Interaction and Functional Cooperation of NF-κB with Smads

TRANSCRIPTIONAL REGULATION OF THE junB PROMOTER*

Received for publication, December 13, 1999, and in revised form, June 16, 2000
Published, JBC Papers in Press, June 28, 2000, DOI 10.1074/jbc.M909923199

Teresa López-Rovira‡, Elisabet Chalaux§, Jose Luis Rosa, Ramon Bartrons, and Franches Ventura¶

From the Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, Feixa Llarga s/n, 08907 L’Hospitalet de Llobregat, Spain

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) family of 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 Mxc2 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 6EF1 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).
The NF-κB/Rel family regulates 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 inducers 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 coregulators, in addition to their role as DNA-binding transcription factors.

**Materials and Methods**

**Recombinant Plasmids**

Luciferase reporter plasmids pB2, pJB2025-2249 and pJB2071-2249 (36), were provided by C. Thompson. pJB2035-2072 and pJB2040-2068CAgA mutant and pJB2040-2068NF-B mutant were generated as follows: oligonucleotides 5′-CTGGCCACAGAGTTCGGCGGGCTTTC-CCCCTGCACC-3′ for wild type; 5′-CACTGGATGCCTGGCCCTTTC-CCCCTGCACC-3′ for CAgA mutant; 5′-CACAGAGTGACCTGGCGCTTTC-CCCCTGCACC-3′ for NF-B mutant, and their respective complementary partner sequences were phosphorylated at 5′ ends, annealed, and ligated with pB2, containing the minimal junB promoter–luciferase gene construct, or with pGL-2fos, containing the minimal c-fos promoter. pNF-B-Luc, which contains two NF-κB sites (5′-AGGGGCATCTTTCCGAGAG-3′) in front of the minimal c-fos promoter, MEKK-1DN, and IκBαDN were kindly provided by P. Muñoz, STP-Lux, Smad1, Myc Smad3, and FLAG Smad4 were provided by J. Massagué. Wild type rhoA, cdk-42, 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. Camañero.

**Cell Culture and Transient Transfection**

Mv1Lu cells 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 histidine. Cells were grown in a 10% CO2 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, and treated with 200 μM human recombinant TGF-β1 (Sigma) or 1 μM 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-β1. 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 MgCl2, 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 μl of ice-cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 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 TTC CCC AGG C-3′ (Promega) was labeled with γ-32P ATP and T4 polynucleotide kinase.

Ten microgram 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 RelC 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 × 106 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 IκBα, 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 bromophenol blue).

**Pull-Down**—Cells were washed twice in cold phosphate-buffered saline 1× after transfection and lysed with 50 μl Tris, pH 7.5, 0.5% NaCl, 0.2% Nonidet P-40, 10% glycerol, 0.1 mM dithiothreitol and 0.25 mg/ml poly(dI-dC). Protein complexes were detected with the ECL detection system (Amersham Pharmacia Biotech). IκBα was 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 coregulators, in addition to their role as DNA-binding transcription factors.

**RESULTS**

Identification of a TGF-β-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.
Interaction and Functional Cooperation of NF-κB with Smads

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. 2A). 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. 2A). In addition, dose-response experiments showed activation at TGF-β concentrations in agreement with the affinities of TGF-β receptors (Fig. 2B).

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 inducer of NF-κB activity. Fig. 2C 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).

NF-κB Transcriptional Activity

The NF-κB transcriptional activity was assayed using 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. 1C).

FIG. 1. Identification of a TGF-β-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.

NF-κB transcriptional activity was assayed using 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. 1C). 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.
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. 3A).

We then examined the time course of κB reporter induction in further details. As shown in Fig. 3B, 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 transcriptional activity 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 IκB. IκB 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. 3C). 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. 3D). 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
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. 4A).

In addition, we also overexpressed a dominant negative mutant of mitogen-activated protein/extracellular signal-regulated 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. 3C). Our results showed that TGF-β did not induce activation of the reporter when cells were treated with the proteasome inhibitor (Fig. 4B). 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 Inducers 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. 5A). 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. 5A).

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. 5B 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 inducers 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. 6A, 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 relB antigens. To assess the functional role of these subunits in transactivation of the NF-κB-driven reporter by
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. 6B). 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 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. 7A). Coexpression of p52 or p65 together with Smads strongly increased the basal ligand-independent transcription, which was further enhanced by growth factor addition (Fig. 7A). These results seem to indicate that, at least Smad3, Smad4, and p65, p52 might be involved in this specific TGF-β transactivation 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 Ni²⁺-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. 7B). 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 flank 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. 2C) or increases in expression of NF-κB subunits (Fig. 3C), 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...
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-
transcription 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. 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 32P-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.](image-url)
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 \textit{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 \textit{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

![Graph](image_url)

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

Acknowledgments—We thank Mónica Valls and Esther Adanero for technical assistance and Drs. X. Bustelo, J. Caamaño, J. Donaldson, J. Massague, K. Matsumoto, P. Muñoz, P. ten Dijke, and C. Thompson for gifts of plasmids, antibodies, and reagents. We also thank Genetics Institute for recombinant BMP-2.

REFERENCES

1. Derynck, R., and Feng, X.-H. (1997) Biochem. Biophys. Acta 1333, F105–F150
2. Massague, J. (1999) Annu. Rev. Biochem. 67, 753–781
3. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471
4. Kretzschmar, M., and Massague, J. (1998) Curr. Opin. Genet. & Dev. 8, 133–111
5. Kawabata, M., and Miyazono, K. (1999) J. Biochem. (Tokyo) 125, 9–16
6. Zhang, Y., and Derynck, R. (1999) Trends Cell Biol. 9, 274–279
7. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) Nature 383, 832–836
8. Zhang, Y., Muñoz, C., and Derynck, R. (1997) Curr. Biol. 7, 270–276
9. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626
10. Hata, A., Lagna, G., Massague, J., and Hemmati-Brivanlou, A. (1998) Genes Dev. 12, 186–197
11. Hayasaka, H., Abdelilah, S., Qia, Y., Cai, J., Xu, Y.-Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L., and Falb, P. (1997) Cell 89, 1165–1173
12. Nakae, A., Arafkite, M., Morén, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) Nature 389, 631–635
13. Dennler, S., Itoh, S., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
14. Shi, Y., Wang, Y. F., Jayaraman, L., Yang, H., Massague, J., and Pavletich, N. P. (1998) Cell 94, 585–594
15. Heoervar, B. A., Brown, T. L., and Howe, P. H. (1999) EMBO J. 18, 1345–1356
16. Li, J.-M., Nichols, M. A., Chandrasekharan, S., Xiong, Y., and Wang, X.-F. (1995) J. Biol. Chem. 270, 26750–26753
17. Datto, M. B., Yu, W., and Wang, X.-F. (1995) J. Biol. Chem. 270, 28623–28628
18. Kieu, T.-T., Bag, A., Yamauchi, D., and Mauviel, A. (1996) J. Biol. Chem. 271, 3722–3728
19. Vindevoghel, L., Kon, A., Leechlader, C. R., Wu, C. W., and Kato, S. (1997) Mol. Cell 1, 611–617
20. Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. (1997) Nature 388, 304–308
21. Chen, X., Rubock, M., and Whitman, M. (1997) Nature 383, 691–696
22. Labbé, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) Mol. Cell 2, 109–120
23. Zhou, S., Zawel, L., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Mol. Cell 2, 121–127
24. Zhang, Y., Feng, X.-H., and Derynck, R. (1998) Nature 390, 909–913
25. Liberati, N. T., Datto, M. B., Frederick, J. P., Shen, X., Wong, C., Rougié-Chapman, E. M., and Wang, X.-F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4844–4849
26. Verachueren, K., Remacle, J. E., Collart, C., Kraft, H., Baker, B., Tyznowski, P., Nelés, L., Wuytens, G., Su, M.-T., Bodmer, R., Smith, J. C., and Huylebroeck, D. (1999) J. Biol. Chem. 274, 20489–20498
27. Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Kato, S. (1999) Science 283, 1317–1321
28. Yang, Y., and Derynck, R. (1999) Annu. Rev. Immunol. 14, 649–681
29. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
30. Matzura, T. (1997) Science 278, 818–819
31. Rasmussen, A., Daub, P., Brown, T. L., and Howe, P. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 875–880
32. Zhou, S., Zawel, L., Dai, H., Miyazono, K., and Taga, T. (1998) Science 281, 479–482
33. Tan, R., Kon, A., Lechleider, R. J., Roberts, A. B., and Uitto, J. (1998) J. Biol. Chem. 273, 10831–10834
34. Perez-Albuere, E. D., Schattmann, G., Sanders, L. K., and Nathans, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 96, 11960–11964
35. Hocevar, B. A., Brown, T. L., and Howe, P. H. (1999) J. Biol. Chem. 275, 6129–6135
36. Vindevoghel, L., Kon, A., Lechleider, R. J., Uitto, J., Roberts, A. B., and Uitto, J. (1998) J. Biol. Chem. 273, 112146–112152
37. Chen, Y., and Derynck, R. (1998) Nature 395, 383–386
38. Vindevoghel, L., Kon, A., Lechleider, R. J., Uitto, J., Roberts, A. B., and Uitto, J. (1998) J. Biol. Chem. 273, 3831–3837
39. Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Kato, S. (1999) Science 283, 1317–1321
40. Baldwin, A. S. Jr. (1996) Annu. Rev. Immunol. 14, 649–681
41. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
42. Matzura, T. (1997) Science 278, 818–819
43. Rasmussen, A., Daub, P., Brown, T. L., and Howe, P. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 875–880
44. Zhou, S., Zawel, L., Dai, H., Miyazono, K., and Taga, T. (1999) Science 281, 479–482
45. Yang, Y., and Derynck, R. (1999) Annu. Rev. Immunol. 14, 649–681
46. May, M. J., and Ghosh, S. (1998) Immunol. Today 19, 80–88
47. Matzura, T. (1997) Science 278, 818–819
48. Rasmussen, A., Daub, P., Brown, T. L., and Howe, P. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 875–880
49. Zhou, S., Zawel, L., Dai, H., Miyazono, K., and Taga, T. (1999) Science 281, 479–482
Interaction and Functional Cooperation of NF-κB with Smads: TRANScriptionAL REGULATION OF THE junBPROMOTER
Teresa López-Rovira, Elisabet Chalaux, Jose Luis Rosa, Ramon Bartrons and Francesc Ventura

J. Biol. Chem. 2000, 275:28937-28946.
doi: 10.1074/jbc.M909923199 originally published online June 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909923199

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 27 of which can be accessed free at http://www.jbc.org/content/275/37/28937.full.html#ref-list-1