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# Interaction and Functional Cooperation of NF-κB with Smads

TRANSCRIPTIONAL REGULATION OF THE *junB* PROMOTER*

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**The transforming growth factor−β (TGF−β) family of cytokines regulates diverse cellular processes through control of the expression of target genes. Smad proteins are a recently identified family of signal transducers for members of the TGF−β family. Smads act as transcriptional regulators through binding to DNA and interacting with a variety of transcription factors. Here, we identified a κB site as a TGF-β-responsive region in the 3′-downstream *junB* promoter region. We also demonstrate that κB sites alone are sufficient to mediate immediate transcriptional activation by TGF-β. Transactivation of κB sites by TGF-β requires an intact NF-κB pathway, cooperates with known activators of this pathway, and is mediated by Smad family members. Furthermore, we show that Smad3 interacts with p52 *in vivo*. These data expand the model in which Smad proteins undergo multiple interactions with several transcription factors that could induce either activation or repression of gene expression.**

Members of the transforming growth factor-β (TGF-β)[1] superfamily play an essential role in the control of proliferation, differentiation, and apoptosis and are therefore important for the development and maintenance of most tissues. Many members of this family have been isolated, and three major subfamilies have been described as follows: TGF-βs, activins/inhibins, and bone morphogenetic proteins (BMPs) (reviewed in Refs. 1 and 2). The effects of these cytokines are executed via ligand-induced heteromeric complex formation of different type I and type II serine/threonine kinase receptors. In the resulting complex, the type II receptor phosphorylates the type I receptor, which then transmits the signal into the cell (2). Smad proteins have recently been defined as the main cytoplasmic transducing molecules in the TGF-β superfamily pathway. On the basis of structural and functional criteria, the Smad family has been divided into three subgroups (2–6). The receptor-regulated or pathway-restricted Smads (R-Smads) are directly phosphorylated by type I receptors. Smad2 and Smad3 propagate TGF-β and activin signals, whereas Smad1, Smad5, and possibly Smad8 are specific for BMP (2–6). Receptor-activated Smads form a hetero-oligomer with Smad4, the common mediator of the TGF-β, activin, and BMP signaling (Co-Smad). Smad4 or DPC4 is the only vertebrate Co-Smad identified to date (7–8). Smad6 (9–10) and Smad7 (11–12) belong to the third class, anti-Smads, and they antagonize signaling by R-Smads and Co-Smads. Activation of receptor-regulated Smads disrupts the autoinhibitory interaction between their N-terminal (MH1) and C-terminal (MH2) domains and allows complex formation between pathway-restricted Smads and Smad4 (2–6). These complexes translocate into the nucleus, where they could directly bind to DNA through their MH1 domains (13–14).

A number of immediate-response genes whose promoters are induced by TGF-β have been identified. They include plasminogen activator inhibitor-1 and fibronectin (13–15), the cyclin-dependent inhibitors p15 and p21 (16–17), and type I and type VII collagen (18–19). Some of them, such as plasminogen activator inhibitor-1, include Smad-binding elements, and multimerization of these elements confers TGF-β inducibility (13). Oligonucleotide screening and the resolution of the crystal structure of Smad3 MH1 domain defined the tetranucleotide CAGA as their minimal Smad DNA-binding element (SBE) (14–20). However, studies of promoters of *Dpp*-responsive genes in *Drosophila*, such as *vestigial* and *tinman*, revealed GC-rich sequences as Mad and/or Medea (*Drosophila* Smad homologs) binding sequences (21). Overall, these data indicate that Smads have relatively low DNA binding affinities and specificity. Furthermore, growing evidence suggests that Smads regulate transcription either by functional cooperation with transcription factors bound to adjacent sites or through direct association with DNA-bound transcription factors. For example, upon ligand stimulation, the forkhead transcription factors FAST1 and FAST2 recruit Smad 2/4 complexes to adjacent sites in the *Mix.2* and *goosecoid* promoters (22–24). Smad3 and -4 interact with the Jun family of transcription factors and synergistically activate AP-1 promoter sequences (25–26). Physical interaction has also been shown between Smads and SIP1, a member of the δEF1 family of transcriptional repressors (27). A similar scenario might occur with cooperative associations between Smad1 and Stat3 or Smad3 and vitamin D receptor. However, in these later cases, interactions require the presence of coactivators such as p300/CBP or steroid receptor coactivator-1, respectively (28–29).

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; BMPs, bone morphogenetic proteins; SBE, Smad DNA-binding element; CBP, CREB-binding protein; LLnL, *N*-acetyl-Leu-Leu-norleucinal; PDB, phorbol dibutyrate; MEKK-1, mitogen-activated protein/extracellular signal-regulated kinase kinase-1; TβR-I (TD), constitutively active TGF-β type I receptor (T204D); TAK-1, TGF-β activated kinase-1; DMEM, Dulbecco's modified Eagle's medium; NTA, nitrilotriacetic acid.



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The NF-κB/*rel* family regulate transcription of several genes, mainly involved in immune or inflammation responses and cell growth control. A wide range of physiological and non-physiological stimuli can activate this transcription factor (30). Five members of the NF-κB/*rel* family have been identified in mammalian cells: p50, p52, p65/*relA, relB,* and c-*rel*. Before activation, NF-κB is in the cytoplasm of most cell types as homo- or heterodimers. Cytoplasmic NF-κB is an inactive complex through its association with the inhibitor proteins, IκBs (30–31). When cells are exposed to inductors of NF-κB, IκB is specifically phosphorylated. This phosphorylation is a signal for ubiquitination and later degradation of IκB by the 26 S proteasome (32). NF-κB is thus free to translocate to the nucleus and activate transcription of target genes. Studies *in vivo* and *in vitro* indicate that different NF-κB dimers have different transcriptional activation properties. Moreover, evidence indicates that interactions between NF-κB and other transcription factors influence the ability of NF-κB to regulate gene expression in a selective manner. Consistent with this, Gerritsen *et al.* (33) have found that p65 and CREB-binding protein (CBP)/p300 interact to activate E-selectin and vascular cell adhesion molecule 1. In addition, steroid receptor coactivator-1 interacts with p50 subunit but not with p65 and coactivates NF-κB (34).

Several enhancer sequences have been implicated in the transcriptional regulation of *junB*, a member of the AP-1 family of transcription factors, both in the 5′- and the 3′-flanking region of this intronless gene (35, 36). Recently, Jonk *et al.* (37) identified two Smad-binding sequences in the mouse *junB* 5′ promoter region that, when multimerized, mediate responsiveness by several members of the TGF-β superfamily. In this report, we have investigated the transcriptional regulation of the immediate early gene *junB* by TGF-β. We analyzed the downstream region of the gene and identified an NF-κB site as a TGF-β− responsive region. We demonstrate that NF-κB sites are sufficient to mediate transcriptional activation by TGF-β. This activation requires an intact NF-κB pathway and is mediated by Smad family members. Finally, we show that Smad proteins interact with NF-κB subunits. These data further support a role for Smads as transcriptional coactivators, in addition to their role as DNA-binding transcription factors.

## MATERIALS AND METHODS

### *Recombinant Plasmids*

Luciferase reporter plasmids pJB, pJB2028-2249 and pJB2071-2249 (36), were provided by C. Thompson. pJB2035-2072 and pJB2040-2068CAGAmut and pJB2040-2068NF-κBmut were generated as follows: oligonucleotides 5′-CTGCCCAG<u>CAGA</u>GTGCGCC<u>GGGGCTTTC-C</u>CCGCTGAC-3′ for wild type; 5′-CA<u>GTTGA</u>GTGCGCC<u>GGGGCTTTC-C</u>CCGC-3′ for CAGAmut; 5′-CAG<u>CAGA</u>GTGCGCC<u>GATACTTTCC</u>CC-GC-3′ for NF-κBmut, and their respective complementary counterparts were phosphorylated at 5′ ends, annealed, and ligated with pJB, containing the minimal *junB* promoter-luciferase gene construct, or with pGL-2fos, containing the minimal c-*fos* promoter. pNF-κB-Luc, which contains two NF-κB sites (5′-A<u>GGGGACTTTCC</u>GAGAGG-3′) in front of the minimal c-*fos* promoter, MEKK-1DN, and IκBαDN were kindly provided by P. Muñoz. 3TP-Lux, Smad1, Myc Smad3, and FLAG Smad4 were provided by J. Massagué. Wild type *rho*A, *cdc42*, and *rac1* and their dominant negative and constitutively active mutants were provided by X. R. Bustelo, whereas *arf6* constructs were obtained from J. Donaldson. Expression vectors for NF-κB subunits p50, p52, p65, c-Rel, and RelB, were provided by J. Caamaño.

### *Cell Culture and Transient Transfection*

Mv1Lu and COS cell lines were cultured in DMEM supplemented with 10% fetal bovine serum. The R1B/L17 cells, provided by J. Massagué, were cultured in minimum Eagle's medium without histidine, supplemented with 10% fetal bovine serum, and 0.5 mM histidinol. Cells were grown in a 10% $CO_2$ atmosphere with 95% humidity.

Cell transfection was performed by the DEAE-dextran method as described previously (19). When different combinations of plasmids were used, total DNA was kept constant by the addition of empty vector.

### *Luciferase Assay*

Mv1Lu cells were split 24 h after transient transfection, cultured in DMEM supplemented with 0.1% fetal calf serum serum, and treated with 200 pM human recombinant TGF-β1 (Sigma) or 1 nM BMP2 (Genetics Institute, Cambridge, MA) for 16 h. Luciferase activities were quantified using the Luciferase Assay System (Promega). Proteasome inhibitor, *N*-acetyl-Leu-Leu-norleucinal (Sigma), or brefeldin A were added 15 min before TGF-β. Luciferase values are expressed as mean ± S.E. of transfection experiments performed in triplicates in three to six independent experiments.

### *Electrophoretic Mobility Shift Assays*

Nuclear extracts were prepared from control and TGF-β- or PDB-treated Mv1Lu as follows. Pelleted cells were resuspended in 400 μl of ice-cold Buffer A (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 25 mM β-glycerophosphate, 2 mM sodium vanadate, 10 mM NaF) by flicking the tube. After a 10-min incubation on ice, cells were vortexed for 30 s and then centrifuged. The pellet was resuspended in 50 ml of ice-cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, protease, and phosphatase inhibitors) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min, and supernatants were aliquoted and stored at −80 °C. The protein content was determined using the Bradford protein concentration assay (Bio-Rad) with bovine serum albumin as standard. The NF-κB oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega) was labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase.

Ten micrograms of the nuclear proteins were diluted to a final volume of 20 μl in a reaction mixture containing 20 mM Tris, pH 7.9, 50 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 1.25 μg of poly(dI-dC). Where indicated, unlabeled oligonucleotides or antibodies (H-119 for p50; K-27 for p52; H-286 for p65; C-19 for RelB and B-6 for c-Rel from Santa Cruz Biotechnology) were added and the samples incubated on ice for 15 min before adding 0.1 pmol of labeled probe (5–10 × $10^4$ cpm). After a 20-min incubation on ice, the reaction mixture was loaded onto a 5% polyacrylamide gel, 0.25× TBE, and 2.5% glycerol and resolved at 20 mA for 2.5 h. Gels were dried and autoradiographed.

### *Western Blot and Pull-down Analysis*

*Total Extracts*—For detection of NF-κB subunits and IκBs, Mv1Lu cells were serum-starved and treated with TGF-β for different times. Then cells were washed twice in cold phosphate-buffered saline and then lysed with sample buffer 1× (62.5 mM Tris, pH 6.8, 10% glycerol, 1% SDS, 100 mM dithiothreitol and 0.25 mg/ml bromphenol blue).

*Pull Down*—Cells were washed twice in cold phosphate-buffered saline 48 h after transfection and lysed with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, and 10 mM imidazole. Cell debris was removed by centrifugation for 1 min. The supernatant fraction was incubated with 30 μl of $Ni^{2+}$-NTA-agarose (Qiagen) for 1 h at 4 °C. Bound proteins were washed four times in lysis buffer, and pellet was resuspended in sample buffer two times. Bound Smad3 was detected by Western blotting using anti-Myc antibody as described above.

*Western Blot*—Protein extracts were resolved on 12% SDS-polyacrylamide gels for detection of IκBs and 10% for Smads and NF-κB subunits, transferred to nitrocellulose membrane, and subjected to Western blot using specific monoclonal or polyclonal antibodies as follows: FLAG-tagged Smad4, Myc-tagged Smad3, and His-tagged p52 were detected by immunoblotting using a 1:1000 dilution of monoclonal antibodies to the FLAG epitope (Sigma), the Myc epitope (9E10), and the His epitope (Amersham Pharmacia Biotech). IκBs were detected with a 1:1000 dilution of antibodies H4 for IκBα, C-20 for IκBβ, and M-364 for IκBε (Santa Cruz Biotechnology) or the antibodies against NF-κB subunits described for electrophoretic mobility shift assays. Immunocomplexes were detected with the ECL detection system (Amersham Pharmacia Biotech).

## RESULTS

*Identification of a TGFβ-responsive κB Site in the junB Promoter*—Previously, we and others (39) have shown that *junB* is an immediate early gene induced by members of the TGF-β family. Preliminary experiments, undertaken to identify regulatory elements upstream of mouse *junB* gene, identified a region that confers a slight response to TGF-β family members

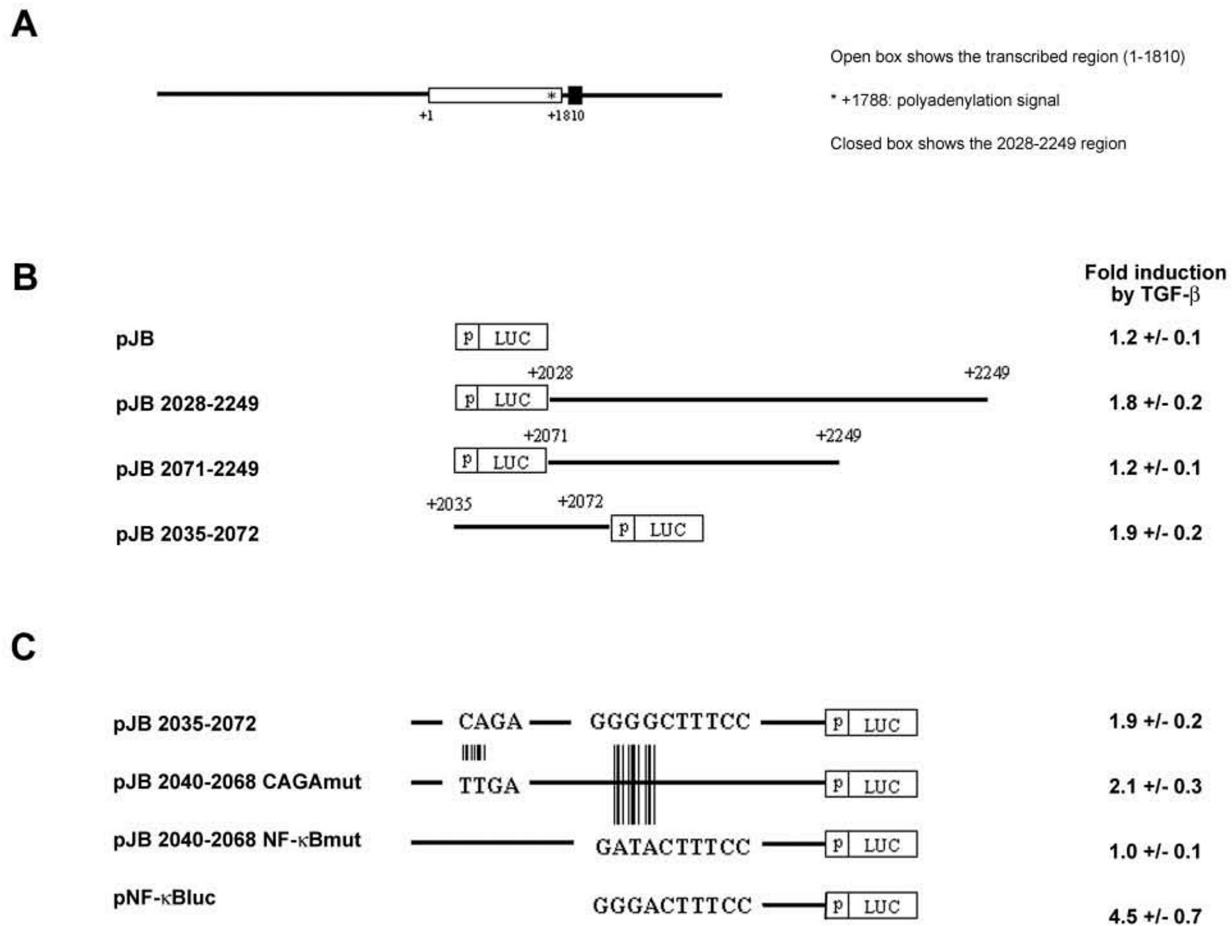
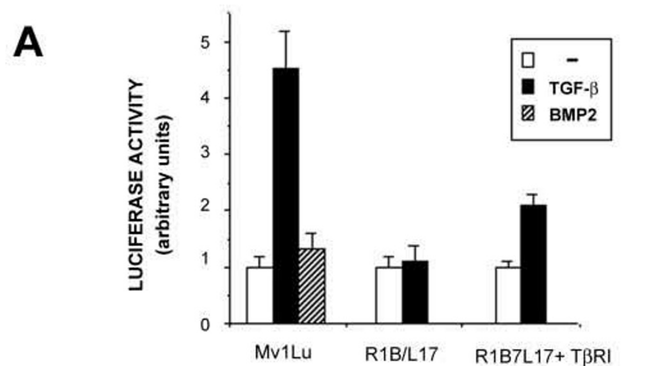


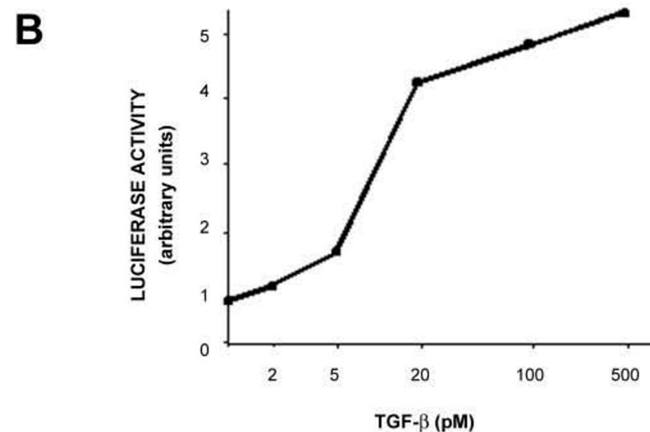

FIG. 1. **Identification of a TGFβ-responsive NF-κB site.** *A,* scheme of the *junB* gene with the location of the 3′-downstream region (*black box*) analyzed in the present report. *B,* analysis of TGF-β response (indicated as fold induction) of partial deletions of the 3′-downstream region and the basal reporter pJB. Mv1Lu cells were transfected with 2 μg/ml of the different constructs. One day later cells were serum-starved and treated with TGF-β, 200 pM, for 16 h. *C,* wild type, mutants for the two DNA motifs (a putative SBE and a NF-κB site) found in the responsive 2035–2072 region and a consensus NF-κB site (present in Igκ or human immunodeficiency virus-long terminal repeat enhancers) were analyzed for TGF-β responsiveness. Mutations on both sites are shown (CAGA to TTGA for CAGAmut and GGGGCTTTCC to GATACTTTCC in NF-κBmut). Data are shown as the mean ± S.E. of triplicates of at least five independent experiments.

(Ref. 39 and data not shown), which might correspond to the responsive enhancer described by Jonk *et al.* (37). We further our study by focusing on the 3′-downstream region of this intronless gene, which has been shown to mediate induction of its expression by serum, growth factors, phorbol esters, or forskolin (35–36). Transfection of PJB 2028-2249-luc, which contains these downstream response elements and the basal *junB* promoter, into Mv1Lu cells elicited a small, but consistent, response to TGF-β (Fig. 1*B*). To localize the minimal effective region, partial constructs were assayed showing that the region from +2035 to +2072 was still fully responsive to TGF-β, whereas the region from +2071 to +2249 did not support significant reporter induction (Fig. 1*B*). Sequence analysis indicates that this responsive region contains both a single SBE and a single κB site. To determine the specific role of NF-κB and SBE sites in the induction of the *junB* expression by TGF-β, we compared constructions containing point mutations of the κB or SBE sites. As shown in Fig. 1*C* mutations of the SBE had no effect on activation by TGF-β, whereas mutations in the κB site completely suppressed reporter inducibility. In addition, a reporter construct containing two consensus NF-κB-binding sites in front of the c-*fos* minimal promoter conferred stronger reporter stimulation by TGF-β (Fig. 1*C*). Thus, these results seem to indicate that a κB site localized at +2054 is sufficient for TGF-β-mediated activation of the *junB* gene in both homologous and heterologous promoter contexts.

*NF-κB Transcriptional Activity Is Rapidly Induced by TGF-β*—To investigate the TGF-β effect through κB sites, we used the reporter driven by κB consensus sequences that confers the strongest response. Whereas TGF-β confers 4–5-fold induction, BMP-2 had no effect on reporter activity in Mv1Lu cells (Fig. 2*A*). To assess whether induction from κB sites required signals initiated by functional TGF-β receptors, we used the Mv1Lu clone R1B/L17, which is unresponsive to TGF-β due to mutations in type I receptor (38). TGF-β only showed effects in R1B cells after reconstitution of type I receptor (Fig. 2*A*). In addition, dose-response experiments showed activation at TGF-β concentrations in agreement with the affinities of TGF-β receptors (Fig. 2*B*).

We also performed electrophoretic mobility shift assays with a radiolabeled NF-κB consensus oligonucleotide incubated with nuclear extracts of cells treated with TGF-β or phorbol dibutyrate (PDB), a known inductor of NF-κB activity. Fig. 2*C* shows that NF-κB binding activity was increased in Mv1Lu cells treated with both factors. However, although the PDB effect was detected after 30 min, TGF-β effect was not detectable until 3 h after growth factor addition. This led us to examine the possibility that TGF-β could induce the release of a cytokine to the extracellular medium, which would be responsible for NF-κB induction. We treated cells with TGF-β and brefeldin A, which is a potent inhibitor of transport of newly synthesized proteins from endoplasmic reticulum to Golgi (40).



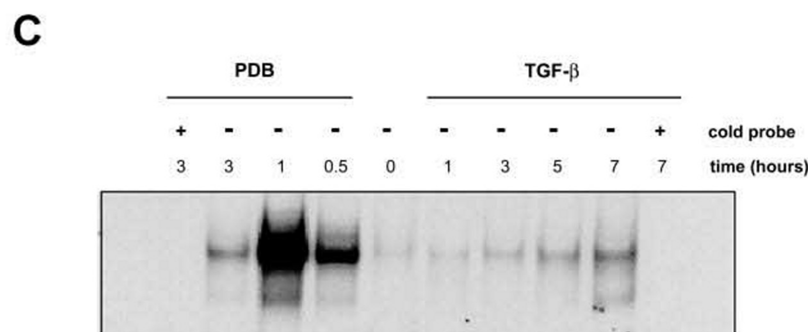




FIG. 2. **TGF-β activates NF-κB transcriptional activity.** R1B/L17 cells, deficient in TGF-β receptor type I, and wild type Mv1Lu cells were transfected with the reporter vector pNF-κB-luc. 24 h later, cells were incubated with TGF-β, 200 pM (*A*), or 2, 5, 20, 100, and 500 pM (*B*) for 16 h, in DMEM supplemented with 0.1% fetal calf serum. Results are shown as the mean ± S.E. of luciferase arbitrary units of triplicates of at least three independent experiments. *C,* nuclear extracts were obtained from Mv1Lu cells treated with or without 100 ng/ml PDB or 1 nM TGF-β in DMEM supplemented with 0.1% serum for different times. Extracts (10 μg of protein) were incubated with $^{32}$P-labeled NF-κB consensus oligonucleotide with or without 20-fold excess of cold probe.

Brefeldin A, either at 1 or 5 μg/ml, had no significant effect on reporter responses for either the κB or the Smad-responsive construct 3TP-lux (Fig. 3*A*).

We then examined the time course of κB reporter induction in further details. As shown in Fig. 3*B*, cells treated with TGF-β or PDB gave the same temporal pattern of induction. A similar pattern was also found with 3TPlux, which binds Smads and confers immediate responses to TGF-β (13). Since the activation profile of either NF-κB or 3TP-lux transcription after PDB or TGF-β addition follows a very rapid pattern, these results suggest that TGF-β induces NF-κB transcriptional activity in an immediate manner.

A number of stimuli increase DNA binding activity through induction of expression of specific NF-κB subunits or by control of their translocation to the nucleus through IκBs. IκBs are phosphorylated by a multiprotein IκB-kinase complex and then ubiquitinated and degraded by the 26 S proteasome (32). To test whether the TGF-β effect proceeds via an increase in expression of NF-κB subunits, we analyzed their expression by Western blotting. No changes were found for either p50, p52, p65, RelB, or c-Rel up to 4 h after TGF-β addition (Fig. 3*C*). To assess the role of TGF-β in IκB degradation, we also analyzed the levels of IκB-α, -β and -ε. Whereas PDB induced a significant decrease in IκB levels, we were unable to detect significant changes caused by TGF-β treatment (Fig. 3*D*). These data suggest that TGF-β increases transactivation activity of NF-κB through a mechanism other than the classic pathway of IκB degradation.

*TGF-β Activation Requires an Intact NF-κB Pathway*—To investigate whether NF-κB activity is required for TGF-β to activate κB sites, we blocked this pathway at two levels. First, we overexpressed a dominant negative mutant of IκB, which

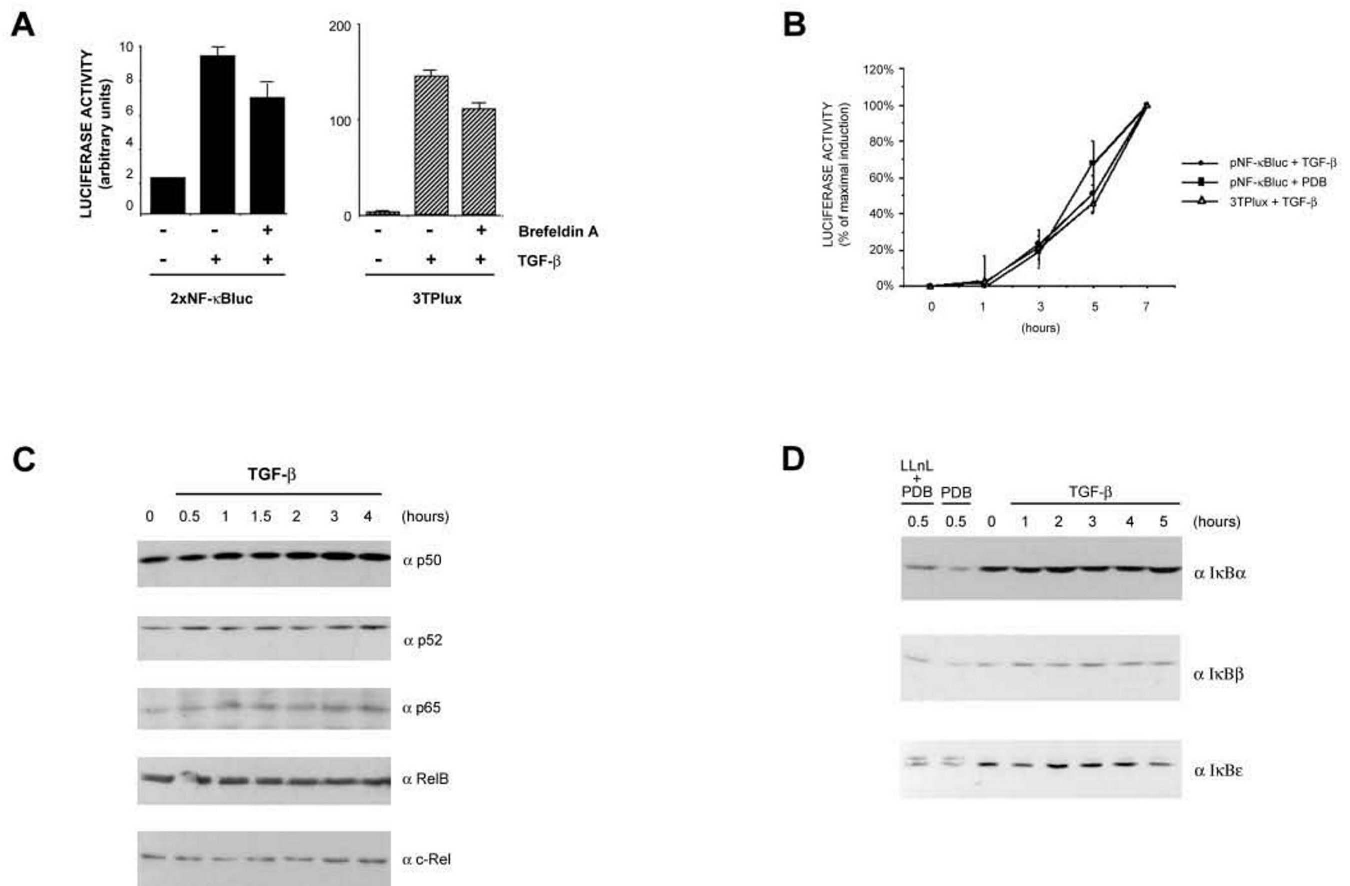



FIG. 3. **Immediate effects of TGF-β do not involve IκB degradation.** *A,* Mv1Lu cells were transfected with pNF-κB-luc or 3TPlux. The day after transfection, cells were treated with or without 200 pM TGF-β and 5 μg/ml brefeldin A as indicated. Results are shown as the mean ± S.E. of triplicates of three independent experiments. *B,* Mv1Lu cells were transfected with the reporter vector pNF-κB-luc. 24 h later, cells were incubated with 200 pM TGF-β for different times, and luciferase activity was assayed. Results are shown as the mean ± S.E. of luciferase activity relative to the induction observed at 7 h after TGF-β addition. *C* and *D,* cell extracts were obtained from Mv1Lu treated for different times with 1 nM TGF-β or 100 ng/ml PDB. Western blot analysis were performed with antibodies against NF-κB subunits (*C*) or IκBs (*D*) as described under "Materials and Methods."

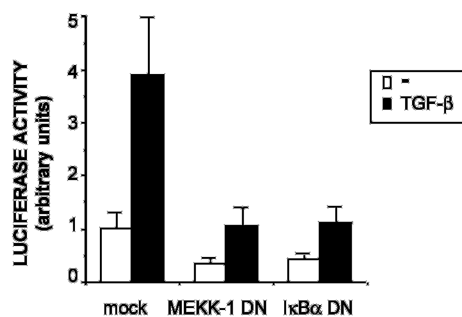
cannot be phosphorylated and thus cannot be recognized by the ubiquitination and degradation machinery. Overexpression of this mutant form significantly reduced both the uninduced and the TGF-β-induced expression of our reporter system (Fig. 4*A*). In addition, we also overexpressed a dominant negative mutant of mitogen-activated protein/extracellular signal-regulated kinase kinase-1 (MEKK-1). MEKK-1 is one of the activators of IKK (41). We found that the induction by TGF-β was also reduced when activation of IKK by MEKK-1 was blocked.

LLnL inhibits the activity of 26 S proteasome and blocks degradation of phosphorylated IκBs (32, Fig. 3*C*). Our results showed that TGF-β did not induce activation of the reporter when cells were treated with the proteasome inhibitor (Fig. 4*B*). There were no differences between control and treated cells when 3TPlux reporter was transfected, suggesting that LLnL has no effect on TGF-β signaling or cell viability. Taken together, these results indicate that TGF-β-induced gene expression from κB binding sites requires NF-κB activity.

*TGF-β Cooperates with Other Inductors of the NF-κB Pathway*—Many stimuli can activate NF-κB, such as inflammatory cytokines, UV irradiation, phorbol esters, and other physiological and non-physiological stimuli. To examine the potential of TGF-β to stimulate NF-κB activity, we studied its effects in combination with PDB. Cells were treated with a fixed dose of PDB and, at the same time, with increasing TGF-β concentrations. Combination of both stimuli had a cooperative effect on reporter gene activity (Fig. 5*A*). When the same assay was performed with a fixed dose of TGF-β and increasing concentrations of PDB, similar enhancement of transcription was obtained (Fig. 5*A*).

During the last few years, several evidences have revealed the implication of the Rho family of small GTPases in signal transduction cascades that activate NF-κB through IκB degradation (42). To investigate the role of these proteins in the TGF-β effect on κB transactivation, we expressed different members of the Rho family and their dominant negative and constitutively active mutants. We also included the GTPase *arf6*, which is involved in vesicle trafficking, as negative control. Fig. 5*B* shows that none of the dominant negative mutants had any significant effect on TGF-β activation. In addition, expression of constitutively active mutants of *rac1* and *cdc42* not only increased basal activation but also strongly enhanced transcriptional induction by TGF-β.

The ability of TGF-β to cooperate with inductors of the NF-κB pathway led us examine which NF-κB subunits were involved in such cooperative effects. First, we preincubated nuclear extracts of cells treated with PDB with antibodies against NF-κB subunits and performed electrophoretic mobility shift assays with an NF-κB consensus probe. As shown in Fig. 6*A*, preincubation of nuclear extracts with antibodies against p52, p65, and RelB revealed a significantly reduced PDB-induced band, whereas no change was obtained with antibodies against p50 or c-Rel. These data suggest that TGF-β cooperates with PDB-induced complexes containing p52, p65 and *rel*B antigens. To assess the functional role of these subunits in transactivation of the NF-κB-driven reporter by



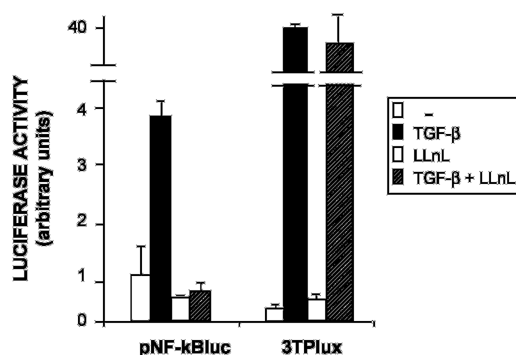




FIG. 4. **TGF-β requires an intact NF-κB pathway.** *A,* Mv1Lu cells were cotransfected pNF-κB-luc in combination with MEKK-1 or IκBα dominant negative mutants or vector alone (*mock*). Luciferase assay was performed 16 h after treatment with 200 pM TGF-β. Results are expressed as mean ± S.E. of triplicates from five independent transfections. *B,* Mv1Lu cells were transfected with pNF-κB-luc or 3TPlux. The day after transfection, cells were split and treated with or without TGF-β and 50 μM LLnL, as indicated. Results are shown as the mean ± S.E. of triplicates of four independent experiments.

TGF-β, we also overexpressed all members of the NF-κB/*rel* family alone and in different combinations. Expression of NF-κB subunits had little effect on basal reporter activity (Fig. 6*B*). More importantly, TGF-β was still able to induce the NF-κB reporter system in cells with ectopically increased levels of NF-κB subunits. In addition, some combinations even potentiated TGF-β responsiveness, especially the combination of p52 and p65/RelA.

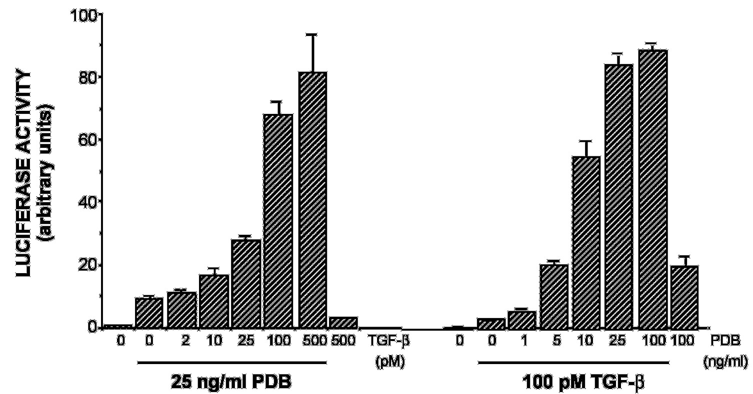
*Involvement of Smads in NF-κB Transcriptional Activation by TGF-β*—Previous studies have shown that, in addition to the regulation of NF-κB by factors which control its nuclear translocation, the specific interaction of NF-κB with other transcription factors is likely to provide an important regulatory step that could determine selective gene activation or repression (30). The above results suggested that Smads, the main transducing molecules of TGF-β signaling, could be involved in such activation. To test this hypothesis, we overexpressed different Smads alone or in combination with NF-κB subunits and analyzed their κB transactivation potential. Smad3, a TGF-β-specific Smad, and Smad4 induced moderate ligand-independent transcription and enhanced responses to TGF-β, whereas Smad1, specific for BMPs, showed no differences compared with control cultures (Fig. 7*A*). Coexpression of p52 or p65 together with Smads strongly increased the basal ligand-independent transcription, which was further enhanced by growth factor addition (Fig. 7*A*). These results seems to indicate that, at least Smad3, Smad4, and p65, p52 might be involved in this specific TGF-β transcriptional activation.

To further examine the involvement of these proteins, we studied the possibility of physical interaction between the NF-κB subunits and the Smads *in vivo*. After overexpression in COS cells, copurification was performed using $Ni^{2+}$-NTA-agarose, followed by Western blotting using anti-Myc antibody. The weak interaction between Smad3 and p52 in the absence of TGF−β was enhanced by cotransfection of constitutively active type I receptor (TβR-I-TD) (Fig. 7*B*). However, in similar assays, we were unable to visualize direct interactions between Smad4 and p52 (data not shown). These results show association of Smad3 and p52 and indicate that nuclear translocation of both Smad3 and p52 may be the key point in the regulation of their transactivation potential.

## DISCUSSION

Previous analysis of the 5′-promoter region of the *junB* gene revealed an SBE that, when multimerized in heterologous promoters, mediates activation by several TGF-β family members (37). However, in the same report, the authors were unable to detect TGF-β inducibility by transient or stable transfection using homologous *junB* promoter constructs. This fact, together with the high level of inducibility of the intact gene, suggested that other responsive elements must be present elsewhere that could mediate full responsiveness in an additive manner. In this study, we identified an immediate-early response element in the 3′-downstream flanking region of this intronless gene. Mutational analysis demonstrated that a single κB site was necessary and sufficient for TGF-β responsiveness both in the minimal *junB* promoter and fused to heterologous reporters. This is consistent with previous results showing TGF-β stimulation of the human immunodeficiency virus 1 enhancer through κB sites or the identification of *schnurri* as a TGF-β superfamily transducer in *Drosophila* (43–44). *schnurri* is homologous to the human MBP-1/2 transcription factors that bind to κB sites, where it has been suggested they may act as transcriptional repressors (45).

We also demonstrated that this rapid activation required NF-κB activity. Either blocking the activators of the pathway or inhibition of degradation of IκB, which results in lower nuclear translocation of NF-κB subunits, diminishes or completely blocks both basal and TGF-β-induced reporter transactivation. Despite this NF-κB requirement, several lines of evidence suggest that this activation differs from the classic mechanisms. First, TGF-β-induced transcriptional activation through κB sites does not involve immediate (up to 3 h) increases in translocation of NF-κB subunits to the nucleus (Fig. 2*C*) or increases in expression of NF-κB subunits (Fig. 3*C*), which is consistent with previous data obtained in HaCaT or HeLa cells (46–47). Second, classical NF-κB signal transduction requires rapid phosphorylation (less than 10 min) and further IκB degradation through the ubiquitin-proteasome pathway to release active NF-κB. Addition of TGF−β does not induce significant disappearance of IκBs or appearance of slower migrating, phosphorylated IκBs in Mv1Lu cells treated with proteasome inhibitors for up to 3 h. Third, although PDB treatment, a known stimulator of IκB kinase, or expression of activated small GTPases promotes reporter activation, TGF−β can augment the response still further (Fig. 5). Similarly, overexpression of NF-κB subunits, alone or in combination, also confers synergy to the TGF-β-induced transactivation activity. Thus, TGF-β signaling seems to converge with the NF-κB pathway at the nuclear level. In this respect, although most of the NF-κB activity remains sequestered by IκBs in the cytoplasm



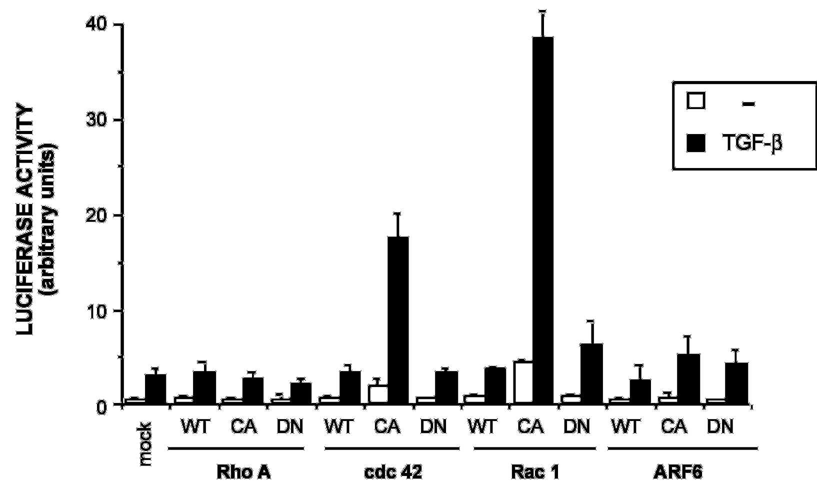
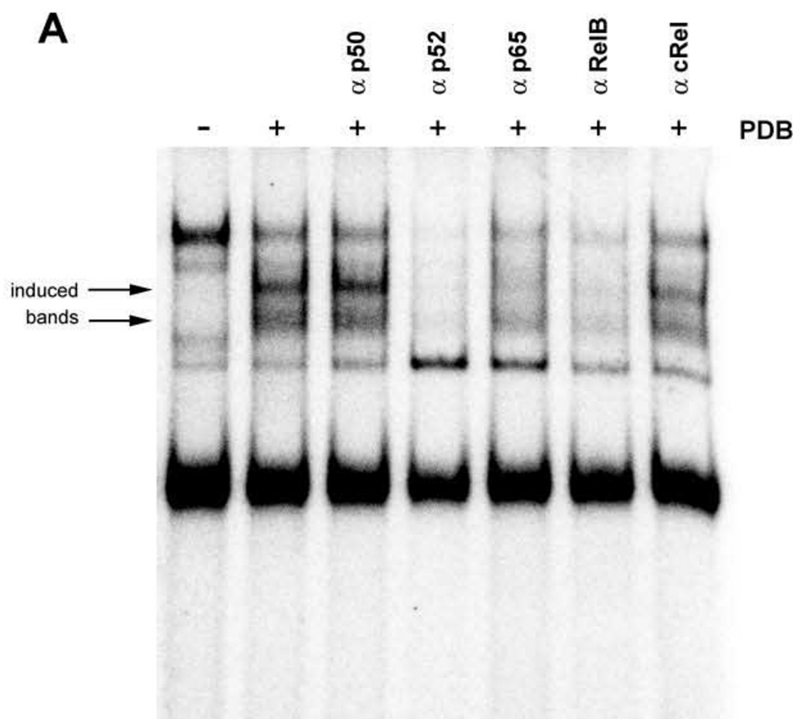




FIG. 5. **TGF-β cooperates with other inductors of the NF-κB pathway.** *A,* Mv1Lu cells were transfected with the reporter pNF-κB-luc. 24 h later cells were split and treated with PDB and/or TGF-β as indicated for 16 h. *B,* Mv1Lu cells were cotransfected with the reporter pNF-κB-luc in combination with different members of the family of small GTPases and their dominant negative (*DN*) or constitutively active (*CA*) forms. *WT*, wild type.

of Mv1Lu cells, it is likely that there is a small constitutive fraction of nuclear activity, as has been described for B cells, corneal keratinocytes, or vascular endothelial muscle cells (30). This small active fraction is likely to be reached by the TGF-β signal, increasing its transactivating activity, whereas signals that increase NF-κB translocation further cooperate in the transcriptional response.

Although we have been unable to detect any binding to κB sites by either bacterially or COS expressed Smad3 or -4 (data not shown), there are still several possible mechanisms to achieve these synergistic effects. It has been postulated that TGF-β may signal some of its cellular responses through activation of members of the mitogen-activated protein kinase family, such as TGF-β activated kinase-1 (TAK-1) or the c-Jun N-terminal kinase (15, 48). These TGF-β-driven kinase pathways could trigger activation of nuclear NF-κB subunits through phosphorylation. However, we did not detect activation of TAK-1 by TGF-β *in vivo,* and overexpression of dominant negative forms of either TAK-1 or SEK-1, an upstream activator of c-Jun N-terminal kinase, did not change reporter inducibility (data not shown). Another possibility would be that physical interactions between Smads and NF-κB subunits are responsible for these cooperative effects. Here, we provide evidence suggesting a role for Smads as transcriptional coactivators through physical interaction with NF-κB subunits. Overexpression of Smad3 and Smad4 enhances transactivation of κB sites in Mv1Lu cells. Moreover, coexpression of Smads together with NF-κB subunits, especially p52 and p65, further increases those responses. We also demonstrate physical interaction of p52 and Smad3 in the absence of DNA, which is enhanced by cotransfection of an active TGF-β receptor. These results are consistent with data reported by Li *et al.* (47) showing that chimeric Gal4-p65 constructs could support TGF-β-dependent activation of a promoter driven by Gal4 DNA-binding sites. Future work is necessary to discern whether interaction upon Smad3 phosphorylation relies on unfolding and increased nuclear localization of Smad3 or whether phosphorylation of C-terminal serines also increases its affinity to bind p52.

These findings add NF-κB subunits to the list of transcrip-



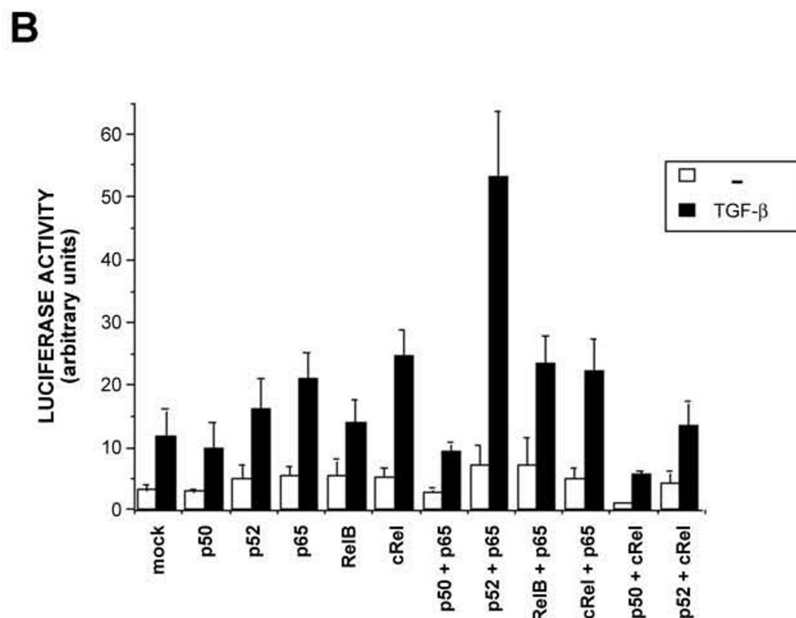
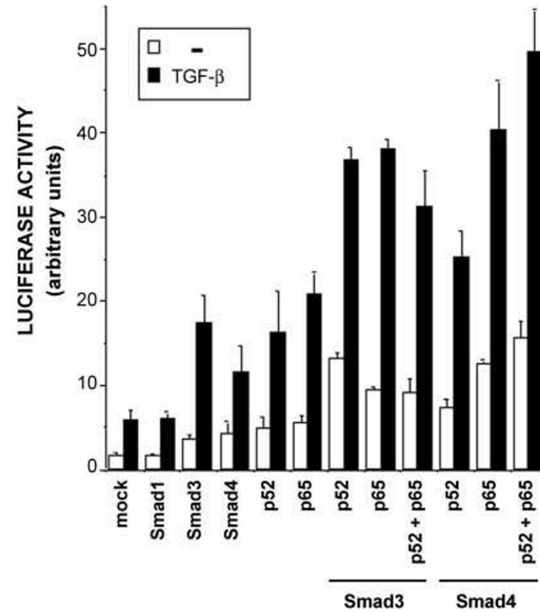




FIG. 6. **Analysis of NF-κB subunits involved in transcriptional activation of NF-κB by TGF-β.** *A,* nuclear extracts were obtained from Mv1Lu cells treated with 100 ng/ml PDB in DMEM supplemented with 0.1% serum. Were indicated, extracts (10 μg of protein) were preincubated with antibodies against NF-κB subunits followed by incubation with $^{32}$P-labeled NF-κB consensus probe. *B,* Mv1Lu cells were cotransfected with the reporter pNF-κB-luc in combination with members of the NF-κB/*rel* family. One day later, transfected cells were incubated with TGF-β 200 pM for 16 h. Results are shown as the mean ± S.E. of triplicates of five independent experiments.

tion factors that physically interact with Smads. Cooperative physical interactions of Smads with transcription factors have been postulated to take place through their binding to adjacent sites on responsive promoters, as described for FAST, TFE3, or Stat3, or through modulation of binding and/or transactivation potential through a single enhancer site, as described for vitamin D receptor, glucocorticoid receptor, AP-1, ATF-2, or Hoxc-8 (23–25, 28–29, 49–50). Data presented here would include NF-κB in the latter case where, without significant changes in NF-κB DNA binding, Smad-dependent enhancement of transcriptional activity requires only κB sites. However, this does not preclude that in the context of other promoters functional cooperativity could rely on separate binding sites. Indeed, cooperativity between Smads and NF-κB has been described in the type VII collagen promoter through two separate SBE and NF-κB enhancers (51).

Besides their interaction with transcription factors, both Smads and NF-κB family members also associate with the closely related transcriptional coactivators p300 and CBP. These coactivators, in addition to their ability to modify the chromatin structure through histone acetylation, interact with several transcription factors through different domains and provide them with a physical link to the basal transcriptional machinery (5–6). The MH2 domain of Smad3 targets the C-terminal domain of both p300 and CBP (52–54). Moreover, the N-terminal domain of p300 and CBP binds to the activation domain of p65 and potentiates its transactivating ability (33). Thus, p300 and CBP could act as a linking module bringing together both transcription factors through separate interaction domains. Involvement of those coactivators is further sup-



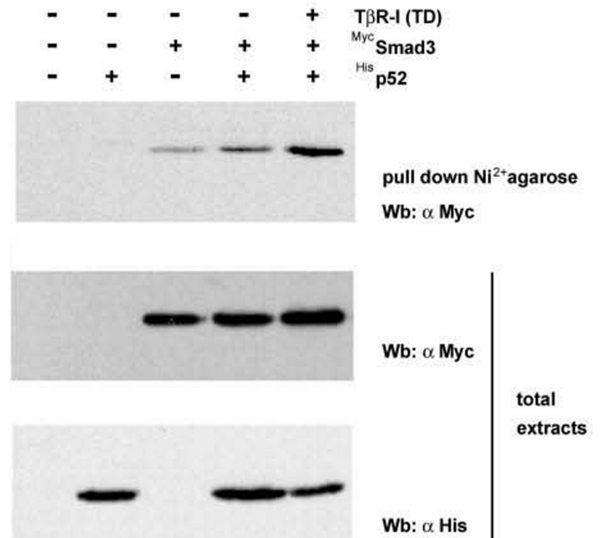




FIG. 7. **Involvement of Smads in NF-κB transcriptional activation by TGF-β.** *A,* Mv1Lu cells were cotransfected with the reporter pNF-κB-luc in combination with Smads and members of the NF-κB/*rel* family. One day later, transfected cells were incubated with 200 pM TGF-β. Results are shown as the mean ± S.E. of triplicates of five independent experiments. *B,* cells were transfected with the indicated combinations of Myc-Smad3, His-p52, and TβR-I(TD). Expression of proteins were determined by direct Western blot (*Wb*) analysis using epitope tags of each protein. Purification in $Ni^{2+}$-NTA-agarose was performed as described under "Materials and Methods" followed by immunoblotting using anti-Myc antibody.

ported by the fact that the viral protein E1A, which binds and sequesters p300 and CBP, has also been reported to block several gene responses to TGF−β. Among them, E1A specifically blocks the induction of *junB* gene expression by TGF−β but not its induction by phorbol esters or serum (55). In addition to their interaction with transcriptional coactivators, Smads could generate transcriptional repressor complexes by binding to factors recruiting histone deacetylases such as TGIF (56) or directly to DNA-binding repressors, such as SIP (27). Thus, the relative levels of Smad coactivators and corepressors would balance the final outcome in transcriptional activity. Therefore, although the interaction between Smads and NF-κB subunits may be essential for control of multiple promoters, whether these interactions result in transcriptional activation or repression of specific targets genes may depend on the cell type. For example, although results presented here in epithelial cells, those reported on collagen VII expression in dermal fibroblasts (51), or in the human immunodeficiency virus-long terminal repeats in HaCaT cells showed cooperative activation (47), most of the effects of TGF−β and cytokines that activate NF-κB are considered to be antagonistic in cells of hematopoietic and lymphoid origin (2).

In conclusion, the present results identified an NF-κB site as a TGF-β-responsive region in the *junB* promoter. We demonstrate that NF-κB sites alone are sufficient to induce immediate transcriptional activation by TGF-β. This activation requires an intact NF-κB pathway and depends on ligand-induced nuclear translocation of Smads where they can associate with NF-κB subunits acting as transcriptional coactivators. These data further expand the notion that Smads undergo

multiple interactions with different transcription factors to induce either activation or repression of gene expression.

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