Repression of transforming-growth-factor- β -mediated transcription by nuclear factor κ B

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Activation of transforming growth factor- β (TGF- β) and activin receptors leads to phosphorylation of Sma- and Mad-related protein 2 (Smad2) and Smad3, which function as transcription factors to regulate gene expression. Smad7 is a regulatory protein which is able to inhibit TGF- β and activin signalling in a negative-feedback loop, mediated by a direct regulation by Smad3 and Smad4 via a Smad-binding element (SBE) in the Smad7 promoter. Interestingly, we found that the Smad7 promoter was also regulated by nuclear factor κ B (NF- κ B), a transcription factor which plays an important role in inflammation and the immune response. Expression of NF- κ B p65 subunit was able to inhibit the Smad7 promoter activity, and this inhibition could be reversed by co-expression of I κ B, an inhibitor of NF- κ B. In addition, the inhibitory activity of p65 was observed in a minimal promoter that contained only the Smad7 SBE and a TATA box, without any consensus NF- κ B binding site. This inhibitory effect appeared to be common to other TGF- β - and activin-responsive promoters, since p65 also inhibited the

forkhead-activin-signal-transducer-2-mediated activation of a *Xenopus* Mix.2 promoter, as well as the Smad3-mediated activation of 3TP-lux which contains PMA-responsive elements and a plasminogen-activator-inhibitor-1 promoter. Activation of endogenous NF- κ B by tumour necrosis factor- α (TNF- α) was also able to inhibit the Smad7 promoter in human embryonic kidney 293 cells. In human hepatoma HepG2 cells, TNF- α was able to inhibit TGF- β - and activin-mediated transcriptional activation. Furthermore, overexpression of the transcription co-activator p300 could abrogate the inhibitory effect of NF- κ B on the Smad7 promoter. Taken together, these data have indicated a novel mode of crosstalk between the Smad and the NF- κ B signalling cascades at the transcriptional level by competing for a limiting pool of transcription co-activators.

Key words: activin, p300, signal transduction, Smad7, transcription factor.

INTRODUCTION

Sma- and Mad-related proteins (Smads) are a family of proteins which mediate signalling for the transforming growth factor- β (TGF- β) superfamily of cytokines, including TGF- β , activin, bone morphogenetic proteins, and many others [1,2]. Structural and functional characterization of Smad proteins has allowed their categorization into three groups: pathway-specific, common mediator and inhibitory. Smads 1–3, 5 and 8 are pathway-specific Smads which are phosphorylated by the type I receptors of the TGF- β superfamily [1,2]. Upon phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with Smad4, the common mediator Smad. These complexes then migrate to the nucleus, and regulate gene transcription through either direct DNA binding, via the MH1 domain of the Smad proteins, or association with other specific transcription factors, such as *Xenopus* FAST-1 (forkhead activin signal transducer-1) or mouse FAST-2 [3–6]. The MH2 domain of Smad proteins contains a transactivating activity that is mediated by interaction with two closely related proteins, CREB-binding protein (CBP) and p300, which function as transcriptional co-activators that link specific transcription factors with the basal transcriptional machinery [7–9]. On the other hand, Smad6 and Smad7 constitute the third group of Smads, the inhibitory Smads. These two

proteins inhibit TGF- β , activin and bone-morphogenetic-protein signalling by interacting with the type I receptors of the TGF- β superfamily, or competing for the complex formation between pathway-specific Smads and Smad4 [1,2].

Nuclear factor κ B (NF- κ B) is a transcription factor which controls expression of a wide range of genes [10,11]. It is mainly involved in cellular functions in immunological and inflammatory responses, as well as control of cell growth and apoptosis. For example, NF- κ B is involved in the cellular response initiated by different proinflammatory cytokines, including interleukins and tumour necrosis factor- α (TNF- α) [12]. In mammalian cells, NF- κ B exists in the cytoplasm as a homodimer or heterodimer composed of variable subunits: p50, p52, p65(RelA), c-Rel, and RelB; although the major form of NF- κ B exists as a heterodimer between p50 and p65 [10,11]. Three of these subunits, p65, RelB and c-Rel, contain a C-terminal transactivation domain, and all five subunits have an N-terminal Rel homology domain, which mediates DNA binding, dimerization, and interaction with an inhibitor of NF- κ B (I κ B). The interaction of NF- κ B with I κ B masks a nuclear localization sequence of NF- κ B, and this interaction sequesters NF- κ B in the cytoplasm. Activation of NF- κ B is initiated when I κ B is phosphorylated and degraded in response to inflammatory cytokines, such as TNF- α , UV radiation, viral infection, potent oxidants, or other substances [10,11].

Abbreviation used: CA-ALK, constitutively active activin-receptor-like kinase; CBP, CREB-binding protein; CMV, cytomegalovirus; FAST, forkhead activin signal transducer; HEK-293 cells, human embryonic kidney 293 cells; NF- κ B, nuclear factor κ B; I κ B, inhibitor of NF- κ B; SBE, Smad binding element; Smad, Sma- and Mad-related protein; TGF- β , transforming growth factor- β ; TNF- α , tumour necrosis factor- α .

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These stimuli release NF- κ B and enable it to translocate into the nucleus, where it regulates transcription of target genes. Similar to other transcription factors including Smads, the transactivating activity of NF- κ B is mediated by an interaction with the common transcription co-activators CBP and p300 which contain an intrinsic acetyltransferase activity [13–15]. Upon activation by associating with other DNA-binding transcription factors, CBP/p300 bridge those transcription factors to the basic transcription apparatus, alter the conformation of the chromatin, and initiate stimulation of transcription.

The TGF- β and NF- κ B signalling pathways may crosstalk with each other. For example, TNF- α and TGF- β 1 can synergistically enhance expression of type VII collagen gene that forms part of the extracellular matrix [16]. On the other hand, treatment of B-cell lymphomas with TGF- β 1 can antagonize the activity of NF- κ B by elevating I κ B transcription, leading to accelerated apoptosis by attenuating the NF- κ B-mediated repression of *c-myc* transcription [17]. Conversely, NF- κ B can block TGF- β 1-induced apoptosis of hepatocytes [18]. These observations indicate that there exists an intrinsic interaction between these two signalling pathways that might be dependent on the cellular context, as well as the nature of gene expression.

We recently reported characterization of the mouse Smad7 promoter [19]. We have found that Smad7 transcription is regulated by TGF- β signalling through direct binding of Smad3 and Smad4 with a Smad-binding element (SBE) present in Smad7 promoter [19]. This observation, in conjunction with recent studies demonstrating an upregulation of Smad7 mRNA upon TGF- β or activin treatment in the cells [20,21], suggests that Smad7 participates in a negative-feedback loop which regulates the length and intensity of the TGF- β and activin signalling. Using the Smad7 promoter and other TGF- β -responsive promoters as model systems, we investigated the functional interaction between TGF- β and NF- κ B signalling pathways at the molecular level, and unveiled a novel crosstalk between them at the transcriptional level.

MATERIALS AND METHODS

Cell culture and cell transfection

The human embryonic kidney 293 cells (HEK-293 cells) and the human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum supplemented with penicillin and streptomycin. Transient cell transfection was performed by calcium phosphate method for HEK-293 cells, and DEAE-dextran method for human hepatoma HepG2 cells [19].

Plasmids

The rat Smad3, the constitutively active activin-receptor-like kinase TGF- β type I receptors (CA-ALK-5), the mouse FAST-2, and the Smad7 promoter constructs have been described previously by us [6,19]. To generate the minimal Smad7 promoter containing the SBE of the Smad7 promoter, an E1B TATA box (5'-TATATAAT-3') in adenovirus type 5 [22] was first engineered into the pGL2-basic vector (Promega). Subsequently, two tandem repeats of the SBE (5'-TCGACAGGGTGTCTAGACGCC-ACG-3') of the Smad7 promoter were subcloned immediately 5' to the TATA box. The human NF- κ B p65 subunit, p50 subunit, and I κ B have been described previously [23,24]. The promoter construct containing three tandem repeats of HIV NF- κ B binding sites (NF- κ B3-luc) has been described previously [25,26]. The *Mix.2* AR3-lux containing three tandem repeats of activin-

responsive elements has been described in [6,27]. The 3TP-lux promoter reporter containing three repeats of PMA-responsive elements and a portion of plasminogen activator inhibitor-1 promoter was originally described by Wrana et al. [28].

Promoter assay

For the promoter assay, about 5×10^4 cells per well in six-well plates were transfected with different combinations of plasmid DNA. An empty vector pcDNA3 (Invitrogen) was used to make up to 5 μ g of total DNA for each transfection. The p cytomegalovirus (pCMV)- β -galactosidase plasmid was co-transfected with other plasmids as an internal control for the transfection efficiency. The cells were harvested at 48 h after transfection by lysis with 400 μ l of Nonidet P40 lysis buffer [29]. In TNF- α -treated groups, cells were treated with TNF- α (2 or 20 ng/ml) for 16 h before harvest. A 20 μ l aliquot of the lysate was used in the β -galactosidase assay as reported previously [30], and 10 μ l of the lysate was used in the luciferase assay with a Promega luciferase assay kit, and counted for 10 s in a FB12 luminometer (Zylux) with the data represented as the relative light unit per second.

RESULTS AND DISCUSSION

Inhibition of Smad7 promoter by NF- κ B

Our initial characterization with the mouse Smad7 promoter indicated the presence of a putative NF- κ B regulatory site at the position of +34bp to +41bp, relative to the major transcription initiation site [19]. To determine whether NF- κ B indeed played a role in regulating the transcription of the *Smad7* gene, we tested the effect of expression of NF- κ B p65 subunit on the Smad7 promoter activity. We used three different promoter constructs containing different lengths of the Smad7 promoter fused to a luciferase reporter gene [19]. When transfected into HEK-293 cells, all three promoter constructs gave rise to a high level of basal transactivating activity. As shown in Figure 1(A), the largest reporter construct, spanning -2.2 kb to +112 bp of the Smad7 promoter, exhibited the highest level of basal promoter activity. The second (-408 to +112 bp) and the third (-408 to -124 bp) promoters showed a little lower basal activity. Interestingly, co-expression of p65 with each of these reporters greatly reduced the basal transcription of the Smad7 promoter. In p65 co-transfected cells, the basal luciferase activity was reduced to approx. 10–15% of the control level for all three Smad7 promoter constructs. This finding was somewhat unexpected, because the putative NF- κ B binding site in the Smad7 promoter was not covered by the third reporter that spans -408 bp to -124 bp of the promoter. Therefore these data not only indicated that NF- κ B was able to inhibit the basal Smad7 transcription, but also suggested that this inhibition was unlikely to be caused by the interaction of NF- κ B with the putative NF- κ B consensus site in the region of +34 bp to +41 bp.

In mammalian cells, the predominant form of NF- κ B is composed of a heterodimer of p65 and p50 [10,11]. When p50 alone was transfected in HEK-293 cells, it had no effect on the basal transcription of Smad7 promoter (results not shown). However, co-expression of p50 with p65 gave the similar level of inhibition as p65 expression alone (Figure 1B). Because p65, but not p50, contains the transactivating domain in the NF- κ B family of transcription factors, this finding suggested that the inhibitory effect might be linked to the presence of the transactivating activity of NF- κ B. In order to further test if the inhibitory effect of NF- κ B p65 on Smad7 promoter was specific, we determined whether or not I κ B was able to reverse the p65

Figure 1 Repression of Smad7 promoter by NF- κ B

(A) Effect of NF- κ B p65 on different lengths of Smad7 promoter. HEK-293 cells were transiently transfected with Smad7 promoter constructs (0.5 μ g), pCMV- β -gal (0.5 μ g) and p65 (2 μ g) as indicated. The absolute change of luciferase activity is shown as means $\pm$ S.D. (B) I κ B reverses the repression effect of p65. HEK-293 cells were transiently transfected with Smad7 promoter -408 to -124 construct (0.5 μ g), pCMV- β -gal (0.5 μ g), p50 (1 μ g), p65 (1 μ g) and I κ B (2 μ g) as indicated. The change of luciferase activity shown as means $\pm$ S.D. was derived by comparing to the value of the vector-transfected cells and normalized by β -galactosidase activity.

effect. I κ B is a specific inhibitor of NF- κ B through masking of the nuclear translocation site of NF- κ B. When I κ B was co-expressed with p65, or p65 plus p50, it was able to completely abrogate the inhibitory effect of NF- κ B on the Smad7 promoter. These data, therefore, provided further evidence that the inhibitory effect that we observed with Smad7 promoter was specifically caused by p65.

Figure 2 Effect of p65 on multiple TGF- β -responsive promoters

(A) Repression of the SBE of the Smad7 promoter by p65. HEK-293 cells were transiently transfected with pCMV- β -gal (0.5 μ g), a control promoter containing only the TATA-box (0.5 μ g), a promoter containing two tandem repeats of the SBE and one TATA-box (0.5 μ g), p65 (1 μ g), I κ B (2 μ g), Smad3 (1 μ g), and the constitutively active ALK-5 (1 μ g) as indicated. The fold change of luciferase activity as compared to the vector-transfected cells (set to 1) is shown as means $\pm$ S.D after being normalized by β -galactosidase activity. (B) p65 inhibited transcription of other TGF- β -responsive promoters. A similar experiment as above was performed by transfection with AR3-lux (0.5 μ g), 3TP-lux (0.5 μ g) or the HIV NF- κ B3-luc reporter (0.5 μ g) instead. The mouse FAST-2 (1 μ g) was co-transfected with AR3-lux.

NF- κ B represses the SBE of the Smad7 promoter

As we have shown before, Smad7 promoter contains a consensus SBE that is able to associate with Smad3 and Smad4. To further determine the regulatory role of NF- κ B on the Smad7 promoter, we generated a luciferase reporter that contained two tandem repeats of this SBE and an E1B TATA box [22]. When the control reporter only containing the TATA-box and the SBE-containing reporter were transfected into HEK-293 cells, both of

them gave rise to a very low basal transactivation, as compared with the longer Smad7 promoter constructs used in Figure 1. However, expression of either CA-ALK-5 or Smad3 was able to significantly stimulate the SBE-containing reporter, but not the TATA-only reporter (Figure 2A). These data provided further evidence that the regulatory function of TGF- β and activin signalling through Smad proteins on Smad7 transcription was mediated by this particular SBE present in the Smad7 promoter.

We next determined the effect of p65 on this shorter form of Smad7 promoter. When p65 was co-transfected with the SBE-containing reporter, it could completely reverse the stimulatory effect of either CA-ALK-5 or Smad3 on the reporter (Figure 2A). These data indicated that the inhibitory activity of p65 could overcome the stimulatory effect of CA-ALK-5 or Smad3 on the SBE of the Smad7 promoter. In addition, we also found that I κ B was able to reverse the p65 effect, further indicating that the inhibitory effect of NF- κ B on the SBE was mediated by p65. It is noteworthy that I κ B appeared to augment the stimulatory effect of CA-ALK5 or Smad3 on the SBE transcription. This might be caused by the possibility that the overexpressed I κ B was able to inhibit endogenous p65 and therefore relieve the basal inhibitory effect of the endogenous p65 on the Smad-mediated transcription.

NF- κ B was able to inhibit other TGF- β -regulated promoters

Our experiments so far have indicated that NF- κ B plays a unique regulatory role on the Smad7 promoter. Is this regulatory function only specific for Smad7 promoter, or common to other promoters that are regulated by TGF- β signalling? To address this issue, we tested two other promoters regulated by TGF- β and activin signalling. AR3-lux is a luciferase reporter that contains three tandem repeats of the activin-responsive elements which are regulated by the FAST group of transcription factors when complexed with Smad proteins following activation of TGF- β or activin signalling [3,31]. Previous studies, as well as our own observations, have indicated that a mouse FAST homologue, FAST-2, was able to stimulate AR3-lux following activation of TGF- β or activin signalling [5,6]. 3TP-lux is another reporter that has been commonly used as an indicator of TGF- β or activin signalling. This reporter contains three tandem repeats of PMA-responsive elements fused with the promoter of plasminogen activator inhibitor-1 [28]. Numerous studies have indicated that 3TP-lux could be activated by TGF- β or activin signalling, possibly through binding of Smad proteins with a CAGA motif in the plasminogen activator inhibitor-1 promoter [32], or interaction of Smads with activator protein-1 that binds PMA-responsive elements [33]. As shown in Figure 2(B), expression of Smad3 in HEK-293 cells was able to stimulate AR3-lux in the presence of the mouse FAST-2. Similarly, Smad3 was also able to elevate the transcription of 3TP-lux. However, the stimulatory effect of Smad3 on both of these promoters was largely abrogated by co-expression of p65. These data clearly indicated that p65 has an inhibitory effect on different types of promoters that are regulated by TGF- β or activin signalling. To prove that p65 was indeed functionally expressed in our experimental system, we tested the effect of p65 on another reporter construct that contains three tandem repeats of NF- κ B binding element present in the long terminal repeat of the HIV genome [25,34]. As shown in Figure 2(B), p65 was able to strongly stimulate this promoter, indicating that p65 was indeed expressed and functioning in these cells. Interestingly, Smad3 expression appeared to further augment the stimulatory effect of p65 on this promoter. This is consistent with a recent report demonstrating

that TGF- β is able to stimulate HIV enhancer through the NF- κ B binding element [35].

Inhibition of TGF- β -mediated transcription by TNF- α

NF- κ B is mainly involved in cellular response to inflammatory and immunological stimuli. One of the prototypic pathways in which NF- κ B plays a pivotal role is TNF- α signalling [12]. Activation of TNF receptors by TNF- α is followed by activation of a whole array of signalling pathways, including activation of NF- κ B through phosphorylation and degradation of I κ B. To see if the regulatory role of NF- κ B we observed here is of any biological significance, we determined whether endogenous NF- κ B could mimic the inhibitory effect of ectopically expressed p65 following TNF- α treatment. To test this possibility, we expressed Smad3 in HEK-293 cells, followed by treatment with different amounts of TNF- α . We observed that TNF- α treatment could significantly inhibit Smad3-induced stimulation of the Smad7 SBE reporter in a dose-dependent manner, and that this effect could be reversed with I κ B expression (Figure 3A). HEK-293 cells do express TNF- α receptors, and this TNF- α effect was presumably due to the activation of endogenous NF- κ B, since this repression could be reversed by I κ B. Taken together, these results indicated that activation of endogenous NF- κ B by biological stimuli may impose an inhibitory effect on the TGF- β /activin-responsive Smad7 promoter in HEK-293 cells. It is interesting to note here that the inhibitory effect of TNF- α on Smad3 activation of the Smad7 promoter was unlikely due to an inhibition of the Smad3 expression by p65. Western-blotting analysis showed that the Smad3 protein level was higher in the cells when it was co-expressed with p65 than in cells which were transfected with Smad3 alone (results not shown). This was probably due to the presence of the NF- κ B binding site in the CMV promoter that drove the ectopic expression of Smad3.

To further delineate the biological significance of our observation that p65 was able to inhibit TGF- β -mediated transcription, we examined the effect of TNF- α treatment on TGF- β - and activin-induced transcription of the AR3-lux reporter derived from the *Xenopus Mix.2* promoter in human hepatoma HepG2 cells. This experiment excluded the use of exogenously expressed p65 or Smad proteins in the experimental system. As shown in Figure 3(B), TGF- β and activin treatment were able to stimulate the AR3-lux transcription by approx. 140- and 20-fold respectively. Furthermore, co-treatment with TNF- α was able to inhibit the TGF- β - and activin-mediated transactivation. These data would indicate that the TNF- α signalling pathway is able to inhibit the transactivating activity of endogenous Smad proteins following stimulation by TGF- β or activin.

Overexpression of p300 overcomes the inhibitory effect of p65

What is the molecular mechanism underlying the inhibitory effect of NF- κ B on TGF- β /activin-regulated promoters? Our findings that p65 could inhibit different types of TGF- β or activin-responsive promoters indicated that this effect is unlikely due to the binding of p65 with a specific sequence element in these promoters, since none of these promoters including the Smad7 SBE, the FAST-binding activin-responsive element, and the 3TP-lux, contain the consensus NF- κ B binding element [36]. On the other hand, recent studies have indicated that the MH2 domain of Smad proteins was able to bind the general transcription co-activators CBP and p300 at the C-terminal region to mediate their transactivating activity [7–9]. Interestingly, NF- κ B is also able to bind the N-terminal and C-terminal regions of

Figure 3 TNF- α inhibited TGF- β -mediated transcription

(A) TNF- α inhibited SBE transcription of the Smad7 promoter. HEK-293 cells were transiently transfected with the SBE-containing Smad7 minimal promoter (0.5 μ g), pCMV- β -gal (0.5 μ g), Smad3 (1 μ g) and I κ B (2 μ g) as indicated. The cells were treated with TNF- α 16 h before the luciferase and β -galactosidase assays. The fold change of luciferase activity is shown as means $\pm$ S.D. after normalization with a β -galactosidase assay. (B) TNF- α inhibited TGF- β - and activin-mediated transcription of AR3-lux derived from *Xenopus Mix.2* promoter. Human hepatoma HepG2 cells were transfected with AR3-lux (0.5 μ g), mouse FAST-2 (1 μ g) and pCMV- β -gal (0.5 μ g). The cells were treated with TGF- β 1 (1 ng/ml), activin (10 ng/ml) or TNF- α (20 ng/ml) for 16 h before luciferase assay. The fold change of luciferase activity normalized by β -galactosidase assay is shown as means $\pm$ S.D.

CBP/p300 [13–15]. There have been some observations demonstrating that NF- κ B is able to repress the transactivating activity of other transcription factors through sequestration of a limiting pool of CBP/p300. For example, NF- κ B and p53

Figure 4 Overexpression of p300 overcame the NF- κ B-mediated repression

HEK-293 cells were transiently transfected with the SBE-containing Smad7 minimal promoter (0.5 μ g), pCMV- β -gal (0.5 μ g), Smad3 (1 μ g), p65 (1 μ g) and different amounts of CBP or p300 as indicated. The fold change of luciferase activity was shown is means $\pm$ S.D. after normalization with β -galactosidase activity.

signalling pathways mutually repress each other's transactivating activity by competing for CBP/p300 [37]. NF- κ B can also repress STAT2-mediated HIV transcription through a similar mechanism [38].

To test if such a transcriptional sequestration of CBP/p300 may underlie the inhibitory effect of NF- κ B on TGF- β /activin- or Smad-mediated transcriptional activation, we determined the effect of overexpression of CBP or p300 in our experimental system. As shown in Figure 4, Smad3 expression was able to stimulate the SBE-containing promoter, and the Smad3 effect was inhibited by co-expressed p65. Both CBP and p300 were able to enhance the stimulation of the promoter by Smad3, consistent with previous reports that CBP and p300 are required for the transactivating activity of Smad proteins [7–9]. Interestingly, expression of p300 was indeed able to abrogate the inhibitory effect of p65 on Smad3-activated Smad7 SBE activation in a dose-dependent manner. CBP could also reverse part of the inhibitory activity of p65, although to a lesser degree. Taken together, these data indicated that p65 may inhibit the Smad-mediated transcriptional control by competing for a limiting pool of CBP/p300. When NF- κ B is overexpressed or activated, it may preferentially bind CBP or p300 and limit the amount of these transcription co-activators available to Smad proteins that are activated by TGF- β or activin signalling.

In conclusion, our results have indicated that the NF- κ B-mediated repression of gene expression via sequestration of the common transcription co-activators may play a role in the transcriptional regulation by Smad proteins. However, it is

noteworthy that NF- κ B may repress or stimulate Smad-mediated transcription depending on the promoter configuration. We hypothesize that, in Smad-regulated promoters without NF- κ B binding sites, NF- κ B may repress gene expression by sequestration of CBP/p300, as exemplified in the present study. On the other hand, if the promoters in which Smad-binding element is in close proximity with NF- κ B binding site, NF- κ B may cooperate with Smad proteins to transactivate the target genes, as observed in the transcriptional regulation of the type VII collagen promoter [16]. In certain cell types, TNF- α treatment was able to increase the mRNA level of Smad7, leading to inhibition of TGF- β signalling [39]. How Smad7 transcription is modulated by NF- κ B might be dependent on the cellular context as well as the configuration of the Smad7 promoter. TNF- α may inhibit the TGF- β -mediated transcription by either an upregulation of the inhibitory Smad or a p65-mediated competition for the common transcription co-activators.

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