Interferon-γ Interferes with Transforming Growth Factor-β Signaling through Direct Interaction of YB-1 with Smad3*

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Transferring growth factor-β (TGF-β) and interferon-γ (IFN-γ) exert antagonistic effects on collagen synthesis in human dermal fibroblasts. We have recently shown that Y box-binding protein YB-1 mediates the inhibitory effects of IFN-γ on COL1A2 transcription through the IFN-γ response element located between −161 and −150. Here we report that YB-1 counter-represses TGF-β-stimulated COL1A2 transcription by interfering with Smad3 bound to the upstream sequence around −265 and subsequently by interrupting the Smad3-p300 interaction. Western blot and immunofluorescence analyses using inhibitors for Janus kinases or casein kinase II suggested that the casein kinase II-dependent signaling pathway mediates IFN-γ-induced nuclear translocation of YB-1. Down-regulation of endogenous YB-1 expression by double-stranded YB-1-specific RNA abrogated the transcriptional repression of COL1A2 by IFN-γ in the absence and presence of TGF-β. In transient transfection assays, overexpression of YB-1 in human dermal fibroblasts exhibited antagonistic actions against TGF-β and Smad3. Physical interaction between Smad3 and YB-1 was demonstrated by immunoprecipitation-Western blot analyses, and electrophoretic mobility shift assays using the recombinant Smad3 and YB-1 proteins indicated that YB-1 forms a complex with Smad3 bound to the Smad-binding element. Glutathione S-transferase pull-down assays showed that YB-1 binds to the MH1 domain of Smad3, whereas the central and carboxyl-terminal regions of YB-1 were required for its interaction with Smad3. YB-1 also interferes with the Smad3-p300 interaction by its preferential binding to p300. Altogether, the results provide a novel insight into the mechanism by which IFN-γ/YB-1 counteracts TGF-β/Smad3. They also indicate that IFN-γ/YB-1 inhibits COL1A2 transcription by dual actions: via the IFN-γ response element and through a cross-talk with the TGF-β/Smad signaling pathway.

Regulation of connective tissue metabolism is under rigorous control by growth factors and cytokines, which act in concert to mesenchymal cell recruitment and proliferation, as well as synthesis and degradation of the extracellular matrix components (1, 2). Disruption of this equilibrium, leading to excessive collagen deposition, is the common hallmark of interstitial fibrotic diseases in various organs. Elucidation of the molecular mechanisms governing the synthesis of type I collagen, the most abundant protein of extracellular matrix components, is essential for understanding not only physiological tissue morphogenesis and homeostasis but also pathological conditions associated with excessive collagen accumulation.

Transforming growth factor-β (TGF-β)\(^1\) is well known to be the principal factor that induces type I collagen gene expression and leads to tissue fibrosis (3–5). Recently, a family of proteins, termed Smad, have been identified as intracellular mediators of the signal transduction pathways of TGF-β superfamily members (6–9). The critical roles of TGF-β/Smad signaling to stimulate transcription of type I collagen genes in fibroblasts have been supported by the following results: (a) TGF-β treatment initiates the nuclear translocation of receptor Smads (Smad2 and Smad3) and Smad4 (10, 11) followed by transcriptional up-regulation of COL1A2 (11), (b) COL1A2 promoter contains a Smad-binding element (SBE) that, together with the adjacent Sp1-binding site, mediates transcriptional up-regulation by TGF-β (12), (c) overexpression of Smad3 markedly induces the COL1A2 promoter activity (11, 13, 14), and (d) transfection with a Smad7 expression plasmid blocks the stimulatory effect of TGF-β on COL1A2 transcription (11, 13). Furthermore, interaction between Smads and coactivators p300/CBP enhances COL1A2 transcription in dermal fibroblasts (15).

The interplay of different signaling pathways is the key to provide cells with an integrated response to total signal inputs. Interferon-γ (IFN-γ), a pleiotropic cytokine produced by T lymphocytes and natural killer cells, is generally antagonistic to TGF-β in the regulation of hematopoietic development and immune cell functions such as inflammation (16). The Janus kinase (Jaks)/signal transducer and activator of transcription (STAT) pathway has been shown to represent the key signal transduction through which IFN-γ regulates gene transcription (17). It has recently been suggested that p300/CBP integrates TGF-β-induced Smad and IFN-γ-induced STAT1 signals that positively and negatively regulate collagen gene transcription, respectively (18). Involvement of STAT1 in the IFN-γ-elicited inhibition of collagen deposition was also shown in STAT1 knockout mouse fibroblasts (19). On the other hand,

\(^{1}\) The abbreviations used are: TGF-β, transforming growth factor-β; SBE, Smad-binding element; IFN-γ, interferon-γ; Jak, Janus kinase; STAT, signal transducer and activator of transcription; IgRE, IFN-γ response element; CK2, casein kinase II; FCS, fetal calf serum; siRNA, small interfering RNA; GST, glutathione S-transferase; MHC-II, major histocompatibility complex class II; CIITA, class II transactivator; CBP, CAMP-responsive element-binding protein-binding protein; CSD, cold shock domain.
several studies have revealed the existence of Jak- or STAT-independent IFN-γ signaling pathways (17, 20, 22). Indeed, several reports have shown that IFN-γ inhibits transcription of collagen genes in human fibroblasts and abrogates the stimulatory effect of TGF-β independent of STAT1-promoter interactions (23–25).

We have previously identified a pyrimidine-rich sequence, entirely different from the IFN-γ activated site, within the human COL1A2 promoter that is critical for transcriptional repression by IFN-γ and designated this region the IFN-γ response element (IgRE) (25). More recently, we have demonstrated that Y box-binding protein YB-1 binds to the IgRE and mediates IFN-γ-elicited COL1A2 inhibition (26). Norman et al. (27) have reported that YB-1 also binds to the proximal promoter sequence of the coordinately expressed β1(I) collagen gene and suppresses gene transcription. In the present study, a combination of the RNA interference technique and cell transfection assays, showed that YB-1 plays critical roles in mediating transcriptional repression of COL1A2 by IFN-γ through the casein kinase II (CK2)-dependent signaling pathway. We demonstrated the functional and physical interactions between YB-1 and Smad3. YB-1 also binds to p300 and interferes with the Smad3-p300 interaction. These findings provide a novel insight into the control of connective tissue remodeling by TGF-β and IFN-γ in physiologic and pathologic conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Plasmids, and Reagents—** Normal human dermal fibroblasts (Clontech, Palo Alto, CA) and human embryonic kidney 293 EBNa cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml of penicillin, and 50 µg/ml of streptomycin. Osteosarcoma KHOS-240S cells (American Type Culture Collection) were grown in minimum essential Eagle’s medium supplemented with 10% FCS, Earle’s salt, nonessential amino acids, 100 units/ml of penicillin, and 50 µg/ml of streptomycin. Human COL1A2 promoter sequence spanning from –342 to +58 or four copies of SBE oligonucleotide (GTCTAGAC) linked to a minimal promoter containing only a TATA box was inserted into a firefly luciferase gene vector, pGL3 Basic (Promega, Madison, WI). Smad3 and YB-1 expression vectors were constructed by ligating their coding sequences into the BamHI/XhoI sites of pCDNA3.1 (+) (Invitrogen). A FLAG-tagged

**FIG. 1. Different signaling pathways are required for IFN-γ-induced nuclear translocation of YB-1 and STAT1.** A, nuclear (NE) or cytoplasmic (CE) extracts were prepared from human fibroblasts left untreated or treated with 100 units/ml of IFN-γ for the indicated lengths of time. They were separated on a 12% SDS-PAGE, electrophoresed, and incubated with antibodies against YB-1, phospho-STAT1, STAT1, lamin A/C, or actin. B, human fibroblasts were pretreated with the indicated kinase inhibitors for 1 h and then treated with 100 units/ml of IFN-γ. Nuclear extracts prepared from cells treated for 6 h with IFN-γ were separated and blotted with YB-1 antibodies, whereas those from cells treated for 0.5 h were incubated with phospho-STAT1 antibodies. C, human fibroblasts were untreated (panels 1, 2, 6, and 7) or pretreated with aurantripyrroloxylic acid (panels 3 and 8), AG490 (panels 4 and 9), or apigenin (panels 5 and 10). One hour later, the cells were left untreated (panels 1 and 6) or treated with 100 units/ml of IFN-γ for 6 h (panels 2–5) or 0.5 h (panels 7–10), fixed, and incubated with YB-1 (panels 1–5) or phospho-STAT1 (panels 6–10) antibodies. Intracellular localization of endogenous YB-1 and phospho-STAT1 was examined under fluorescence microscopy. Note that nuclear translocation of YB-1 by IFN-γ was inhibited by pretreatment of cells with aurantripyrroloxylic acid or apigenin, whereas rapid nuclear accumulation of phospho-STAT1 by IFN-γ was blocked by aurantripyrroloxylic acid or AG490.
Fig. 2. Both YB-1 and STAT1 are necessary to mediate transcriptional repression of COLIA2 by IFN-γ. A, KHOS-240S cells were transfected with 2 μg of double-stranded siRNA and incubated for 48 h. After additional transfection with 2 μg of siRNA for 48 h, the cells were treated with 100 units/ml of IFN-γ. Nuclear extracts prepared from cells treated for 6 h with IFN-γ were separated and blotted with YB-1 or STAT1 antibodies, whereas those from cells treated for 0.5 h were incubated with phospho-STAT1 antibodies. B, KHOS-240S cells were transfected with 2 μg of double-stranded siRNA and incubated for 48 h. After additional transfection with 2 μg of siRNA for 48 h, the cells were treated with 5 ng/ml TGF-β and/or 1 units/ml IFN-γ in the presence of 2 μg of siRNA for 40 h. Promoter activities were expressed in relative units compared with untreated cells transfected with control siRNA (mean ± S.D., n = 6). The asterisk signifies that the values are significantly different between the groups. NS, not significant.

YB-1 expression plasmid was obtained by subcloning the entire coding sequence into a pcDNA3-FLAG vector. A FLAG-tagged p300 expression vector (28) and an Sp1 expression plasmid (29) were generously provided by Drs. M. Kawabata and G. Elder, respectively. Bacterial expression plasmids were prepared by ligating respective coding sequence into the BamHI/XhoI sites of pGEX-4T-1 (Amersham Biosciences) or pET28a (+) (Novagen, Madison, WI). Jak inhibitors auranincarbonyl acid and AG490 were purchased from Calbiochem (San Diego, CA) and Sigma, respectively, and a CK2 inhibitor, apigenin, was obtained from Calbiochem.

Preparation and Transfection of Small Interfering RNA (siRNA)—Twenty-one double-stranded siRNAs were synthesized and purified by Dharmacon Research Inc. (Lafayette, CO). The siRNA sense sequences used for targeting YB-1 and STAT1 were GGUCAUCGGCAAC-GAAGGGUuTdTdT and GCUUCUUGGCUUACGCGCtTdTdT, respectively. A nonspecific siRNA, scramble (B-Bridge, San Jose, CA), was used as a control. KHOS-240S cells were transfected with 2 μg of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours later, further transfection with 1 μg of siRNA, and 3 μg of reporter plasmid was carried out. The cells were incubated with 5 ng/ml TGF-β (Peprotech, Rocky Hill, NJ) and/or 1 units/ml IFN-γ (Roche Applied Science) in the presence of 2 μg of siRNA for another 36 h. Transfection efficiency of siRNAs was estimated by a Silencer siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer’s protocol.

Transient Transfection and Dual Luciferase Assay—Human fibroblasts were transfected using Lipofectin reagent (Invitrogen) according to the manufacturer’s protocol. Total amount of DNA in each transfection was kept constant by the addition of appropriate empty vectors. Following incubation with or without 5 ng/ml TGF-β in media with 0.1% FCS, the cells were rinsed twice with phosphate-buffered saline, and dual luciferase assays were performed according to the manufacturer’s protocol (Promega). Transcriptional activities of reporter constructs were normalized against those of cotransfected pRLCMV (Promega).

Real Time Reverse Transcriptase-PCR Assay—The amounts of targeting mRNA was quantified as previously described (26). Briefly, 50 ng of total RNA was reverse transcribed using ImProm-II reverse transcriptase (Promega). The relative mRNA expression levels of COLIA2 gene were normalized against those of glyceraldehyde-3-phosphate dehydrogenase gene in the same RNA preparation.

Electrophoretic Mobility Shift Assay—Four copies of SBE oligonucleotide were end-labeled using T4 polynucleotide kinase and used as probes to examine the binding of recombinant YB-1 to the Smad3-DNA complex (30). DNA-protein complexes were separated from unbound oligonucleotide probe on 4% polyacrylamide gels in 0.5 TBE as previously described (25). In some instances, a His6-tagged Smad3 (1–210) was preincubated with anti-pentahistidine antibodies (Qiagen) for 1 h at 4°C.

Bacterially Expressed Recombinant Proteins—The His6-tagged proteins were produced using the pET system (Novagen) as previously described (26). The full-length YB-1 or Smad3 as well as their deletion mutants fused to glutathione S-transferase (GST) were expressed in Escherichia coli and purified according to the manufacturer’s protocol. Briefly, bacteria were grown in LB medium and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 20°C. The GST fusion proteins were isolated using glutathione-Sepharose 4B (Amersham Biosciences), washed three times with phosphate-buffered saline, and eluted by 10 mM glutathione. Then the buffer was changed to phosphate-buffered saline supplemented with 0.5 mM phenylmethylsulfonyl fluoride using Centricron Plus-20 (Millipore, Bedford, MA). The protein concentration was determined using a commercial assay kit.

GST Pull-down Assay—Lysates of 293 EBNA cells transfected with YB-1 or Smad3 expression plasmid were prepared in 600 μl of 1× lysis buffer (10 mM Tris, 30 mM Na4P2O7, 50 mM NaCl, 50 mM NaF, 1% Triton X-100 at pH 7.1, 1 mM dithiothreitol, and inhibitors (20 μg/ml aprotonin, 20 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na3VO4). GST fusion proteins bound to glutathione-Sepharose beads, 30 μl of cell lysates were incubated for 2 h at 4°C, and the bound proteins were detected by Western blotting using either anti-Smad3 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-YB-1 polyclonal antibodies prepared as previously described (31).

Western Blot Analysis—Nuclear or cytoplasmic proteins and total cell lysates were prepared from KHOS-240S cells or human fibroblasts. Immunoblotting was performed as previous described (26) using anti-YB-1, anti-STAT1 (Transduction Labs, Lexington, KY), anti-phospho-STAT1 (Upstate Biotechnology, Inc., Lake Placid, NY) or anti-type I collagen.
Polyclonal antibodies. 7.5% SDS-PAGE, electroblotted, and incubated with type I collagen antibodies (Polysciences, Warrington, PA). Filters were depolymerized with nitrocellulose membranes for 2 h. Human dermal fibroblasts were transfected with 150 ng of either pcDNA3.1(+)- or YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS and incubated for another 40 h in the presence of TGF-β. Total RNA was extracted, and quantitative analysis of COL1A2 mRNA expression was performed by real time reverse transcriptase-PCR (Tagman). Relative expression levels of COL1A2 were normalized against those of glyceraldehyde-3-phosphate dehydrogenase measured in the same total RNA preparation (mean ± S.D., n = 6). B, human dermal fibroblasts were transfected with 150 ng of either pcDNA3.1(+) or YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS and incubated for another 64 h in the presence of TGF-β (5 ng/ml). The total cell lysates were separated on a 12% SDS-PAGE, electroblotted, and incubated with type I collagen polyclonal antibodies.

collagen antibodies (Polysciences, Warrington, PA). Filters were depolymerized with 2 M glycine-HCl (pH 2.8) for 1 h. Equal loading of protein was confirmed by anti-lamin A/C antibodies (Santa Cruz) for nuclear proteins or anti-actin antibodies (Santa Cruz) for cytoplasmic proteins or total cell lysates. An ECL detection system (Amersham Biosciences) was used to detect immunoreactive proteins.

Immunoprecipitation and Western Blot Analysis—293 EBNA cells transfected with the indicated expression vectors were lysed in 600 μl of 0.5× lysis buffer as previously described (32). Immunoprecipitations were performed using mouse monoclonal anti-FLAG-agarose (Sigma) or anti-p300 monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA) and protein G-Sepharose beads (Amersham Biosciences). The proteins bound to beads were washed with lysis buffer and resolved by 12% SDS-PAGE. The gels were blotted onto nitrocellulose membranes and subjected to immunoblotting with anti-Smad3 or anti-YB-1 polyclonal antibodies.

Immunofluorescence—Human fibroblasts were fixed with cold methanol, incubated with primary antibodies for 1 h, followed by fluorescein isothiocyanate-labeled secondary antibodies (Santa Cruz). Intracellular localization of endogenous YB-1 or phospho-STAT1 was confirmed by anti-lamin A/C antibodies (Santa Cruz) for nuclear proteins or anti-actin antibodies (Santa Cruz) for cytoplasmic proteins or total cell lysates. An ECL detection system (Amersham Biosciences) was used to detect immunoreactive proteins.

Immunofluorescence—Human fibroblasts were transfected with 150 ng of either pcDNA3.1(+) or YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS and incubated for another 64 h in the presence of TGF-β (5 ng/ml). The total cell lysates were separated on a 12% SDS-PAGE, electroblotted, and incubated with type I collagen polyclonal antibodies.

RESULTS

Different Signaling Pathways Mediate IFN-γ-induced Nuclear Translocation of YB-1 and STAT1—It has been previously reported that IFN-γ-induced phosphorylated STAT1 antagonizes TGF-β/Smad signaling by interfering with the interaction between Smad3 and p300/CBP transcriptional co-activators. Western blot analyses of nuclear and cytoplasmic proteins prepared from IFN-γ-treated human fibroblasts indicated that IFN-γ initiates nuclear translocation of both YB-1 and STAT1 but with different kinetics (Fig. 1A). As previously described, phosphorylated STAT1 was observed in the nucleus as early as 0.5 h after IFN-γ treatment (33), whereas IFN-γ-induced nuclear translocation of YB-1 was observed 6 h later (26). The amounts of YB-1 and STAT1 present in cytoplasmic extracts were reciprocally decreased (Fig. 1A). It has been recently reported that IFN-γ stimulates CRK activity and subsequently increases the expression of inducible cyclic AMP early repressor gene (34). In addition, YB-1 and other Y box proteins readily undergo phosphorylation by CRK in a variety of cells (35). We therefore investigated whether IFN-γ stimulates nuclear translocation of YB-1 through the CRK pathway. For this purpose, human fibroblasts were preincubated for 1 h with aurintricarboxylic acid (36), AG490 (34), or apigenin (34), which inhibits Jak1/Jak2, Jak2, and CK2, respectively. The amount of nuclear phosphorylated STAT1 induced by IFN-γ was markedly decreased by Jak inhibitors aurintricarboxylic acid and AG490 but not by CK2 inhibitor apigenin (Fig. 1B). In contrast, incubation of human fibroblasts with aurintricarboxylic acid or apigenin decreased the amount of nuclear YB-1 induced by IFN-γ, whereas it was not affected by AG490 (Fig. 1B). Differential signaling pathways required for nuclear translocation of STAT1 and YB-1 were further confirmed by immunofluorescence experiments localizing subcellular distribution of endogenous STAT1 and YB-1. Consistent with the results of Western blot analyses, IFN-γ-induced nuclear accumulation of endogenous YB-1 was prevented by aurintricarboxylic acid (Fig. 1C, panel 3) or apigenin (Fig. 1C, panel 5) but not by AG490 (Fig. 1C, panel 4). Although cell cycle-dependent nuclear localization of YB-1 has been shown (37), IFN-γ-induced nuclear translocation of YB-1 was observed independently from the cell culture conditions (data not shown). In contrast, the rapid nuclear translocation of STAT1 following IFN-γ treatment was completely abolished by aurintricarboxylic acid (Fig. 1C, panel 8) or AG490 (Fig. 1C, panel 9) but not by apigenin (Fig. 1C, panel 10). Treatment of cells with kinase inhibitors alone did not alter the subcellular distribution of endogenous STAT1 or YB-1 (data not shown). Taken together, these results suggested that IFN-γ initiates nuclear translocation of both STAT1 and YB-1 but through the different signaling pathways.

Both YB-1 and STAT1 Are Necessary for COL1A2 Repression by IFN-γ—To examine the functional roles of YB-1 and STAT1, as well as their independence from each other, in mediating the inhibitory effects of IFN-γ on COL1A2 transcription, we employed the RNA interference technique using the YB-1- or STAT1-specific double-stranded RNAs. Those siRNA duplexes were directed against YB-1 (nucleotides 285–303) and STAT1α (nucleotides 1926–1944, STAT1β has same sequence), respectively, and transfected into KHOS-240S cells. Transfection efficiency of siRNA was almost 100%, and down-regulation of YB-1 or STAT1 had no effect on the cell number and morphology under the experimental conditions used (data not shown). As shown in Fig. 2A, transfection with YB-1 siRNA, STAT1 siRNA, or both significantly decreased the expression of respective protein. In contrast, those siRNAs had no effect on the expression of each other (Fig. 2A). Treatment of control or YB-1 siRNA-transfected KHOS-240S cells with IFN-γ induced rapid phosphorylation of STAT1 in the nucleus, whereas phosphorylated STAT1 was hardly observed in the nucleus after IFN-γ treatment of STAT1 siRNA-transfected cells (Fig. 2B). On the other hand, treatment of control or STAT1 siRNA-transfected cells with IFN-γ for 6 h increased the amount of nuclear YB-1.
Cross-talk between IFN-γYB-1 and TGF-β/Smad Signaling

Fig. 4. YB-1 inhibits TGF-β- and Smad3-stimulated COL1A2 transcription. A and D, human dermal fibroblasts were cotransfected with 4 μg of either –342COL-Luc (A) or SBE4-Luc (D) reporter construct, 0.5 μg of pRLCMV, and 50 ng of YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS in the presence of TGF-β (5 ng/ml). Dual luciferase activity was determined 40 h later. B and E, human dermal fibroblasts were cotransfected with 4 μg of either –342COL-Luc (B) or SBE4-Luc (E), 0.5 μg of pRLCMV, 50 ng of Smad3 expression vector, and various amounts (10–50 ng) of YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS, and dual luciferase activity was determined 40 h later. C, human dermal fibroblasts were cotransfected with 4 μg of –342COL-Luc, 0.5 μg of pRLCMV, 50 ng of Sp1 expression vector, and various amounts (10–50 ng) of YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 0.1% FCS, and dual luciferase activity was determined 40 h later. In all cases, pcDNA3.1(+)+ control vector was used to ensure an equal amount of DNA in each sample. Promoter activities were expressed in relative units compared with untreated cells transfected with control expression vector (mean ± S.D., n = 6).

about 3-fold (Fig. 2B). Under this experimental condition, we examined whether YB-1 and/or STAT1 mediate the inhibitory effects of INF-γ on COL1A2 transcription in the absence or presence of TGF-β. The basal and TGF-β-stimulated promoter activity remained unchanged by knockdown of either YB-1 or STAT1 expression (Fig. 2C). Consistent with the results of previous studies (18, 23), IFN-γ significantly inhibits the basal promoter activity and counter-repressed TGF-β-stimulated COL1A2 transcription. Down-regulation of YB-1 or STAT1 significantly suppressed these IFN-γ actions in the absence and presence of TGF-β (Fig. 2C). In addition, a complete diminution of IFN-γ-elicited repression of COL1A2 transcription was observed by knockdown of both YB-1 and STAT1 (Fig. 2C). Taken together, these results indicated that IFN-γ induces nuclear translocation of YB-1 and STAT1, both of which are necessary for the full IFN-γ activity to inhibit basal and TGF-β-stimulated COL1A2 transcription.

YB-1 Antagonizes TGF-β- and Smad3-stimulated Collagen Expression—We next examined the effects of YB-1 overexpression on type I collagen gene expression and protein production using real-time reverse transcriptase-PCR assays and Western blot analyses, respectively. As shown in Fig. 3A, overexpression of YB-1 not only decreased steady-state levels of endogenous COL1A2 mRNA but also reduced the elevated COL1A2 mRNA levels by TGF-β. This diminution in gene expression was reflected in reduction of collagen protein production as determined by Western blot analyses (Fig. 3B). However, overexpression of YB-1 did not affect the expression of STAT1 (data not shown). In addition, overexpression of these results suggested that YB-1 inhibits both basal and TGF-β-induced collagen synthesis, which is likely to be mediated by inhibition of collagen gene transcription.

To test the possibility that IFN-γ-YB-1 functionally counteracts TGF-β/Smad signaling, human dermal fibroblasts were transiently transfected with the –342COL-Luc construct together with a YB-1 expression vector and incubated with or without TGF-β. Consistent with our previous data (26), COL1A2 transcription was significantly decreased by overexpression of YB-1 (Fig. 4A). Furthermore, YB-1 also inhibited COL1A2 transcription stimulated by TGF-β (Fig. 4A). Similarly, antagonistic effects of YB-1 expression on Smad3-stimulated COL1A2 transcription were observed in a dose-dependent manner (Fig. 4B). In contrast, YB-1 had no effects on the activation of COL1A2 transcription by Sp1 (Fig. 4C). Then the same experiments were conducted using the minimal promoter containing only the SBE. Cotransfection of human dermal fi-
His6-Smad3 without the MH2 domain in the presence of GST-beled SBE oligonucleotide probe and the truncated version of YB-1 and His6-Smad3. FLAG-tagged YB-1 and His6-Smad3 were overexpressed in human embryonic kidney 293 EBNA cells. Immunoprecipitations were performed with anti-FLAG monoclonal antibody, and coimmunoprecipitated Smad3 protein was detected by Western blotting with anti-Smad3 polyclonal antibodies. As shown in Fig. 5A, Smad3 was detected in association with YB-1.

**Identification of the Interacting Regions between YB-1 and Smad3**—To define the interacting regions between YB-1 and Smad3 in more detail, each protein was partly deleted to produce overlapping mutants. They were linked to GST and expressed as fusion proteins in bacteria. YB-1 protein has three independent domains: an amino-terminal region (N), a DNA-binding region called a cold shock domain (CSD), and a carboxyl-tail region (C) (Fig. 6A). The CSD is a highly conserved nucleic acid recognition domain, whereas the carboxyl-tail region is thought to interact with other cellular proteins (38). The GST-fused YB-1 derivatives (Fig. 6B) were bound to glutathione-Sepharose beads and incubated with 293 EBNA cell lysates in which the full-length Smad3 was expressed. The full-length YB-1-GST fusion protein showed an interaction with Smad3, whereas GST alone did not (Fig. 6C). Although the CSD+C region of YB-1 showed a weak interaction with Smad3, deletion of some carboxyl terminus region (CSD+ΔC) led to no binding to Smad3. Similarly, the C region alone or the N+CSD region of YB-1 failed to bind to Smad3.

Smad3 has highly conserved amino (MH1) and carboxyl (MH2) domains that are separated by a less conserved proline-rich linker region (L) (Fig. 7A). The GST-fused Smad3 derivatives (Fig. 7B) were bound to glutathione-Sepharose beads and incubated with cell lysates in which the full-length YB-1 was expressed. The full-length Smad3-GST fusion protein interacted with YB-1, whereas GST alone did not (Fig. 7C). Although both MH2 and MH2+L regions failed to bind to YB-1, MH1 region of Smad3 showed a stronger interaction with YB-1 than did the full-length protein. Taken together, our data indicate that an association of these two proteins is mediated through the CSD+C region of YB-1 and the MH1 domain of Smad3.

**YB-1 Interferes with the Binding between Smad3 and p300**—It has been shown that Smad3 interacts with transcriptional coactivators p300/CBP and mediates the stimulation of COLIA2 transcription by TGF-β (15). The ability of YB-1 to repress TGF-β/Smad3-stimulated COLIA2 transcription (Fig. 4) raised the possibility that YB-1 may disrupt the interaction between Smad3 and p300. We therefore examined the physical interaction of YB-1 with the Smad3-p300 complex. For this purpose, 293 EBNA cells were transfected with expression plasmids encoding each protein, and protein-protein interactions were analyzed by immunoprecipitation-Western blot analysis. In agreement with the results of a previous study (39), TGF-β treatment significantly accelerated the binding of Smad3 to p300 (Fig. 8). Interestingly, YB-1 also interacted with p300 irrespective of TGF-β stimulation. Furthermore, YB-1 potentially inhibited the interaction of Smad3 with p300 by preferential association with p300 (Fig. 8). Altogether, YB-1 interferes with the interaction between Smad3 and p300 by its ability to bind to both Smad3 (Fig. 5B) and p300 (Fig. 8).
YB-1 interferes with the synergistic transactivation of IFN-β, as shown in previous studies demonstrating that YB-1 binds to the IFN-β promoter to mediate the inhibitory effect of IFN-β on transcription stimulated by Smad3 and p300. As shown in Fig. 9A, YB-1 exerted an antagonistic effect on COL1A2 transcription stimulated by Smad3 and p300 in a dose-dependent manner. Similarly, coexpression of Smad3 and p300 synergistically enhanced transcription of the SBE4-luciferase gene, and YB-1 abrogated this synergistic activation in a dose-dependent manner (Fig. 9B). Collectively, these data suggest that YB-1 competes with Smad3 for available p300 in the nucleus.

**DISCUSSION**

The present study has shown, for the first time, that YB-1 antagonizes TGF-β and Smad3-elicited COL1A2 stimulation. YB-1 interferes with the synergistic transactivation of COL1A2 by Smad3 and p300 through its direct binding to Smad3 and p300 followed by intervention of the Smad3-p300 interactions. This mechanism of cross-talk, together with the results of our previous study demonstrating that YB-1 binds to the IFN-γ response element of COL1A2 promoter to mediate the inhibitory effect of IFN-γ, provides a novel insight into the physiological control of connective tissue remodeling by TGF-β and IFN-γ during wound healing and tissue repair processes.

So far, a number of studies have shown that the transcription of COL1A2, one of the best characterized responses in fibroblasts, is stimulated by TGF-β and inhibited by IFN-γ (13, 14, 23–25). Although an antagonistic action of inhibitory Smad7 against TGF-β/Smad signaling was initially proposed using fibrosarcoma-derived U4A cell lines (16), recent studies have excluded the role of Smad7 in mediating suppression of TGF-β responses by IFN-γ in human dermal fibroblasts (18). It has been reported that STAT1 and Smad3 interact with the amino- and carboxyl-terminal regions of p300, respectively, and that these interactions contribute to transcriptional activation of target genes (32, 39, 40). Based on these findings, Ghosh et al. (18) have suggested that the ability of IFN-γ to inhibit Smad3 transactivation resulted from a competition for p300 by STAT1α or other transcriptional factors induced by IFN-γ. Although they did not show direct evidence that overexpression of STAT1α resulted in repression of COL1A2 transcription, involvement of STAT1 in the inhibition of collagen deposition by IFN-γ was shown by others using STAT1 knockout mouse fibroblasts (19). On the other hand, several studies have revealed the existence of Jak- or STAT-independent IFN-γ signaling pathways (17, 20, 22). In addition, recent studies reported a novel mechanism for IFN-γ/CK2-mediated transcriptional regulation (34, 41). In the present study, using several protein kinase inhibitors, we have demonstrated an alternative IFN-γ signal transduction leading to transcriptional repression of COL1A2, namely the Jak1/CK2/YB-1 pathway. Experiments using the double-stranded specific RNAs indicated that both YB-1 and STAT1 are necessary for transcriptional repression of COL1A2 by IFN-γ. In addition, knockdown of YB-1 or STAT1 expression by transfecting with the respective siRNA did not affect the expression or nuclear translocation of each other. Thus, it is possible that both YB-1 and STAT1 contribute independently to transcriptional repression through the competition with the common Smad3 for the cellular p300.

It should be noted that IFN-γ initiated nuclear translocation of both YB-1 and STAT1 but with different kinetics (Fig. 1A).

**FIG. 6. Smad3 interacts with DNA-binding domain of YB-1.** A. YB-1 deletion mutants used in the following experiments are schematically shown. B, different subregions of YB-1 were expressed as GST fusion proteins. Approximately equal amounts of proteins were immobilized on glutathione-Sepharose beads prior to incubation with cell lysates from 293 EBNA transfected with an Smad3 expression vector. The gel stained with Coomassie Brilliant Blue shows the presence of GST and GST-YB-1 proteins marked with asterisks with Coomassie Brilliant Blue shows the presence of GST and GST-YB-1 proteins marked with asterisks with Coomassie Brilliant Blue shows the presence of GST and GST-YB-1 proteins marked with asterisks with Coomassie Brilliant Blue shows the presence of GST and GST-YB-1 proteins marked with asterisks. C. Western blot analysis of Smad3 was performed as described under "Experimental Procedures." Input represents one-fifth of the lysates used for GST-pull-down assays.
The Jak/STAT pathway represents an extremely rapid membrane-to-nucleus signal transduction (36). Ligand-activated IFN-γ/H9253 receptor recruits the Jak1 and Jak2, which selectively stimulate the phosphorylation of STAT1. STAT1 dimers then rapidly migrate to the nucleus and regulate gene expression through the IFN-γ/H9253-activated site. On the other hand, although YB-1 was originally identified as a transcription factor bound specifically to the Y box sequence containing a CCAAT motif within the promoter of major histocompatibility complex class II (MHC-II), it has also been shown to bind to mRNA and proteins (38). Several lines of evidence indicate that YB-1 is a major general translation regulator through mRNA structure arrangement and packaging (35). Phosphorylated Y box proteins make a complex with mRNA, whereas dephosphorylation destabilizes the binding of Y box proteins to RNA and initiates translation (42). In addition, a previous study has implied the existence of an anchoring protein responsible for the cytoplasmic retention of YB-1 (43). A recent study also revealed that interaction with a splicing factor SRp30c confers nuclear YB-1 shuttling (44). These complicated associations of YB-1 with mRNA and proteins present in the cytoplasm are probably responsible for the relatively late nuclear translocation of YB-1 following IFN-γ treatment.

Multiple mechanisms of transcriptional repression have been proposed (45). The simplest mechanism of repression is activator displacement (46, 47). In addition, a zinc finger transcriptional factor YY1 exhibits transcriptional repressor activity through its acetylation by p300 (48). E1A, an adenoviral oncoprotein, is associated with both Smad3 and p300 followed by the impedance of their interaction and also directly inhibits the histone acetyltransferase activity of p300 (28). While suppressing collagen expression, IFN-γ/H9253 stimulates MHC-II gene expression. Three factors are known to regulate MHC-II gene expression in immune cells: class II transactivator (CIITA), X box-binding proteins, and a Y box-binding protein (49). CIITA is a non-DNA-binding transactivator that functions as a molecular switch to control gene expression by interacting with

**FIG. 7.** YB-1 interacts with the MH1 domain of Smad3. A, Smad3 deletion mutants used in the following experiments are schematically shown. B, different subregions of Smad3 were expressed as GST fusion proteins. Approximately equal amounts of proteins were immobilized on glutathione-Sepharose beads prior to incubation with cell lysates from 293 EBNA transfected with a YB-1 expression vector. The gel stained with Coomassie Brilliant Blue shows the presence of GST and GST-Smad3 proteins marked with asterisks on the left. C, Western blot analysis of YB-1 was performed as described under “Experimental Procedures.” Input represents one-fifth of the lysates used for GST-pull-down assays.

**FIG. 8.** YB-1 interferes with the interaction between Smad3 and p300. Interactions between YB-1 and p300 or between Smad3 and p300 in vivo were examined by immunoprecipitation (IP) followed by immunoblotting (Blot) in 293 EBNA cells. Immunoprecipitations were performed with anti-p300 monoclonal antibody, and coimmunoprecipitated YB-1 and Smad3 proteins were detected by Western blotting with anti-YB-1 and anti-Smad3 polyclonal antibodies, respectively.

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DNA-binding proteins specific for the gene promoter or with coactivators p300/CBP (49–51). A previous study using a cell line that is selectively defective in CIITA expression strongly suggested that transcriptional repression of COLIA2 by IFN-γ is mediated through the Jak/STAT pathway-dependent CIITA expression (50). In addition, a recent study demonstrated that a regulatory factor for X box 5 recruits CIITA at the COLIA2 transcription start site and represses transcription (51). These results therefore suggest that the common transcriptional factors or coactivators are involved in transcriptional regulation of collagen and MHC genes. Although direct evidence for the interaction between CIITA and YB-1 has not been shown, thyrotropin receptor suppressor element-binding protein-1, which is a homolog of YB-1, regulates CIITA action (49). The cooperative effects of YB-1 and CIITA on transcriptional repression of COLIA2 are currently under investigation.

Our recent study showed that a Y box consensus sequence mediates transcriptional repression of COLIA2 by YB-1 in human dermal fibroblasts (26). Combined with the results of the present study, we propose here a hypothetical model that transcriptional repression of COLIA2 by IFN-γ is exerted through the two distinctive pathways illustrated in Fig. 10.

Once translocated into the nucleus by IFN-γ, YB-1 interacts with IgRE within the proximal promoter region of COLIA2. Meanwhile, YB-1 is associated with Smad3 bound to the upstream TGF-β-responsive element (52) and with p300 coactivator, which directly interferes with the TGF-β/Smad3 signaling. Because the Smad3/YB-1/p300 heterotrimer complex was not observed (Fig. 8), YB-1 probably interacts with the same region of p300 that binds to Smad3. Precise mapping of the interacting regions between YB-1 and p300 are currently under investigation. In the absence of TGF-β stimulation, inactive Smad3 specifically binds to a protein named SARA (Smad anchor for receptor activation) in the cytoplasm. Immunoprecipitation and peptide microsequence analyses showed that YB-1 is indirectly or directly associated with RNA helicase and poly(A)-binding proteins, which are components of messenger ribonucleoprotein particles, in the cytoplasmic fraction of normal human fibroblasts.2 However, we could hardly identify Smads as YB-1 interacting proteins under the same experimental condition. In addition, it has been shown that pretreatment of fibroblasts with IFN-γ fails to prevent TGF-β-induced nuclear translocation of Smad3 (18). We therefore speculate that each anchoring protein might block the direct association between Smad3 and YB-1 in the cytoplasm. Once translocated into the nucleus, both transcription factors might encounter each other and antagonistically control gene transcription.

In summary, the present study provides an alternative explanation for antagonistic regulation of COLIA2 transcription by TGF-β and IFN-γ. Our results establish that YB-1 mediates IFN-γ signal through the IgRE and via interactions with Smad3 and p300. These results lead us to better understanding of molecular mechanisms responsible for connective tissue remodeling in physiologic and pathologic conditions.

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Interferon-γ Interferes with Transforming Growth Factor-β Signaling through Direct Interaction of YB-1 with Smad3

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