



Induction of the AP-1 members c-Jun and JunB by TGF- β /Smad suppresses early Smad-driven gene activation

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Smad proteins transduce signals from TGF- β receptors and regulate transcription of target genes. Among the latter are c-jun and junB, which encode members of the AP-1 family of transcription factors. In this study, we have investigated the functional interactions of the Smad and AP-1 transcription factors in the context of Smad-specific gene transactivation in both fibroblasts and keratinocytes. We demonstrate that overexpression of either junB or c-jun prevents TGF- β - or Smad3-induced transactivation of the Smad-specific promoter construct (SBE)₄-Lux. Inversely, Smad-driven promoter transactivation by TGF- β /Smad is significantly enhanced when c-jun expression is abolished in HaCaT keratinocytes, and when junB expression is prevented in fibroblasts, consistent with the cell-type specific induction of jun members by TGF- β . We also demonstrate that Smad-specific gene transactivation in junB^{-/-} mouse embryonic fibroblasts is significantly higher than in embryonic fibroblasts from the control parental mouse line, and that this difference is abolished by rescuing junB expression in junB^{-/-} cells. Finally, we have determined that off-DNA interactions between Smad3 and both c-Jun and JunB result in the reduction of Smad3/DNA interactions. From these results, we provide a model in which jun expression in response to the initial Smad cascade represents a negative feed-back mechanism counteracting Smad-driven gene transactivation. *Oncogene* (2001) 20, 2205–2211.

Keywords: TGF- β ; signaling; Smad; AP-1; Jun; gene expression

Introduction

Transforming growth factor- β (TGF- β) superfamily members (Activin, Bone Morphogenic Proteins, TGF- β s, Decapentaplegic) are multifunctional cytokines, which affect cell proliferation, interactions with the extracellular matrix, and/or survival (Letterio and

Roberts, 1997; Massagué, 1998). The TGF- β s signal via serine/threonine kinase transmembrane receptors which phosphorylate cytoplasmic mediators of the Smad family (Piek *et al.*, 1999; Massagué and Chen, 2000). The ligand-specific Smad1, Smad2, Smad3 and Smad5, interact directly with, and are phosphorylated by, activated TGF- β receptor type I (Derynk *et al.*, 1998). Upon phosphorylation at their SXS carboxy-terminal motif, they form heteromeric complexes with Smad4, a common mediator for all Smad pathways (Derynk *et al.*, 1998). The resulting Smad hetero-complexes are then translocated into the nucleus where they activate target genes, binding DNA either directly or in association with other transcription factors (Massagué and Wotton, 2000). The inhibitory Smads, Smad6 and Smad7, prevent phosphorylation and/or nuclear translocation of receptor-associated Smads (Piek *et al.*, 1999). Interestingly, it has been shown that Smad7 expression may, in certain cell types, represent an autoinhibitory feedback loop downstream of the Smad signaling cascade (Nakao *et al.*, 1997).

The AP-1 family of transcription factors has also been implicated in various gene regulatory activities, either negative or positive, of TGF- β (Kerr *et al.*, 1990; Kim *et al.*, 1990; Mauviel *et al.*, 1993, 1996; Chung *et al.*, 1996; Tang *et al.*, 1998). Modulation of AP-1/Jun expression by TGF- β is a cell-type specific phenomenon, as TGF- β activates c-jun expression only in epithelial cells, whereas it induces junB in mesenchymal cells (Mauviel *et al.*, 1993, 1996). These Jun family members exhibit different functional properties as transcription factors. Specifically, JunB is known to be a weak AP-1 transactivator as compared to c-Jun, and to even antagonize some of the transcriptional activities of c-Jun (Chiu *et al.*, 1989; Schütte *et al.*, 1989; Mauviel *et al.*, 1993; Deng and Karin, 1993).

Following the discovery of Smads as early transducing molecules downstream of the TGF- β receptors, it has been shown that activation of both c-jun and junB promoters by TGF- β involves Smad-dependent mechanisms (Jonk *et al.*, 1998; Wong *et al.*, 1999). These results are consistent with the respective time courses of activation of Smad and Jun proteins by TGF- β . Specifically, using cultured human dermal fibroblasts, we have previously shown that Smad/DNA interactions occur rapidly and maximally within 10 min

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following TGF- β addition, persists at least 1 h, and are no longer detectable 3 h post-stimulation (Vindevooghel *et al.*, 1998a). On the other hand, induction of *c-jun* and *junB* expression occurs after Smad translocation, with maximal mRNA levels observed 1–6 h after TGF- β stimulation (Mauviel *et al.*, 1993, 1996).

In this study, we have examined the role of TGF- β -induced AP-1 expression on Smad-specific gene transcription downstream of TGF- β . We provide evidence for an inhibitory role of AP-1 members *c-Jun* and *JunB* on Smad-specific gene transactivation, a phenomenon which represents a novel suppressive feedback mechanism of Smad signaling.

Results

Overexpression of *junB* or *c-jun* prevents Smad-driven gene transactivation

As a first approach to determine the role played by TGF- β -induced Jun expression on Smad-dependent gene activation, the effect of *junB* and *c-jun* overexpression was examined on the activation by either exogenous TGF- β or by Smad3 overexpression of the artificial Smad-specific construct (SBE)₄-Lux. This reporter consists of four consensus Smad3/4 binding sequences cloned upstream of the SV40 promoter and driving the expression of the luciferase reporter gene (Zawel *et al.*, 1998). As shown in Figure 1, neither *junB* nor *c-jun* alone had an effect on the basal activity of (SBE)₄-Lux, whereas TGF- β induced its activity about threefold. The latter induction was totally prevented by overexpression of either *c-jun* or *junB*. A similar inhibitory activity of *c-jun* and *junB* overexpression was observed on Smad3-driven transactivation of (SBE)₄-Lux (not shown). These results indicate that both *c-Jun* and *JunB*, members of the AP-1 family, directly antagonize Smad3-driven, TGF- β -induced, gene transactivation.

Antisense *c-jun* and *junB* expression enhance TGF- β -induced Smad-specific gene transactivation in human dermal fibroblasts and HaCaT keratinocytes

To examine the role played by endogenous Jun members on Smad-dependent gene activation, antisense *c-jun* and *junB* expression vectors (Mauviel *et al.*, 1993, 1996) were utilized to block the expression of *c-jun* or *junB*, and their activation by TGF- β in either human dermal fibroblast or HaCaT keratinocyte cultures. First, the effect of the antisense vectors was examined on (SBE)₄-Lux. As expected, TGF- β addition led to a significant upregulation of (SBE)₄-Lux in both fibroblasts and HaCaT keratinocyte cultures (Figure 2a,b, respectively). In fibroblasts, antisense *c-jun* expression had little, if any, effect on TGF- β -induced transactivation, whereas antisense *junB* significantly potentiated TGF- β effect (Figure 2a). Inversely, antisense *c-jun* potentiated TGF- β effect in HaCaT keratinocytes whereas antisense *junB* did not (Figure 2b).

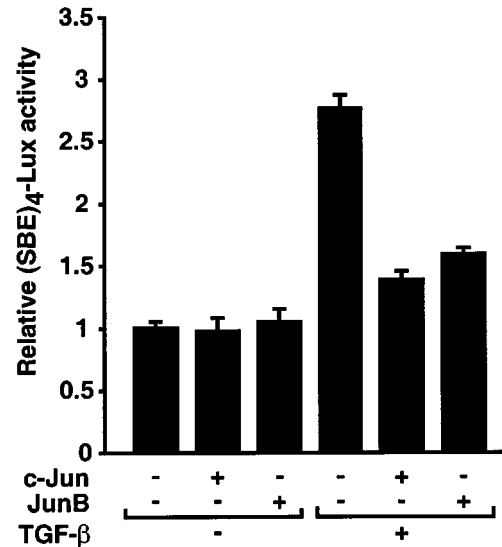


Figure 1 Overexpression of *c-jun* and *junB* prevents TGF- β -induced Smad-dependent activation of (SBE)₄-Lux. Human dermal fibroblasts in late logarithmic growth phase were co-transfected with 3 μ g of (SBE)₄-Lux, with 6 μ g of either pRSV/*junB* or pRSV/*c-jun* expression vectors. Empty pRSVe was used to maintain equivalent amounts of transfected DNA in each plate. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Three hours later, TGF- β (10 ng/ml) was added where indicated and incubations were continued for 24 h, at which time reporter gene activity was determined. Luciferase activity (mean \pm s.d.) of three independent experiments performed in duplicate is shown in the form of bar graphs

From these experiments, it appears that Jun family members directly counteract Smad-specific transcription downstream of the TGF- β receptors in a cell-type specific manner: *c-Jun* plays a major role in epidermal keratinocytes only, whereas *JunB* is a potent inhibitor of Smad-specific gene expression downstream of TGF- β in dermal fibroblasts. These data, indicating differential effects of the antisense vectors used depending on the cell type are consistent with the cell-type specific induction of Jun components by TGF- β . Specifically, we have previously demonstrated that TGF- β selectively induces *junB*, and not *c-jun*, in dermal fibroblasts, whereas it potently enhances *c-jun* expression in epidermal keratinocytes (Mauviel *et al.*, 1993, 1996). It appears, therefore, that *c-jun* activation in keratinocytes, and *junB* activation in fibroblasts likely serve as a negative control mechanism against the rapid Smad-driven gene transactivation.

To test this hypothesis in the context of a natural promoter, we examined the effects of both antisense *c-jun* and *junB* vectors on the upregulation of the human type VII collagen gene (*COL7A1*) promoter by TGF- β , previously identified as a direct Smad target (Vindevooghel *et al.*, 1998a,b). Since *COL7A1* is expressed in both fibroblasts and keratinocytes in the skin (Marinkowich *et al.*, 1993), it represents an ideal target to validate the results obtained using the artificial (SBE)₄-Lux construct. As shown in Figure 2c, antisense *junB* significantly elevated *COL7A1* promoter activation by TGF- β in fibroblasts. Similarly, antisense *c-jun* en-

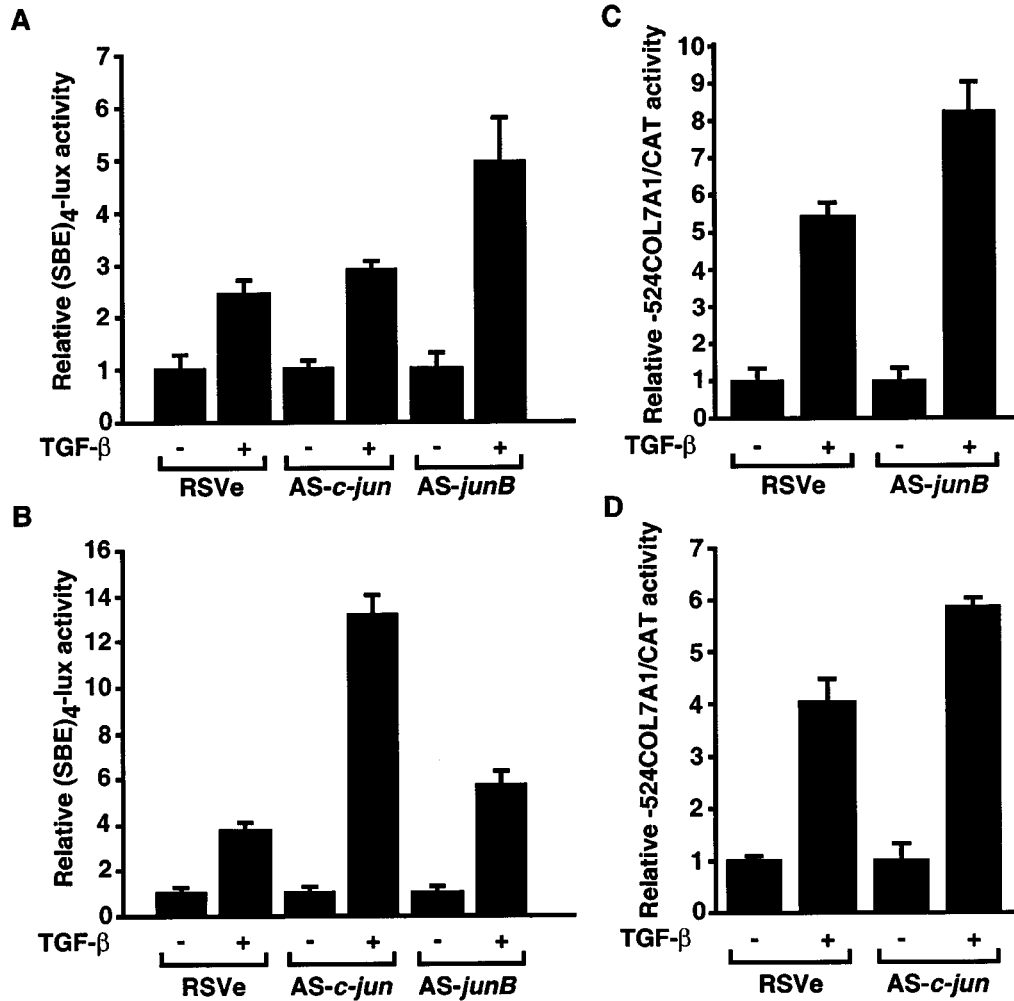


Figure 2 Antisense *c-jun* and *junB* expression vectors enhance TGF- β -induced gene transactivation in a cell-type specific manner. Human dermal fibroblasts (a,c) and HaCaT keratinocytes (b,d) were co-transfected with 3 μ g of either (SBE)₄-Lux (a,b) or -524COL7A1/CAT (c,d) promoter/reporter gene constructs, together with 6 μ g of either empty RSVe vector, AS-*c-jun* or AS-*junB* antisense vectors. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Eighteen hours later, the cultures were treated for 24 h with 10 ng/ml of TGF- β , where indicated. Incubations were continued for 24 h and reporter gene activity was determined. Results represent reporter gene activity (mean \pm s.d.) of three independent experiments performed in duplicate and shown in the form of bar graphs

hanced TGF- β -induced *COL7A1* transactivation in HaCaT keratinocytes (Figure 2d). These results which parallel those obtained with the artificial (SBE)₄-Lux construct (see above), further demonstrate the negative control exerted by Jun family members on Smad-driven gene transactivation downstream of TGF- β .

JunB^{-/-} mouse embryo fibroblasts exhibit increased TGF- β /Smad responsiveness

To further examine the modulatory role played by Jun family members on Smad-driven transcription, the TGF- β responsiveness of wild-type and *junB^{-/-}* immortalized 3T3 cells (Schorpp-Kistner *et al.*, 1999; Szabowski *et al.*, 2000) was measured using (SBE)₄-Lux as a Smad target construct. As shown in Figure 3, transactivation by TGF- β of (SBE)₄-Lux in *junB^{-/-}* fibroblasts was twice that observed in wild-type

fibroblasts. The difference is consistent with the observations made in normal human fibroblasts in which blockade of *junB* expression resulted in similarly increased Smad-dependent transcriptional response (see Figure 2a,c). Rescue of *junB* expression by transfection of a *junB* expression vector into *junB^{-/-}* fibroblasts strongly inhibited TGF- β response of (SBE)₄-Lux, to levels identical to those observed in the wild-type fibroblasts, confirming that lack of *junB* expression is responsible for the increased Smad-dependent gene response in *junB^{-/-}* fibroblasts.

c-Jun and JunB physically interact with Smad3 in solution and reduce Smad/DNA interactions

To investigate the mechanisms by which Jun proteins antagonize Smad-mediated gene transactivation, we first examined the possibility that Smad3 and Jun

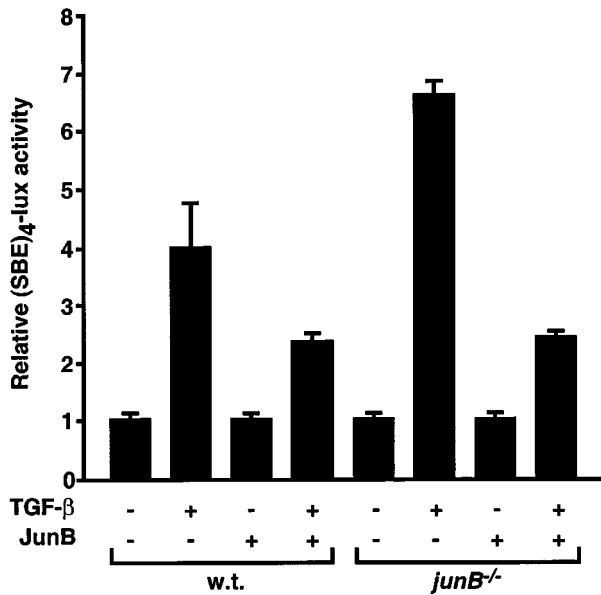


Figure 3 *junB*^{-/-} fibroblasts exhibit increased TGF-β/Smad responsiveness. Wild-type (w.t.) and *junB*^{-/-} mouse embryonic fibroblasts were transfected in parallel with (SBE)₄-Lux, in the absence or presence of pRSV-*junB* expression vector. After glycerol shock, the cells were placed in medium supplemented with 1% FCS. Three hours later, the cultures were treated for 24 h with 10 ng/ml of TGF-β, where indicated. Luciferase activity (mean ± s.d.) of three experiments performed in duplicate is shown in the form of a bar graph

proteins undergo protein-protein interactions. To this end, 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 antibody, followed by Western blot analysis with anti-Myc and anti-HA antibodies. As shown in Figure 4a, both Smad3-Myc and c-Jun-HA were efficiently expressed in our experimental system (lane 1). When Smad3-Myc was expressed in COS-7 cells, it was efficiently immunoprecipitated by the anti-Myc antibody (lane 2). As expected, the latter antibody did not, however, immunoprecipitate overexpressed c-Jun-HA (lane 3). When both c-Jun-HA and Smad3-Myc were co-expressed, immunoprecipitation of Smad3-Myc led to co-precipitation of c-Jun-HA (lane 4), indicating direct c-Jun/Smad3 interaction. Alternatively, COS-7 cells were transfected with Smad3-Flag and JunB-Myc expression vectors. In the latter case, co-immunoprecipitation of Smad3-Flag with JunB-Myc was observed (not shown). These data indicate that c-Jun and JunB share the capacity to form heterocomplexes with Smad3 off-DNA, corroborating recent findings (Zhang *et al.*, 1998; Wong *et al.*, 1999; Liberati *et al.*, 1999).

We next tested the ability of c-Jun and JunB 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 Smad-specific 3 × CAGA probe (Dennler *et al.*, 1998). *In vitro* transcribed c-Jun

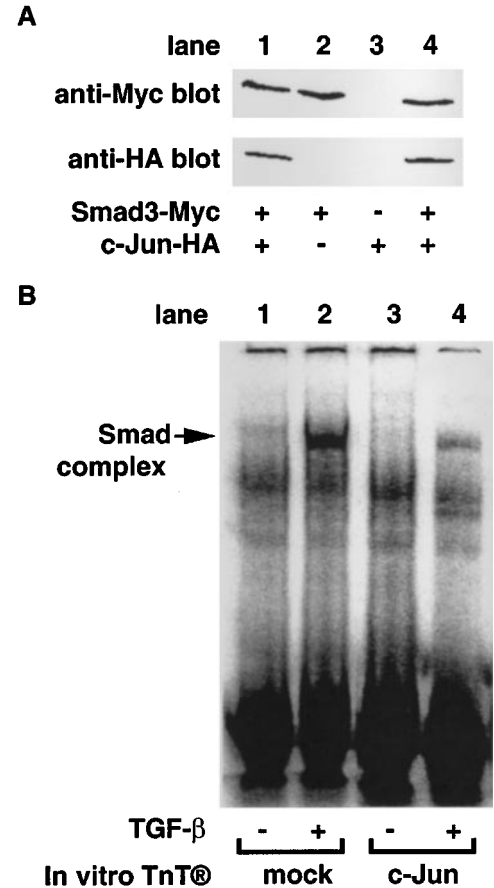


Figure 4 Off-DNA Smad3/c-Jun interactions reduce Smad3/DNA complex formation. (a) COS-7 cells were transfected with an activated TGF-β type I receptor expression vector together with Smad3-Myc and c-Jun-HA expression vectors. 40 h later, cell extracts were immunoprecipitated with an anti-Myc antibody (lanes 2, 3 and 4) or not (lane 1). The immunoprecipitates were separated electrophoretically in 10% acrylamide/SDS gels and immunoblotted with anti-HA or anti-Myc antibodies, as described in Materials and methods. (b) Electrophoretic mobility shift assays were performed using the Smad3/4-specific 3XCAGA oligonucleotide (26) 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 (lanes 3 and 4), as indicated. Mock TnT (with empty vector) reaction mix was used in lanes 1 and 2

or JunB were added to the binding reactions and their effects on TGF-β-induced Smad/DNA complex formation was determined. As shown in Figure 4b, TGF-β induced the formation of a unique Smad/DNA complex (lane 2 vs lane 1), consistent with our previous observations (Vindevooghel *et al.*, 1998a,b). 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). A similar reduction in Smad/DNA complex formation was observed when JunB was added to the Smad/DNA binding reaction instead of c-Jun (not shown).

These experiments suggest that Smad3/Jun interactions in solution 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.

Discussion

It has been shown previously that induction of c-Jun and JunB components of the AP-1 family of transcription factors by TGF- β occurs downstream of the initial Smad cascade, as Smad-specific *cis*-elements are required for TGF- β -dependent activation of both *c-jun* and *junB* promoters (Jonk *et al.*, 1998; Wong *et al.*, 1999). In this report, we have evidenced that secondary *jun* expression downstream of Smad signaling functions as a mechanism of suppression of Smad-specific gene transactivation. A schematic representation of the model is provided in Figure 5. The model finds its bases in (i) the kinetics of Smad/DNA interactions and (ii) the kinetics of *jun* genes activation by TGF- β , as we determined previously (Mauviel *et al.*, 1993; 1996; Vindevoghel *et al.*, 1998a). It is supported by the following new observations: first, expression of an antisense *junB* vector in fibroblasts, and of an antisense *c-jun* vector in keratinocytes, respectively, leads to enhanced response of a Smad-specific reporter construct to TGF- β . These results were corroborated by experiments using *junB*^{-/-} MEFs which exhibited significantly higher responsiveness to TGF- β /Smad transactivation than parental control MEFs. Also, antisense *junB* expression in fibroblasts, and that of antisense *c-jun* in keratinocytes, potentiated the effect of TGF- β on the human *COL7A1* promoter, previously identified as a Smad target (Vindevoghel *et al.*, 1998a,b). Secondly, using immunoprecipitation/Western blotting techniques, we have demonstrated the existence of direct protein-protein interactions between Smad3 and Jun family members. Thirdly, we have shown that Jun proteins have the ability to decrease Smad/DNA complex formation. The latter mechanism likely results in decreased transcriptional activity from Smad-specific *cis*-elements. These results are in agreement with the demonstration that interactions between Smad3 and Jun proteins occur between the MH1 domain of Smad3 and a 20 amino acids region close to the leucine zipper portion of c-Jun and JunB (Zhang *et al.*, 1998; Liberati *et al.*, 1999). Because the MH1 domain of Smad3 is also its DNA binding domain, it is likely that off-DNA interactions with Jun proteins are not compatible with simultaneous DNA binding of Smad3, consistent with both our observations and with the deductions made from the crystal structure of the MH1 domain of Smad3 (Shi *et al.*, 1998).

In addition to preventing Smad3/DNA interactions, another possible mechanism for the inhibitory effect of Jun proteins on Smad-specific transcription could be that they sequester p300, a known transcriptional co-activator for both Smads and Jun proteins (Kamei *et al.*, 1996; Janknecht *et al.*, 1998; Feng *et al.*, 1998; Shen *et al.*, 1998), whose availability within the nucleus is limited (Kamei *et al.*, 1996; Hottiger *et al.*, 1998). Such

sequestration of p300 by Jun family members would likely result in reduced Smad3-dependent transcription (Verrecchia *et al.*, 2000). Similarly, competition for p300 has been proposed to explain the antagonistic effects of E1A and RelA on c-Jun- and Smad3-mediated transcription (Lee *et al.*, 1996; Maggirwar *et al.*, 2000).

In conclusion, this is the first demonstration for a role of Jun/AP-1 family members as suppressors of Smad-dependent gene transactivation in the context of a regulatory feedback loop controlling initial transcriptional responses to TGF- β . This phenomenon is complementary to the previously described mechanism by which TGF- β may block Smad signaling via induction of inhibitory Smad7 (Nakao *et al.*, 1997). The latter prevents accessibility of the TGF- β receptors to substrate Smads, and prevents translocation of Smad3/Smad4 complexes into the nucleus (Nakao *et al.*, 1997; Piek *et al.*, 1999), whereas the mechanism of suppression exerted by Jun proteins appear to occur directly at the level of Smad/DNA interactions, as described in this study.

Materials and methods

Cell cultures

junB^{-/-} mouse embryos fibroblasts (MEFs), obtained from the outgrowth of 9-day-old embryos in which targeted disruption of the *junB* gene has been performed (Schorpp-Kistner *et al.*, 1999), and wild-type MEFs were immortalized according to the 3T3 protocol (Schreiber *et al.*, 1995). Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskins, HaCaT keratinocytes (Boukamp *et al.*, 1988) and MEFs 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 μ g/ml streptomycin-G and 0.25 μ g/ml FungizoneTM). Human recombinant TGF- β 1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). It is referred to as TGF- β throughout the text.

Plasmid constructs

(SBE)₄-Lux (Zawel *et al.*, 1998) was a kind gift from Dr Scott Kern, Johns Hopkins University, Baltimore, MD. For *c-jun* and *junB* expression, we used full-length human cDNAs cloned into pRSVe expression vector (Chiu *et al.*, 1989). The human type VII collagen gene (*COL7A1*) promoter construct 524COL7A1/CAT, a Smad3/4 responsive natural promoter, has been described previously (Vindevoghel *et al.*, 1998a,b). Tagged Smad3, c-Jun and JunB expression vectors, were obtained by subcloning the entire coding sequences into either pCDNA3-Flag, pCDNA3-Myc or pCDNA3-HA (Verrecchia *et al.*, 2000). Antisense *c-jun* and *junB* vectors have been described previously (Mauviel *et al.*, 1993, 1996). Integrity of all constructs was verified by automated sequencing (ABI).

Transient cell transfections and reporter assays

Transient cell transfections were performed with the calcium phosphate/DNA co-precipitation procedure using a commer-

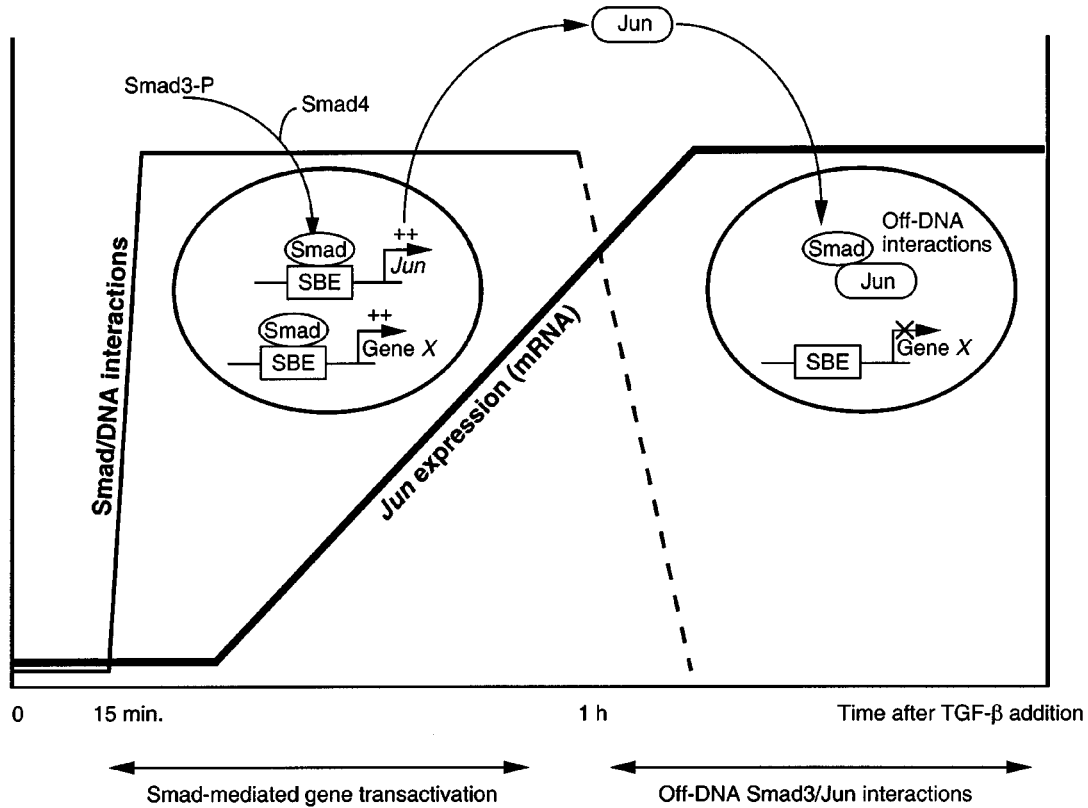


Figure 5 Schematic representation of the inhibitory effect of Jun on Smad-dependent gene transactivation. TGF- β , through interaction with its membrane receptors, rapidly activates the Smad cascade, resulting in Smad/DNA interactions within minutes (Vindevoghel *et al.*, 1998a), leading to activation of direct target genes (X), including *jun* genes (mRNA expression profile deduced from Mauviel *et al.* (1993, 1996)). As a consequence of *jun* gene expression, the initial transactivation of Smad-dependent genes (X) is abrogated by sequestration of Smad3 by Jun proteins which prevents its binding to specific DNA elements

cial assay kit (Promega Corp., Madison, WI, USA). Following appropriate incubation periods (see Figure legends), the cells were rinsed once with phosphate buffered saline, harvested by scraping, and lysed in 200 μ l 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). CAT activity was measured using [14 C]chloramphenicol as substrate, followed by thin layer chromatography. Quantitation was performed with a phosphorimager (Amersham-Pharmacia Biotech, Uppsala, Sweden).

In vitro protein synthesis

T7 promoter-driven transcription and translation of c-Jun-HA and JunB-Flag proteins were performed in a single tube assay (TnT[®], Promega Corp., Madison, WI, USA) according to the manufacturer's protocol.

Electrophoresis mobility shift assays

A 3 \times CAGA Smad-specific oligonucleotide (Dennler *et al.*, 1998) was used as a probe to determine Smad/DNA interactions. Nuclear extracts were isolated using a small

scale preparation (Andrews and Faller, 1991), aliquoted in small fractions to avoid repetitive freeze–thawing, and stored at -80°C until use. Binding mixtures were separated electrophoretically on 4% acrylamide gels in 1 \times Tris-Acetate-EDTA buffer, pH 8.0.

Immunoprecipitations and Western blotting

COS-7 cells were transfected with an activated T β RI expression vector, together with tagged Smad3-Myc and c-Jun-HA expression vectors. 40 h later, cells were solubilized to perform immunoprecipitations and Western analyses using anti-Myc or anti-HA antibodies (Sigma), as described previously (Verrecchia *et al.*, 2000).

Abbreviations

CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; SBE, Smad binding element; TGF- β , transforming growth factor- β .

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