (Fig. 6*B*) in the presence of antagonistic IFN-γ. In contrast, a mutant form of p300 defective in histone acetyltransferase activity failed to relieve IFN-γ repression (data not shown); indicating that modulation of *COL1A2* transcription involved the histone acetylase function of p300. p300 enhanced transactivation of a minimal *GAS* promoter by low concentrations (10 units/ml) of IFN-γ, suggesting a significant *in vivo* functional interaction between p300 and activated endogenous Stat1α in fibroblasts (Fig. 6*C*). To examine if the antagonistic effect of IFN-γ on TGF-β-stimulated *COL1A2* transactivation could be due to down-regulation of cellular p300 levels, immunoblotting was performed. The results showed that the pool of endogenous p300 was slightly increased in IFN-γ-treated fibroblasts, indicating that IFN-γ interfered with the function, and not the amount, of p300 in these cells (Fig. 6*D*). Consistent with this notion, we also found that IFN-γ had no detectable effect on the levels or subcellular distribution of endogenous p300 (data not shown). Taken together, these results suggested that p300 was present in limiting amounts in fibroblasts, and the ability of IFN-γ to inhibit Smad3 transactivation resulted from competition for p300 by Stat1α or other transcription factors induced by IFN-γ.

To directly examine the *in vivo* interaction of endogenous Smad3 with p300, fibroblasts were transfected with a *p300* expression plasmid and treated with TGF-β alone, or together with IFN-γ for 30 min, a time point sufficient for induction of p300 interaction with both Stat1 and Smad3 (36, 45). At the end of the incubations, whole cell lysates from fibroblasts transfected with *p300* expression plasmid and treated with

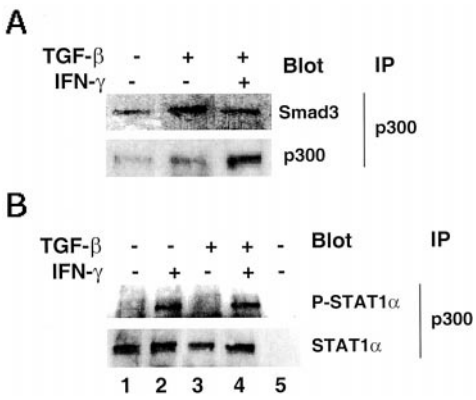

FIG. 7. **Ligand-induced interaction of p300 with activated cellular Smad3 or Stat1*α* *in vivo*.** Confluent fibroblasts were transfected with *p300* expression plasmids. Following incubation in the presence or absence of IFN-γ or TGF-β for 30 min, whole cell lysates were prepared and immunoprecipitated with antibodies to p300. Equal amounts of immunoprecipitated proteins were analyzed by immunoblot with antibodies to: *A,* Smad3 and p300; or *B,* tyrosine-phosphorylated Stat1α or Stat1α. *Last lane*, control IgG.

IFN-γ and/or TGF-β for 30 min were immunoprecipitated with p300 antibody and following electrophoresis, examined by immunoblot with antibodies to Smad3. As shown in Fig. 7*A*, the TGF-β-induced interaction of p300 with endogenous Smad3 appeared to be modestly reduced upon treatment of the fibroblasts with IFN-γ. Next, the interaction of Stat1α with p300 was examined. Immunoprecipitated proteins were examined by immunoblot with antibodies to Stat1α or tyrosine-phosphorylated Stat1α. As shown in Fig. 7*B*, IFN-γ strongly enhanced the interaction of p300 with tyrosine-phosphorylated Stat1α, and this interaction was not altered by TGF-β. These results demonstrate that in primary fibroblasts, p300 interacted with both activated Smad3 and activated Stat1α *in vivo*; in a ligand-dependent manner. Whereas IFN-γ reduced the interaction of p300 with endogenous Smad3, TGF-β was unable to modulate the association of p300 with activated Stat1α.

DISCUSSION

The production of ECM proteins by fibroblasts must be coordinated and strictly regulated. During dynamic processes of tissue remodeling such as wound healing, fibroblasts are subject to simultaneous signaling by distinct combinations of cytokines, growth factors, and other regulatory molecules. These extracellular signals provide fibroblasts with information that synergistically or antagonistically influence the expression of target genes. Type I collagen is the major structural component of the connective tissue of the skin and other organs. Because its excessive accumulation results in fibrosis, the dynamic equilibrium between signals that stimulate and those that inhibit collagen synthesis must be carefully maintained. The transcription of *COL1A2*, one of the best characterized responses in fibroblasts, is stimulated by TGF-β and inhibited by IFN-γ (47–51). Therefore, the intracellular cross-talk between the signaling pathways activated by these two functionally antagonistic cytokines is of substantial interest.

The results presented here provide evidence for the nuclear integration of TGF-β and IFN-γ signaling at the level of the shared cofactors p300/CBP. Stat1α, a critical transducer of IFN-γ responses, is one of a large group of transcription factors that employ p300/CBP to bring about their effects on transcription. Interactions between Stat1α and p300/CBP can be ligand-dependent or constitutive, indicating that both unphosphorylated monomeric, and phosphorylated dimeric Stat1α can interact with p300/CBP. We have previously demonstrated that in primary fibroblasts, endogenous p300/CBP is required

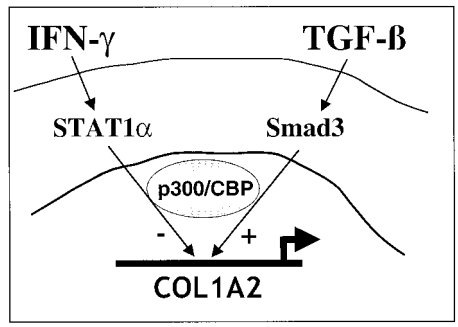

FIG. 8. **Antagonistic regulation of *COL1A2* transcription in primary fibroblasts by TGF-β (+) and IFN-γ (−).** The two cytokines exert opposing effects on gene expression through competition between Smads and STAT1 for limiting amounts of the shared cellular coactivators p300/CBP.

by activated Smad3 to stimulate *COL1A2* transcription (42). TGF-β induces direct binding of Smad3 to p300/CBP (36–42). In fibroblasts exposed to TGF-β and IFN-γ simultaneously, activated Stat1α and Smad3 are therefore likely to compete with each other for interaction with p300/CBP. Because p300/CBP is present in limiting amounts in these cells, the net effect of simultaneous signaling by the two antagonistic cytokines on target gene transcription appears to be determined by the levels of the coactivators, and the relative affinity of their interaction with Smad3 and IFN-γ signal transducers. Although we cannot exclude the possibility that integration of antagonistic signals at the level of p300 involves direct inhibition of p300 function by IFN-γ/Stat1α, rather than its simple sequestration, the functional synergism between p300/CBP and IFN-γ shown in Fig. 6*C* suggests that this is not the case. Further studies to examine the effect of IFN-γ on p300/CBP histone acetyltransferase activity are currently in progress. The model for integration of antagonistic transcriptional signals through their competition for nuclear coactivators is illustrated in Fig. 8. This model proposed here is reminiscent of the regulation of the scavenger receptor gene by IFN-γ and macrophage colony-stimulating factor via p300/CBP (45). The well documented antagonistic effect of nuclear receptors on AP-1-mediated signaling likewise is ascribed to competition for limiting amounts of cellular cofactors (67). Furthermore, while this paper was under review, several reports have demonstrated that antagonistic regulation of Smad-mediated transcriptional responses by TNF-α is also mediated through competition for cellular p300 (68, 69).

In contrast, a distinct model has been proposed to account for the suppression of other TGF-β-induced transcriptional responses by IFN-γ (65) or TNF-α (66). In examining the antagonistic regulation of *3TP-lux* activity by TGF-β and IFN-γ, Ulloa *et al.* (65) concluded that IFN-γ suppression of Smad-mediated responses was mediated by endogenous Smad7 functioning as autocrine inhibitor of TGF-β signaling. Similarly, the opposing activities of TNF-α on TGF-β-induced transactivation of *3TP-lux* or *SBE4-luc* were shown to be mediated by Smad7 (66). In the present studies using primary skin fibroblasts, we were unable using a variety of approaches to detect induction of Smad7 expression by IFN-γ; furthermore, down-regulation of endogenous Smad7 levels by antisense failed to abrogate the inhibitory effects of IFN-γ on TGF-β-stimulated transcription in these cells. The findings suggest that expression and autoregulatory function of ligand-induced Smad7 may show cell-lineage specific differences.

Two additional lines of evidence are provided here to exclude the role of antagonistic Smad7 in mediating suppression of TGF-β responses by IFN-γ. First, we demonstrated that IFN-γ was capable of abrogating *COL1A2* transcriptional stimulation

induced by Smad3, indicating that the antagonistic cross-talk between TGF-β and IFN-γ occurred downstream of the ligand-bound TGF-β receptors. Furthermore, treatment of the fibroblasts with IFN-γ did not prevent TGF-β-induced nuclear accumulation of pathway-restricted Smads, indicating that, in distinct contrast to the endogenous Smad7 autoinhibition model proposed by Ulloa *et al*. (65), negative regulation of Smad signaling by IFN-γ in fibroblasts occurred distal to Smad3 activation or Smad3-Smad4 complex nuclear translocation. Our observations are consistent with a model whereby competition of regulated transcription factors for p300/CBP interaction, which occurs within the nucleus, but not with Smad7-mediated blockade of the activation of pathway-restricted Smads by TGF-β receptors. Finally, endogenous Smad7 induction by TGF-β abrogated the stimulation of *COL1A2* promoter activity (15), indicating that antagonistic Smad7 serves to limit positive regulation of transcription, rather than mediating ligand-induced negative regulation, in primary fibroblasts.

In summary, the present results provide compelling evidence for the role of p300/CBP in antagonistic regulation of *COL1A2* transcription by TGF-β and IFN-γ. The results indicate that in normal skin fibroblasts, the intracellular signaling pathways triggered by TGF-β and IFN-γ are integrated at the nuclear level, and appear to involve a competition between activated Stat1α and Smad3 for interactions with limiting amounts of cellular p300/CBP. Further characterization of the molecular basis by which IFN-γ inhibits SMAD-mediated transcriptional activity in fibroblasts will facilitate the design of specific inhibitors of Smad signaling, which may be therapeutically useful in scleroderma and other fibrotic disease characterized by excessive TGF-β responses. Taken together with recent reports, our observations suggest that antagonistic signals regulating cell functions are integrated by distinct mechanisms that are context- and lineage-specific.

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Antagonistic Regulation of Type I Collagen Gene Expression by Interferon-γ and Transforming Growth Factor-β: INTEGRATION AT THE LEVEL OF p300/CBP TRANSCRIPTIONAL COACTIVATORS

Asish K. Ghosh, Weihua Yuan, Yasuji Mori, Shu-jen Chen and John Varga

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