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Tumor Necrosis Factor-α Inhibits TGF-β/Smad Signaling in Human Dermal Fibroblasts via AP-1 Activation

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Abbreviations: FCS, fetal calf serum ; SBE, Smad binding element; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α

Running title : TNF-α Inhibition of Smad Signaling is mediated by AP-1

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ABSTRACT

Understanding the molecular mechanisms underlying the antagonistic activities of tumor necrosis factor-α (TNF-α) against transforming growth factor-β (TGF-β) is of upmost importance, given the physiopathological implications of these cytokines. In this report, we demonstrate that TNF-α prevents TGF-β-induced Smad-specific gene transactivation, without inducing detectable levels of inhibitory Smad7 in human dermal fibroblasts. On the other hand, c-Jun and Jun-B, both induced by TNF-α, block Smad3-mediated transcription. Expression of antisense c-Jun mRNA prevents TNF-α inhibition of TGF-β/Smad signaling whereas that of dominant-negative IKK-α or antisense Smad7 does not. We provide evidence for off-DNA interactions between Smad3 and both c-Jun and Jun-B, accompanied with reduced Smad3/DNA interactions. Finally, we show that overexpression of the transcriptional co-activator p300 prevents TNF-α/AP-1 inhibition of TGF-β/Smad signaling. These data suggest that TNF-α interferes with Smad signaling through the induction of AP-1 components, the latter forming off-DNA complexes with Smad3 and preventing its binding to specific *cis*-element(s). In addition, Jun members compete with Smad3 for the common transcription coactivator p300. These two mechanisms are likely to act in concert to decrease Smad-specific transcription.

INTRODUCTION

The TGF-β family of growth factors comprise activins, bone morphogenic proteins, and the TGF-βs, multipotent cytokines that control various aspects of cell growth and differentiation, and play an essential role in embryonic development, tissue repair or immune homeostasis (1, 2). The TGF-βs signal via serine/threonine kinase transmembrane receptors which phosphorylate cytoplasmic mediators of the Smad family (3, 4). The Smad protein family can be divided into three groups. The receptor-associated Smads, such as Smad1, Smad2, Smad3 and Smad5, interact directly with, and are phosphorylated by, activated TGF-β receptor type I (TBRI or BMPRI), and are ligand specific (5). Upon phosphorylation, they form heteromeric complexes with Smad4, which is a common mediator for all Smad pathways (5). These heterocomplexes are then translocated into the nucleus where they function as transcription factors, binding DNA either directly or in association with other proteins (6). Finally, a third group of Smad proteins, the inhibitory Smads, such as Smad6 and Smad7, prevents phosphorylation and/or nuclear translocation of receptor-associated Smads (3).

Tumor necrosis factor-α (TNF-α) is a potent pro-inflammatory cytokine implicated in the pathogenesis of degenerative diseases such as rheumatoid arthritis, as well as in HIV reactivation, graft versus host disease, scleroderma and shock (7, 8). Prior to its activation, the 26 kDa TNF-α pro-peptide is proteolytically converted to its active 17 kDa form. After subsequent trimerization, TNF-α binds and activates two distinct membrane bound receptors, the 55 kDa type I receptor (TNFR1) and the 75 kDa type II receptor (TNFR2), found on most cell types. Most effects are transduced by TNFR1 and the most characterized transcription factor families activated by TNF-α are NF-κB and AP-1.

NF-κB consists of dimers of proteins of the Rel family (9-11). Prior to activation, homo- and hetero-dimeric members of the Rel family are held latent in the cytoplasm by Iκ-B family members. Upon appropriate stimulation, a series of phosphorylation events

occurs, terminating on Iκ-B via the Iκ-B kinase-α (Ikk-α) (11), leading to its degradation and to the nuclear translocation of NF-κB.

Expression and activation of the AP-1 family of transcription factors represents another essential pathway by which numerous biological effects of TNF-α are mediated (7, 8). They consist of homo- and heterodimers of the protein products of the *Fos* and *Jun* gene families, characterized by a leucine zipper structure allowing dimerization and subsequent DNA binding to the consensus DNA sequence TGAGTCA (12, 13). AP-1 transcription factors have also been implicated in some of the TGF-β regulatory effects on gene expression, such as the modulation, either negative or positive, of interstitial collagenase (MMP-1), plasminogen activator inhibitor-1 (PAI-1), or transin (rat MMP-3) (14-16). In the context of tissue remodeling, TGF-β essentially plays an anabolic role favoring extracellular matrix deposition. On the other hand, TNF-α is known to antagonize most of TGF-β effects on gene expression (16-19). We have previously demonstrated that TNF-α, as well as c-Jun overexpression, prevents TGF-β activation of the α2 type I collagen gene (COL1A2) promoter (18). To date, however, despite numerous evidences for the antognonistic activities of TGF-β and TNF-α, it is not clear whether TNF-α directly interferes with Smad signaling, and , if so, how it does it. It has been recently suggested that TNF-α, through NF-κB activation, can either induce or inhibit Smad7 expression, a molecule that interferes with Smad phosphorylarion by TBRI and subsequent translocation into the cell nucleus. (20, 21). TNF-α alters inhibitory Smad7 expression in a cell type-specific manner, as RelA translocation induces Smad7 expression and subsequent blocade of TGF-β signaling in mouse embryo fibroblasts (20), whereas in human embryonic kidney 293 cells, NF-κB activation inhibits Smad7 gene expression (21).

In this report, we have examined the relative contributions of both AP-1 and NF-κB

pathways in mediating TNF-α effects on TGF-β/Smad signaling in human dermal fibroblasts. We provide evidence for an essential role of AP-1 (c-Jun and Jun-B) downstream of TNF-α in blocking Smad signaling. We also demonstrate that NF-κB activation by TNF-α does not lead to significant expression of Smad7 in this cell type, and that blockade of either the NF-κB pathway or Smad7 expression does not alter TNF-α effect on Smad signaling, as opposed to blockade of c-Jun expression. Our data suggest that TNF-α repression of Smad signaling may be achieved through repression of the transcriptional effects of Smad3 within the cell nucleus, by c-Jun and Jun-B, which directly interfere at the level of Smad3/DNA interactions and subsequent gene transactivation.

MATERIALS AND METHODS

Cell Cultures-Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskins, were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μg/ml streptomycin-G and 0.25 μg/ml Fungizone™), and utilized in passages 3-8. Human recombinant TGF-β1 and TNF-α were from R&D Systems Inc.(Minneapolis, MN).

Plasmid Constructs-$(SBE)_4$-Lux (22) was a kind gift from Dr. Scott Kern, Johns Hopkins University, Baltimore, MD. For c-Jun and Jun-B expression , we used a full-length human cDNAs in pRSVe expression vector (23, kindly provided by Dr. M. Karin, La Jolla, CA). Tagged Smad2, Smad3, c-Jun and Jun-B vectors, were obtained by subcloning the entire coding sequences into either pCDNA3-Flag, pCDNA3-Myc or pCDNA3-HA. G5E1b-Lux containing five Gal4 binding sites driving the expression of luciferase and Gal4BD-Smad3 fusion protein expression vector, containing the full-length Smad3 cDNA excised from pGEX-Smad3 (kind gift from Dr. R. Derynck, UCSF, San Francisco, CA) and subcloned in frame with the Gal4-DNA binding domain into pSG24, has been described previously (24). p300 expression vector was a kind gift from Dr; T. Shioda, Boston, MA. Integrity of all constructs was verified by automated sequencing (ABI).

Transient Cell Transfections and Reporter Assays-Transient cell transfections of human dermal fibroblasts were performed with the calcium phosphate/DNA co-precipitation procedure using a commercial assay kit (Promega Corp., Madison, WI). Following appropriate incubation periods (see Figure legends), the cells were rinsed once with phosphate buffered saline, harvested by scraping, and lysed in 200 μl of Reporter Lysis

Buffer (Promega). pRSV-β-galactosidase was co-transfected in every experiment, and the β-galactosidase activities were used to monitor transfection efficiency. Aliquots corresponding to identical β-galactosidase activity were used for each reporter assay. Luciferase activity was determined with a commercial assay kit according to the manufacturer's protocol (Promega).

In vitro protein synthesis- T7 promoter-driven transcription and traduction of Smad2-Flag, Smad3-Myc, c-Jun-HA and Jun-B-Flag proteins were performed in a single tube assay (TnT®, Promega Corp., Madison, WI) according to the manufacturer's protocol.

Electrophoresis Mobility Shift Assays- A 39bp Smad-specific probe (3xCAGA, 25) was used to determine the effect of TNF-α on TGF-β-induced Smad/DNA interactions. Nuclear extracts were isolated using a small scale preparation (26), aliquoted in small fractions to avoid repetitive freeze-thawing, and stored at -80°C until use. The protein concentration in the extracts was determined using a commercial assay kit (Bio-Rad). Binding mixtures were separated electrophoretically on 4% acrylamide gels in 0, 5 x Tris-Borate-EDTA, as previously described (27, 28).

Immunoprecipitations and Western blotting-COS-7 cells were transfected with TGF-β receptors expression vectors together with tagged Smad3 and c-Jun expression vectors. 40 h later, cells were washed twice with cold PBS, scraped, and solubilized in a buffer containing 20 mM Tris -HCl, pH8, 150 mM NaCl, 5 mM $MgCl_2$, 0, 5% NP-40, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 20 μg/ml aprotinine and 20 μg/ml leupeptine. Lysates were cleared of debris by centrifugation and incubated with anti-Myc 9E10 antibody (Santa Cruz Biotech, Santa Cruz, CA), 2 h at 4°C, followed by incubation with protein G-Sepharose beads at 4°C for 1 h (Amersham Pharmacia Biotech, Uppsala, Sweden). After

5 washes with solubilization buffer, the immunoprecipitates were eluted by boiling for 3 min in SDS sample buffer (100 mM Tris-HCl, pH 8, 8, 0, 01% bromophenol blue, 36% glycerol, 4% SDS) and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose filters, immunoblotted with appropriate anti-HA, -Flag and –Myc antibodies (Santa Cruz Biotech.), and revealed using a chemiluminescence detection system (Amersham-Pharmacia).

RESULTS

TNF-α prevents TGF-β-induced Smad-mediated gene transactivation but does not induce detectable levels of Smad7 in human dermal fibroblasts

We have previously established that TNF-α is a potent antagonist of TGF-β-induced COL1A2 promoter activity (18). Activation of the latter promoter by TGF-β has recently been shown to involve Smad3/4 (29). To determine precisely whether TNF-α may exert an inhibitory activity on Smad-specific transcription, and to circumvent any interference of additional, non Smad-specific *cis*-elements, we examined the modulation of $(SBE)_4$-Lux, a reporter construct in which four tandem repeats of a consensus Smad3/4 binding element (SBE, 22) drive the expression of the luciferase gene. As shown in Fig. 1, and as expected from the literature, TGF-β enhanced $(SBE)_4$-Lux activity approximately 3-fold above control levels. Interestingly, this induction was completely abolished by addition of TNF-α 1 h prior to TGF-β.

A recent report has shown that Smad7 induction by TNF-α via NF-κB activation may be responsible for its ability to interfere with the Smad signaling pathway in mouse fibroblasts (20). Also, Smad7 was recently identified as a direct Smad3/4 target, downstream of TGF-β (30). However, Smad7 promoter activity is inhibited by TNF-α, via NF-κB, in 293 cells (21). Consistent with the known inhibitory activity of Smad7 on TGF-β/Smad signaling, we observed that overexpression of Smad7 efficiently blocked TGF-β effect on $(SBE)_4$-Lux activity (not shown). Therefore, we tested whether Smad7 was rapidly induced by TNF-α, and by TGF-β, in our experimental system, and could therefore account for the inhibitory activity of TNF-a on Smad-driven gene transactivation. For this purpose, confluent fibroblast cultures were treated for 1 h with TNF-α prior to addition of TGF-β. Three hours later, total cell extracts were subjected to Western blot analysis using

an anti-Smad7 antibody. As shown in Fig. 2A, neither TNF-α nor TGF-β induced detectable levels of Smad7. In contrast, c-Jun levels were markedly elevated by TNF-α, but not by TGF-β, consistent with our previous observations (16, 31). Functionality of the anti-Smad7 antibody was verified using cell extracts from COS-7 cells transfected with a Smad7 expression vector, pGFP–Smad7, in the presence or absence of the antisense Smad 7 vector pGFP-ASSmad7 (Fig. 2B : a strong Smad7 signal was detected in Smad-7-transfected COS-7 cell extracts, and this signal was significantly lowered when the antisense vector was co-transfected. These data establish both the functionality of the anti-Smad7 antibody to recognize its antigen and the efficacy of the antisense Smad7 vector to block Smad 7 expression.

C-Jun and Jun-B block Smad3-mediated transactivation

We have previously shown that transactivation of human COL1A2 promoter by TGF-β is abolished by c-Jun overexpression (18). Since, c-Jun and Jun-B are both potently induced by TNF-α in dermal fibroblasts (31), we examined the effects of their overexpression on Smad3-mediated transactivation of the reporter constructs $(SBE)_4$-Lux. As shown in Fig. 3, neither Jun-B nor c-Jun had an effect on the basal activity of the construct, whereas Smad3 overexpression induced its activity several times. When co-expressed with Smad3, Jun-B abbrogated Smad3-dependent transactivation of $(SBE)_4$-Lux in a dose-dependent manner, reaching 80% when identical amounts of Jun and Smad3 expression vectors were used. Similarly, c-Jun overexpression did not modify the basal activity of the construct but strongly inhibited Smad3-mediated transactivation. These results indicate that both c-Jun and Jun-B members of the AP-1 family directly antagonize Smad3-driven gene transactivation.

Expression of antisense c-Jun mRNA prevent TNF-α inhibition of TGF-β/Smad signaling whereas that of a dominant-negative Ikk-α or antisense Smad7 does not

To determine the relative contributions of the AP-1 and NF-κB pathways on TNF-α inhibition of TGF-β/Smad signaling, $(SBE)_4$-Lux was co-transfected in parallel with either an antisense c-Jun vector, pRSV-ASc-Jun, or a dominant-negative Ikk-α expression vector, pD/N-Ikk-α, or pGFP-ASSmad7. As shown in Fig. 4, pRSV-ASc-Jun expression prevented TNF-α inhibitory effect on $(SBE)_4$-Lux transactivation by TGF-β, whereas empty pRSV had no effect. Contrary to pRSV-ASc-Jun, neither pD/N-Ikk-α nor pGFP-ASSmad7 had an effect on the inhibitory activity exerted by TNF-α on TGF-β-induced Smad $(SBE)_4$-Lux transactivation. Efficient NF-κB activation/translocation by TNF-α was verified by electrophoretic mobility shift assay using a consensus NF-κB oligonucleotide as a probe and nuclear extracts from parallel control or TNF-α-treated (30 min) fibroblast cultures (not shown). Together, these data suggest that c-Jun, not NF-κB/Smad7 is responsible for TNF-α inhibition of Smad signaling in human dermal fibroblasts.

c-Jun and Jun-B interact with Smad3 off-DNA and reduces Smad/DNA interactions

To investigate the mechanisms by which c-Jun antagonizes Smad-mediated gene transactivation, we first examined the ability of Smad3 and Jun proteins to participate in off-DNA protein-protein interactions. For this purpose, COS-7 cells were transfected with Smad3-Myc and c-Jun-HA expression vectors. Immunoprecipitations of the cell lysates were performed with an anti-Myc, followed by Western blot anaylsis with anti-Myc and anti-HA or anti-Flag antibodies. As demonstrated in Fig. 5, c-Jun interacts with Smad3 off-DNA, as evidenced by the co-precipitaion of c-Jun-HA together with Smad3-Myc. Alternatively, COS-7 cells were transfected with Smad3-Flag and Jun-B-Myc expression

vectors. In the latter case, we observed co-immnunoprecipitation of Smad3-Flag with Jun--Myc (not shown).

These data establish the capacity of Smad3 to form heterocomplexes with Jun family members off-DNA, and corroborate recent observations (32, 33).

We next tested the ability of c-Jun to interact with Smad3/4, in the context of TGF-β-induced Smad/DNA complexes. For this purpose, EMSA experiments were performed, using nuclear extracts from TGF-β-stimulated fibroblast cultures incubated with a radiolabeled 3x CAGA probe. *In vitro* transcribed c-Jun or Jun-B were added to the binding reactions and their effects on TGF-β-induced Smad/DNA complex formation was determined. As shown in Fig. 6A, and as expected from our previous observations (27, 28), TGF-β induced the formation of a unique Smad/DNA complex (lane 2 vs. lane 1). Addition of *in vitro* synthesized c-Jun to the binding reaction strongly diminished Smad interactions with DNA, as compared to addition of an identical amount of mock TnT® reaction (lane 4 vs. lane 2). Quantitation with a phosphorimager of the radioactivity present in the Smad/DNA complexes in lanes 2 and 4 indicated 80% reduction in Smad/DNA complex formation upon c-Jun addition, whereas no change was induced by addition of an equal amount of Smad2 (lane 6). A similar reduction in Smad/DNA complex formation was also observed when Jun-B was added to the Smad/DNA binding reaction instead of c-Jun, although slightly less marked (approximately 50% reduction in Smad/DNA complex formation, Fig. 6B, lane 4 vs. lane 2).

From these experiments, it is suggested that off-DNA Smad3/AP-1 interactions may sequester some of the available Smad3 and compete against Smad binding to its cognate *cis*-element, a phenomenon which likely inhibits Smad3-dependent transcription.

p300 overexpression prevents c-Jun inhibition of Smad signaling

Another possibility for AP-1 members to interfere with the Smad pathway would be to compete for certain co-factors necessary for optimal transcriptional responses. One such factor is p300. Because the amount of p300 is limiting within the cell nucleus (34), formation of c-Jun/p300 complexes may reduce the amount of p300 available to Smad3 for optimal transcription. If this scenario is correct, then, overexpression of p300 should overcome the inhibitory effect of TNF-α/c-Jun on TGF-β/Smad3 activation of the $(SBE)_4$-Lux construct. To test this hypothesis, we first measured the effect of p300 overexpression on c-Jun inhibition of $(SBE)_4$-Lux transactivation by Smad3. As shown in Fig. 7A, p300 overexpression prevented the inhibitory effect of c-Jun. Similarly, p300 overexpression overcame the inhibitory effect of TNF-α on TGF-β-mediated $(SBE)_4$-Lux transactivation (Fig. 7B).It should also be noted that p300 overexpression resulted in increased $(SBE)_4$-Lux activity in response to both TGF-β stimulation and Smad3 overexpression, a result which is consistent with the concept of limited availability of p300, and which confirms the recently described role of p300 as a Smad3 coactivator (35-37).

The role of p300 as a Smad3 coactivator with limited availability was further examined in a Gal4-Smad3 fusion protein-based transactivation assay. As shown in Fig. 7C, this Gal4 fusion-based assay fully recapitulated the data obtained with the $(SBE)_4$-Lux construct. Specifically, Gal4-Smad3, but not c-Jun, activated the reporter gene containing five Gal4 binding sites upstream of the luciferase gene. This activation by Gal4-Smad3 was repressed by c-Jun and enhanced by p300 overexpresion, the latter preventing the inhibitory effect of c-Jun.

Together, these data demonstrate that c-Jun competes against Smad3 for the available p300 in the cell nucleus. The latter mechanism, in turn, would act in concert with direct off-DNA Smad3/Jun interactions to decrease Smad3-specific gene transcription.

DISCUSSION

We report a mechanism of suppression of TGF-β/Smad3 signaling by TNF-α, which involves the transcription factors c-Jun and Jun-B. These AP-1 components are key factors in the transmission of signals from various pro-inflammatory cytokines known to antagonize TGF-β in the context of tissue repair and maintenance of tissue homeostasis. This phenomenon is complementary to a recently uncovered mechanism by which TNF-α may block Smad signaling. Specifically, it has been shown that, in certain situations, RelA/NF-κB can induce the expression of the inhibitory Smad7, and stabilize the association of the latter with activated TGF-β type I receptors (20). Thus, Smad7 induction by TNF-α via RelA activation could represent a mechanism by which TNF-α antagonizes TGF-β signaling at the level of TGF-β type I receptor function, by preventing Smad2 and Smad3 phosphorylation and subsequent translocation into the nucleus. This is, however, not a universal mechanism, as other reports indicate (a), that TNF-α, or NF-κB overexpression, inhibits Smad7 promoter activity human embryonic 293 kidney cells (21) ; and (b), that neither TNF-α nor TGF-β induce Smad7 expression in endothelial cells (38) and in human dermal fibrobalsts (this report). The latter observations also diverge from the recent demonstration that TGF-β may induce Smad7 expresion in certain cell types (30, 39), a phenomenon that is Smad3/4-dependent (30). It appears therefore that the control of Smad7 expression by cytokines is cell-type specific. It should be noted that the lack of induction of Smad7 expression in human dermal fibroblasts occurred despite efficient NF-κB/RelA activation and subsequent nuclear translocation upon TNF-α treatment (31, 40), and despite rapid activation of Smad signaling and Smad-dependent transcription by TGF-β (our data, 27, 28). These observations indicate that Smad7 gene

regulation by either TNF-α or TGF-β requires mechanisms other than just RelA and/or Smad3 activation.

In this report, we have demonstrated that TNF-α, via AP-1 activation, interferes with Smad signaling within the cell nucleus. First, we suggest the possibility of a direct Smad3 squelching mechanism, where Jun proteins bind Smad3 off-DNA and prevent its binding to specific DNA binding sites. In suport of these findings, it has been shown that the interaction between Smad3 and Jun-B occurs between the MH1 domain of Smad3 and a 20 amino acids region close to the leucine zipper portion of c-Jun and Jun-B (32, 33). Since the MH1 domain is also the DNA binding domain of Smad3, it may be speculated that off-DNA interactions with Jun proteins are not compatible with simultaneous DNA binding of Smad3, consistent with both our observations and deductions from the crystal structure of the MH1 domain of Smad3 (41). Secondly, we have demonstrated that p300 overexpression overcomes the inhibitory effect of TNF-α/AP-1 on TGF-β/Smad3-specific transcription from the $(SBE)_4$-Lux construct. These results were confirmed in a Gal4-Smad3 fusion protein assay system. A possible mechanism for the inhibitory effect of TNF-α/c-Jun would be that c-Jun sequesters p300, a known transcriptional co-activator for both Smads and Jun proteins (35-37, 42), whose availability within the nucleus is limited (34, 42). Such sequestration of p300 by c-Jun likely reduces p300/Smad3 interactions and resulting Smad3-dependent transcription. A similar working model of competition for p300 has been proposed to explain the antagonistic effects of E1A and RelA on c-Jun- and Smad3-mediated transcription (13, 43, 44).

Despite the characterization of several Smad-responsive natural promoters in the literature, such as those for COL1A2, COL7A1 or PAI-1, we chose to focus our study on the artifcial, Smad-specific, $(SBE)_4$-Lux construct. The reasons for this choice are as

follows. First, we have previously shown that the direct inhibitory effect of TNF-α on COL1A2 gene transcription is an NF-κB-dependent mechanism, through direct binding of RelA/NF-κB1 (p65/p50) complexes to a *cis*-element immediately adjacent to the COL1A2 TGF-β response element (39). TNF-α may also exert an antagonistic activity against TGF-β effect on COL1A2 promoter via c-Jun induction (18), a phenomenon which may reflect c-Jun-mediated inhibition of the Smad3/4 pathway, the latter participating in COL1A2 gene transactivation (29). However, due to the direct inhibitory effect of NF-κB on COL1A2 promoter activity, the use of the latter as a tool to study the transcriptional outcome of Smad/AP-1 interactions specifically seemed inappropriate. Regarding the COL7A1 gene, we have shown that TNF-α and TGF-β synergistically activate its promoter via distinct Smad- and NF-κB-specific *cis*-elements (45). Somewhat similarly, the PAI-1 promoter, which contains at least three AP-1 sites distant from three CAGA boxes, is stimulated by both Smad- and AP-1 pathways (15, 25). Therefore, the presence of multiple cytokine-responsive elements within natural promoters known to be Smad-responsive prevented us from using them in this study, as additional, non Smad-dependent, regulatory mechanisms would have participated in the outcome of the experiments.

In conclusion, the present report establishes that the AP-1 pathway downstream of TNF-α directly interferes with TGF-β/Smad3 signaling at the level of Smad3-mediated gene transcription. Direct c-Jun/Smad3 or Jun-B/Smad3 interactions off-DNA may reduce the ability of available Smad3 to reach its DNA targets. Alternatively, Jun components may recruit the transcriptional co-activator p300, reducing its availability for Smad3. Both mechanisms likely contribute simultaneously to the inhibitory effect of TNF-α on TGF-β/Smad3 signaling.

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LEGENDS TO FIGURES

FIG . 1. **TNF-α inhibits TGF-β-induced transactivation of $(SBE)_4$-Lux**. Fibroblasts in late logarithmic growth phase were transfected with 6 μg of $(SBE)_4$-Lux promoter/reporter gene construct. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Eighteen hours later, TNF-α (10 ng/ml) was added, followed by TGF-β (10 ng/ml) 1 h later. Incubations were continued for 24 h and reporter gene activity was determined.

FIG. 2. **TGF-β and TNF-α do not induce early expression of Smad7 in human dermal fibroblasts** . *A*, sub-confluent fibroblasts were incubated for 24 h in fresh medium supplemented with 1% FCS. TNF-α (10 ng/ml) was then added, followed by TGF-β (10 ng/ml) 1 h later. Incubations were continued for 3 h and total protein extracts were subjected to Western blot analysis using anti-Smad7 and anti-c-Jun antibodies. *B*, COS-7 cells were transfected with activated TGF-β receptor type I expression vector and pRSV-β-galactosidase, with either empty CMV expression vector or Smad7 expression vector, in absence or presence of antisense Smad7 vector. Twenty four hours later, Amounts of cell extracts corresponding to identical β-galactosidase activity were subjected to Western blot analysis using a Smad7 antibody.

FIG. 3. **C-Jun and Jun-B inhibit Smad3-dependent activation of $(SBE)_4$-Lux**. Fibroblasts in late logarithmic growth phase were co-transfected with 3 μg of $(SBE)_4$-Lux, without or with 6 μg of pcDNA-Smad3-Flag and either pRSV/Jun-B (0.5, 2, 6 μg) or pRSV/c-Jun (6 μg) expression vectors. Empty pRSV was used to maintain equivalent amounts of transfected DNA in each plate. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Incubations were continued for 40 h and reporter gene activity was determined.

FIG. 4. **Antisense c-Jun prevents TNF-α inhibition of Smad-dependent activation of $(SBE)_4$-Lux by TGF-β**. Sub-confluent fibroblasts were co-transfected with 3 μg of $(SBE)_4$-Lux , together with either pRSV-ASc-Jun, or pD/N-Ikk-α, or antisense Smad7 expression vectors. Empty pRSV was used to maintain equivalent amounts of transfected DNA in each plate. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Incubations were continued for 40 h and luciferase activity was determined. Note that only the antisense c-Jun vector blocked TNF-α effect on TGF-β-driven transactivation of $(SBE)_4$-Lux.

FIG. 5. **Evidence for Smad3/c-Jun and Smad3/Jun-B interaction off-DNA**. COS-7 cells were transfected with TGF-β receptor type I expression vector together with tagged Smad3-Myc and c-Jun-HA expression vectors. 40 h later, cell extracts were immunoprecipitated with an anti-Myc antibody. The immunoprecipitates were subjected to Western blot analysis using anti-HA or anti-Myc antibodies.

FIG. 6. **c-Jun and Jun-B reduce Smad3/DNA interactions.** EMSAs were performed using the Smad3/4-specific 3x CAGA oligonucleotide (Dennler et al. 1998) as a probe, together with nuclear extracts from control and TGF-β-treated (30 min.) fibroblast cultures, in the absence or presence of *in vitro*-synthesized full length c-Jun or Smad2 (A), or Jun-B (B), as indicated. Mock TnT® reaction mix (without expression vector) was used in lanes 1 and 2.

FIG.7. **p300 overexpression prevents TNF-α/c-Jun inhibitory effect on TGF-β/Smad3 transactivation.** *A*, Sub-confluent fibroblasts were co-transfected with 3 μg of $(SBE)_4$-Lux, without or with p300 expression vector (5 μg), in the presence or absence of 5 μg of either Smad3 or c-Jun expression vectors. After glycerol shock, the cells were placed in medium

supplemented with 1% FCS, and luciferase activity was detemined 24 h later. *B*, Fibroblast cultures were co-transfected with 3 μg of $(SBE)_4$-Lux, without or with p300 expression vector (8 μg). After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Eighteen hours later, TNF-α (10 ng/ml) was added, followed by TGF-β (10 ng/ml) 1 h later. Incubations were continued for 24 h and reporter gene activity was determined. *C*, confluent fibroblast cultures were co-transfected with 2 μg of Gal4-Lux reporter construct, without or with 5 μg of Gal4-Smad3 fusion protein expression vector, in the presence of 5 μg of either c-Jun of p300 expression vectors, alone or in combination. 24 h later, luciferase activity was determined. In all cases, empty pCMV expression vector was used to equalize the amount of transfected DNA between plates.

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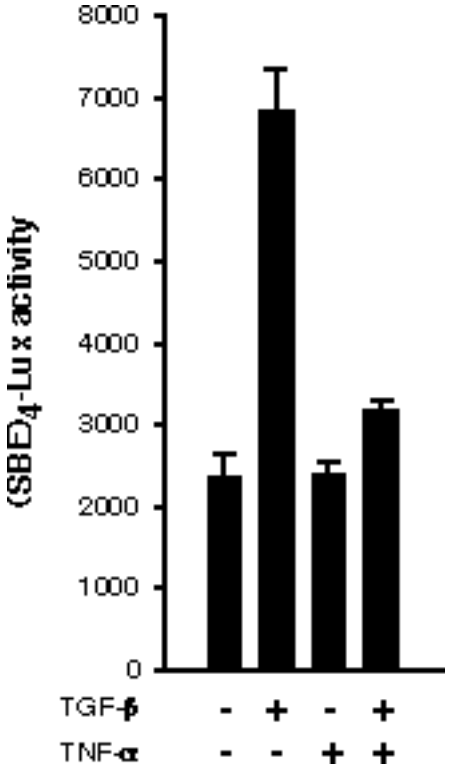

Fig.1

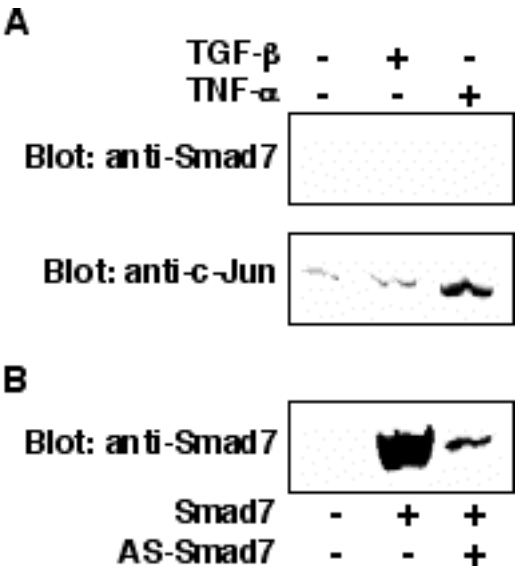

Fig. 2

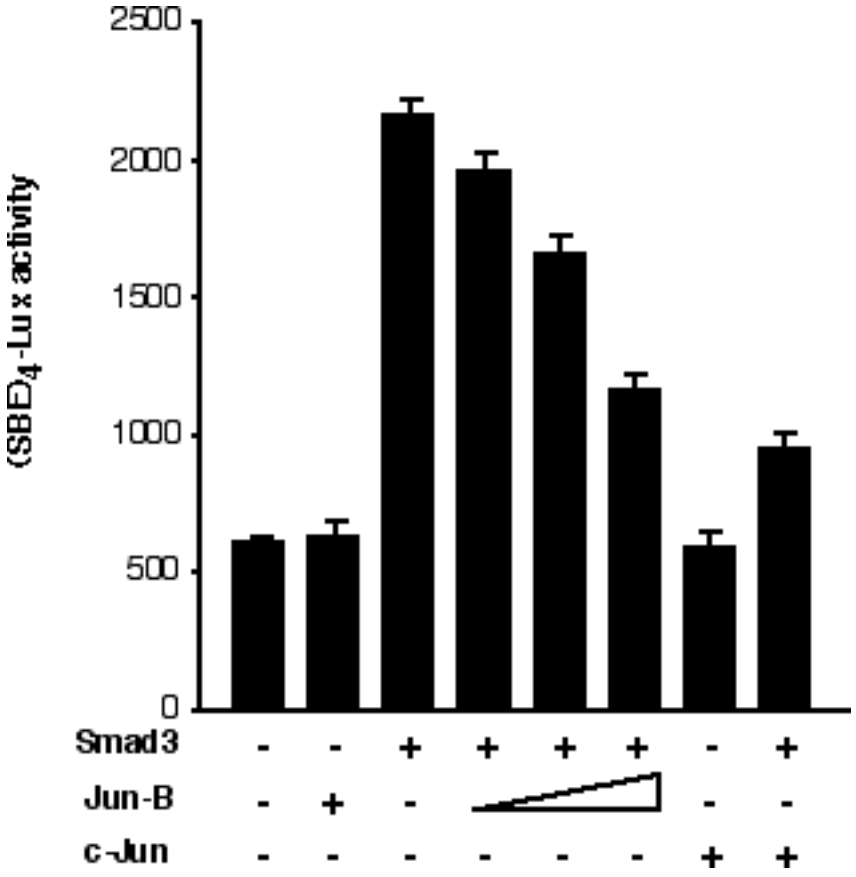

Fig. 3

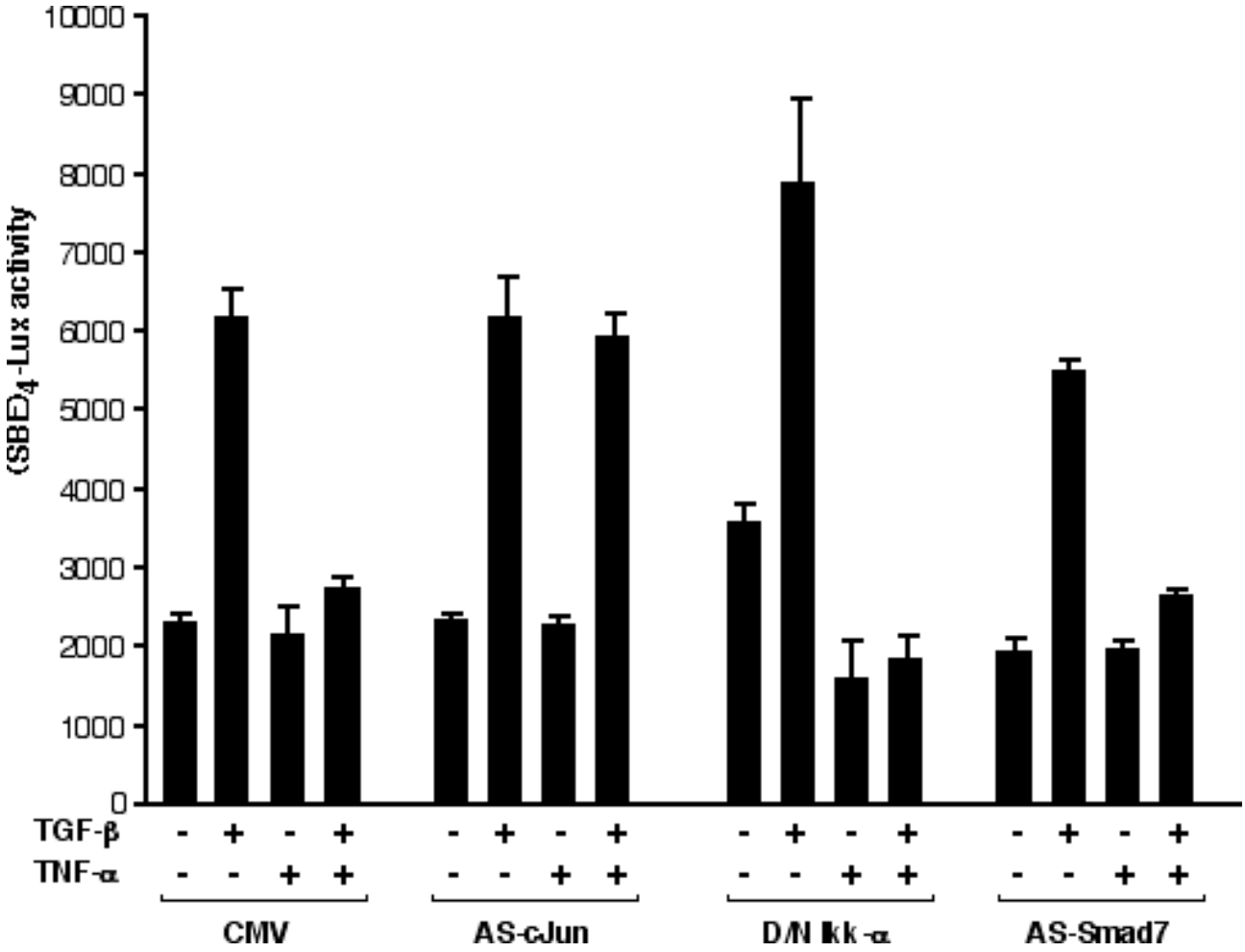

Fig. 4

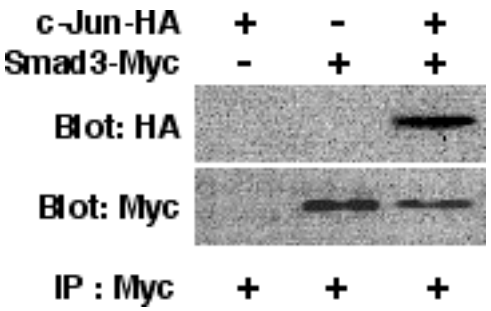

Fig. 5

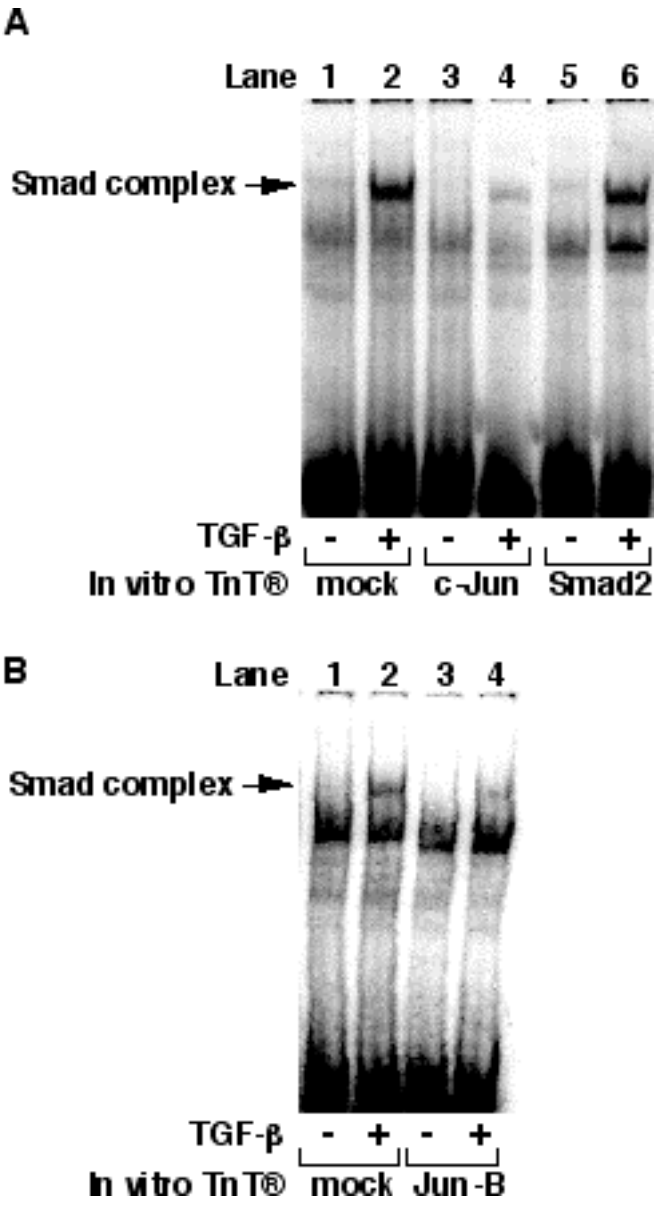

Fig. 6

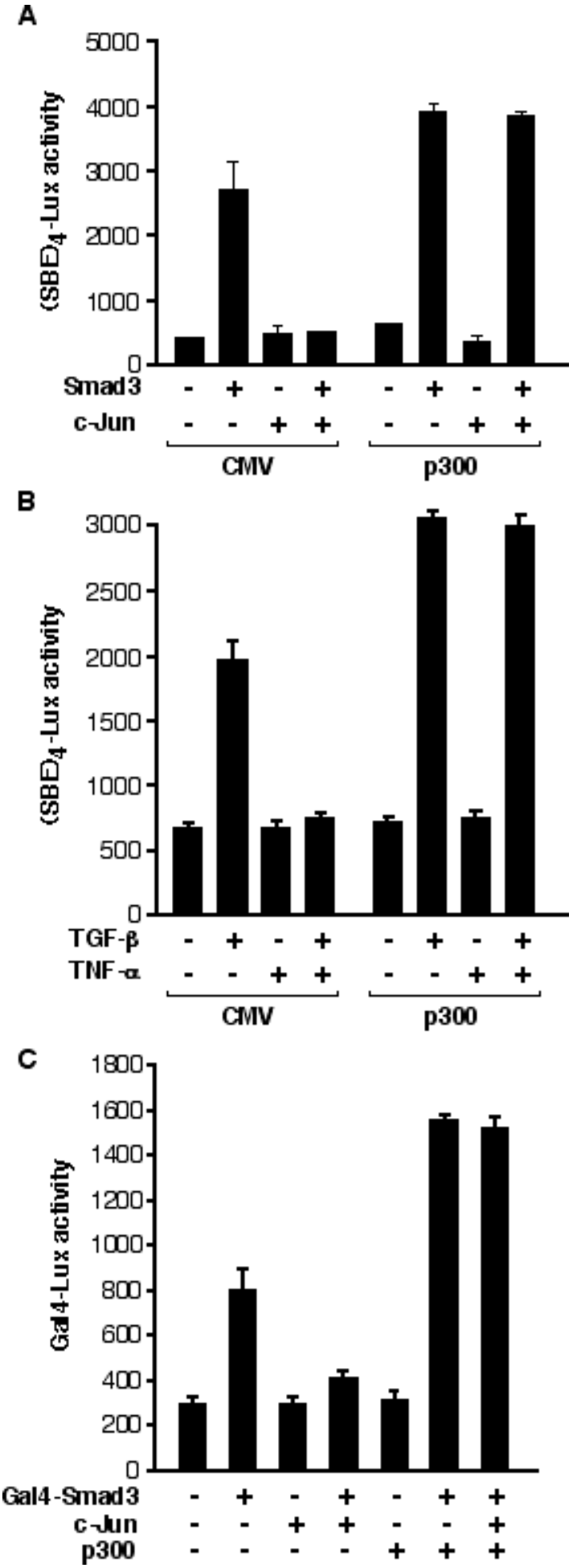

Fig. 7