Repression of Smad2 and Smad3 transactivating activity by association with a novel splice variant of CCAAT-binding factor C subunit

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Activation by transforming growth factor-β (TGF-β)/activin receptors leads to phosphorylation of Smad2 (Sma- and Mad-related protein 2) and Smad3, which function as transcription factors to regulate gene expression. Using the MH2 domain (Mad homologue domain of Smad proteins 2) of Smad3 in a yeast two-hybrid screening, we isolated a novel splice variant of CAATT-binding factor subunit C (CBF-C), designated CBF-Cb, that associated with Smad3. CBF-C is one of the subunits that form a heterotrimeric CBF complex capable of binding and activating the CAATT motif found in the promoters of many eukaryotic genes. CBF-Cb is 62 amino acids shorter than the wild-type CBF-C in the N-terminal region. In addition, CBF-Cb is expressed ubiquitously in various mouse tissues. By an immunoprecipitation assay, we detected an *in vivo* association of CBF-Cb with Smad2 and Smad3, independent of signalling by activated TGF-β type I receptors. In transient transfection experiments, overexpression of CBF-Cb was able to repress the transactivating activity of Smad2 and Smad3, mediated either by direct binding to the Smad-responsive element or through their association with the Smad-interacting transcription factor FAST-2 (forkhead activin signal transducer-2). The Smad-mediated transcriptional response after TGF-β receptor activation was also inhibited by overexpression of unspliced CBF-C. In addition, the repressive activity of CBF-Cb on Smad2- and Smad3-mediated transcriptional regulation was abrogated by co-expression of the general transcription activator p300. The association of CBF-Cb with Smad2 was competitively inhibited by overexpression of p300. These data indicate a novel mechanism for modulation of the transcriptional activity of Smad proteins, whereby the interaction of CBF-Cb, as well as canonical CBF-C, with the MH2 domain of Smads may prevent the association of Smads with transcriptional co-activators.

Key words: activin, CBF, NF-Y, transforming growth factor-β (TGF-β).

INTRODUCTION

The Smads (Sma- and Mad-related proteins) are a family of proteins that mediate signalling for the transforming growth factor β (TGF-β) superfamily of cytokines, which includes TGF-β, activin, bone morphogenetic proteins (BMPs), and many others [1,2]. Both functional and biochemical studies have indicated that Smad2 and Smad3 are activated by TGF-β and activin receptors. On the other hand, Smad1, Smad5 and Smad8 function as signalling proteins downstream of BMP receptors. Upon phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with Smad4, the common mediator Smad. These complexes then migrate to the nucleus and activate gene transcription through either direct DNA binding by the Smad proteins or association with other sequence-specific transcription factors. The DNA-binding ability of Smad proteins is achieved mainly by their MH1 domain (Mad homologue domain of Smad proteins 1), as indicated by the crystal structure of the Smad MH1 domain [3]. For example, Smad3 and Smad4 have been shown to be able to associate with a consensus palindromic motif, GTCTAGAC [4]. The MH1 domains of *Drosophila* Mad and mammalian Smad1 and Smad5 could associate with a GC-rich motif [5,6]. Smad proteins may also stimulate transcription by associating with other sequence-specific transcription factors. For example, Smad2 and/or Smad3, when complexed with Smad4, may associate with *Xenopus* FAST-1 (forkhead activin signal transducer-1) to regulate *Mix.2* gene transcription, or with mouse FAST-2 to regulate the goosecoid promoter [7,8]. The transactivating activity of Smad proteins is achieved by their MH2 domain, which interacts with two closely related transcriptional co-activators, CAATT-binding factor (CBF) and p300, that link specific transcription factors with the basal transcriptional machinery [9–11].

In addition to transcriptional activation activity, Smad proteins may function as transcriptional repressors by different mechanisms. In the absence of TGF-β signalling, the proto-oncoproteins SnoN and c-Ski are able to associate with Smad2, Smad3 and Smad4 and prevent the transcription-activating activity of Smads in the nucleus [12,13]. Phosphorylation of Smad proteins by TGF-β signalling causes rapid degradation of SnoN and c-Ski and relieves their repressive activities [14]. Furthermore, c-Ski may also repress Smads directly by recruiting the transcriptional repressor N-CoR that is associated with the histone deacetylase (HDAC) complex [12]. The involvement of HDAC in Smad-mediated transcriptional repression is further exemplified by the finding of the association of Smad2 with a homeodomain protein TG-interacting factor (TGIF) in a TGF-β-inducible manner [15]. This interaction recruits TGIF to Smad-responsive elements and leads to transcriptional repression, partly due to in the interaction of TGIF with HDAC. Interestingly, the MH1 domain of Smads may interact directly with HDAC,

Abbreviations used: ALK, activin receptor-like kinase; ARE, activin-responsive element; BMP, bone morphogenetic protein; CA-ALK5, constitutively active ALK5; CBF (or NF-Y), CAATT-binding factor; CBF-C, C subunit of CBF; CBF-Cb, splice variant of CBF-C; FAST, forkhead activin signal transducer; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HEK293, human embryonic kidney 293; HDAC, histone deacetylase; MH domain, Mad homologue domain of Smad proteins; P/CAF, p300/CREB-binding protein (where CREB is cAMP response element-binding protein); RT-PCR, reverse transcription–PCR; SBE, Smad-binding element; Smad, Sma- and Mad-related protein; TGF-β, transforming growth factor-β; TGIF, TG-interacting factor.

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indicating further the importance of HDAC in Smad-mediated transcriptional repression [16]. In addition, the transcription-repressing activity of Smad proteins may be mediated by interaction with other repressors. Smad-interacting protein 1 (SIP1), a two-handed zinc-finger/homeodomain protein, is able to associate with Smads and repress their transcriptional activities [17]. On the other hand, Smad6 can associate directly with Hoxc-8/Hoxa-9 to repress Smad1-mediated BMP signalling in the osteopontin promoter [18].

CBF (also known as NF-Y) is a heteromeric complex composed of three subunits, CBF-A, CBF-B and CBF-C, that specifically binds the CCAAT sequence motif in the promoter and enhancer of many eukaryotic genes [19–21]. CBF is expressed ubiquitously in different tissues, and all three subunits are required for specific DNA binding and transcriptional activation [22]. The transcriptional activity of the complex is mediated by two activation domains, one in CBF-B and the other in CBF-C, both of which are able to interact with the histone acetyltranferase P/CAF (p300/CREB-binding protein, where CREB is cAMP response element-binding protein) [23] and with the co-activator dTAFII110 of the *Drosophila* TFIID complex [24]. The transactivating activity of this complex in mammalian cells is due partly to the interaction of CBF-B with the general transcriptional co-activator p300 [25].

In order to further delineate transcriptional regulation by Smad proteins, we used the MH2 domain of Smad3 as bait in yeast two-hybrid screening. As a result, we isolated a novel splice variant of CBF-C, designated CBF-Cb, and found that it is able to repress the transcriptional activities of the Smad proteins downstream of TGF-β signalling. Our data also suggested that the repressive activity of this CBF-C variant is likely to be due to it preventing association of the MH2 domain of Smad proteins with the general transcription co-activator p300.

MATERIALS AND METHODS

Cell culture, cell transfection and promoter assay

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum supplemented with penicillin and streptomycin. Transient cell transfection was performed by the calcium phosphate method for HEK293 cells [26]. Luciferase and β-galactosidase assays with the transfected cells were performed as described previously [27].

Yeast two-hybrid screening

A DNA fragment encoding the final 100 amino acids of rat Smad3 protein (residues 326–425) was generated by PCR and cloned into the bait vector pGBKT7 (Clontech) in-frame with the Gal4 DNA-binding domain. A mouse brain Matchmaker cDNA library in pACT2 was screened with the bait according to the manufacturer's instructions (Clontech). The isolated mouse CBF-Cb clone was sequenced completely from both strands with an automatic DNA sequencer (PerkinElmer-ABI).

Plasmids

The Smads and the constitutively active TGF-β type I receptor [constitutively active ALK5 (CA-ALK5), where ALK is activin receptor-like kinase] and the TGF-β-responsive Smad-binding element (SBE) luciferase reporter (SBE-luc) used here have been described previously by us [26–28]. Flag-epitope-tagged CBF-Cb was generated by cloning the coding region of isolated CBF-Cb into a pRC/CMV vector (Invitrogen) with a Flag tag sequence at the 5′ end (G. Gaudriault, personal communication). The first ATG of CBF-Cb was removed during the cloning so that transcription would start from the KOZAK sequence in the front of the Flag tag.

Reverse transcription–PCR (RT-PCR)

Total RNA from mouse tissues and from mouse breast cancer 4T1 cells was isolated using the GenElute Mammalian Total RNA Kit (Sigma). The RNA was treated with RNase-free DNase I (Gibco BRL) to remove the residual DNA, and then reverse transcribed with an oligo(dT) primer using the Superscript Preamplification System (Gibco BRL) to generate first-strand cDNA. The products were diluted and used in PCR with oligonucleotide primers to detect the expression of two isoforms of CBF-C and of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), using *Taq* DNA polymerase (Promega). The sequences of the PCR primers were as follows: 5′-AGCAGCAGTGATGCCCAGCAAA-3′ and 5′-CGCTTTGGAGGTTTCAGTTCATC-3′ for CBF-C; 5′-GTCTTCACCACCATGGAGAAGG-3′ and 5′-ATGAGGTCCACCACCCTGTTGC-3′ for G3PDH. The PCR products were separated on 3 % (w/v) agarose gels and visualized by staining with ethidium bromide. In addition, RT-PCR was used to isolate the cDNA clone of the unspliced form of CBF-C with two primers that cover the entire coding region of mouse CBF-C. The CBF-C coding sequence was then cloned into pRC/CMV for expression studies. The sequence integrity of the cloned mouse CBF-C was confirmed by sequencing with an automatic DNA sequencer.

Immunoprecipitation and immunoblotting assays

For co-immunoprecipitation, the cell lysate after transfection was incubated with 9E10 mouse anti-Myc antibody (1 μg/ml) and with rabbit anti-mouse antibody (2 μg/ml) in the presence of Protein A–Sepharose (Pharmacia). After incubation at 4 °C for 4 h, the Sepharose beads were washed three times with 1 × lysis buffer and separated by SDS/8 %-PAGE, as described previously [28]. The protein was then transferred to a nitrocellulose membrane and blotted with 9E10 anti-Myc antibody or M2 mouse anti-Flag antibody (Sigma), followed by incubation with a secondary horseradish peroxidase-conjugated antibody (Bio-Rad), and detected by Chemiluminescence Reagent Plus (NEN). For Western blotting, the cell lysate was separated on SDS/8 %-PAGE and detected by the M2 anti-Flag antibody.

RESULTS

Isolation by yeast two-hybrid screening of a novel CBF-C that interacts with the MH2 domain of Smad3

Using yeast two-hybrid screening, we set out to characterize proteins that may interact physically with the MH2 domain of Smad3. The final 100 amino acids of Smad3 were fused with the Gal4 DNA-binding domain, and the fusion protein was used as bait in the screening. This portion of Smad3 comprises approximately the C-terminal two-thirds of the MH2 domain, since the full-length MH2 domain of Smads has transactivating activity when fused with the Gal4 DNA-binding domain [29]. When this Smad3 MH2 bait was transformed into yeast, it was not able to activate Gal4-controlled gene expression (results not shown). Approx. 1×10^6 independent clones from a mouse brain cDNA library were screened with the bait, and 22 positive clones were isolated and sequenced. Partial sequencing indicated that one of the positive clones was identical with CBF-C [30], one of the subunits that form the heterotrimeric CBF (NF-Y), and was therefore subjected to further characterization.

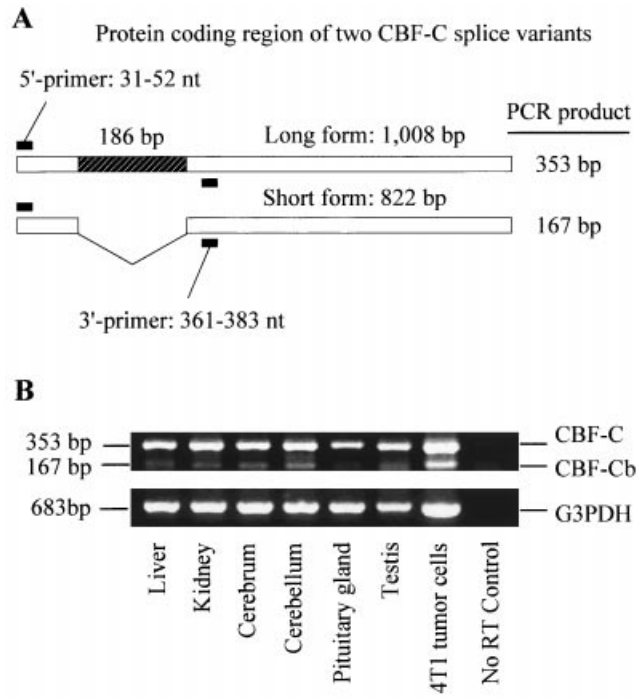

Figure 1 Structure and tissue expression of CBF-Cb

(**A**) Structure of CBF-Cb (short form) as compared with that of wild-type CBF-C (long form). The positions of the primers used to detect the splice variant (CBF-Cb) are illustrated. (**B**) Expression of CBF-C and CBF-Cb in different mouse tissues and a mouse cell line. Total RNA from mouse tissues and cells was prepared and used in RT-PCR to determine full-length CBF-C and the splice variant. The PCR products were separated on a 1.5% (w/v) agarose gel and stained with ethidium bromide. The molecular sizes of the PCR products are indicated on the right. The same RNA samples were also used in RT-PCR with G3PDH-specific primers to normalize the amount of RNA used in the reaction.

The CBF-C clone isolated from the yeast two-hybrid screening contained the full-length cDNA sequence. Further sequencing analysis, however, found that this clone is probably a splice variant of CBF-C, designated here as CBF-Cb. The CBF-Cb cDNA clone encodes a 273-amino-acid protein that is 62 amino acids shorter than wild-type CBF-C; it lacks residues 36–97 of CBF-C, as shown in Figure 1(A). In order to confirm if CBF-Cb is indeed expressed in mouse tissues, we applied an RT-PCR experiment to determine the expression and relative levels of the long form (CBF-C) and the short form (CBF-Cb). Two oligonucleotide primers outside the splice region found in CBF-Cb were used in the experiment, with total RNA isolated from different mouse tissues and a mouse cell line. The predicted PCR product from CBF-Cb was 186 bp shorter than that of CBF-C. As shown in Figure 1(B), we detected the presence of the short form of CBF-C in various mouse tissues, as well as in the mouse 4T1 breast cancer cell line [31]. It appears that both CBF-C and CBF-Cb are ubiquitously expressed in different mouse tissues, and that CBF-Cb comprises approx. 5–10% of the total CBF-C transcripts. This experiment, therefore, provided evidence that CBF-Cb is biologically present in the cells, rather than being a result of random cloning errors while constructing the cDNA library.

In vivo interaction of CBF-Cb with Smad2 and Smad3

Next we determined if CBF-Cb is able to associate *in vivo* with TGF-β- and activin-specific Smad proteins, using an immunoprecipitation assay. Smad2 and Smad3 are the pathway-specific

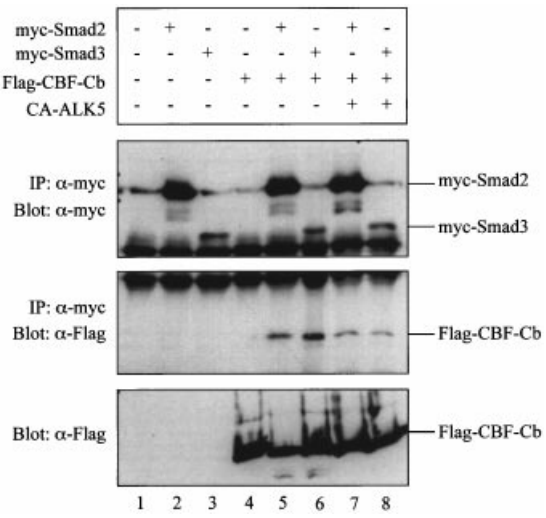

Figure 2 *In vivo* association of CBF-Cb with Smad2 and Smad3

CBF-Cb, Smad2, Smad3 and CA-ALK5 were expressed in HEK293 cells as indicated. The cell lysate was immunoprecipitated (IP) with an anti-Myc antibody, followed by Western blotting with either an anti-Flag antibody (to detect Flag-tagged CBF-Cb) or an anti-Myc antibody (to detect Myc-tagged Smad2 and Smad3). The lysate was also used in straight Western blot analysis to determine the level of expression of CBF-Cb.

Smad proteins that specifically transduce signalling by TGF-β and activin receptors [1,2]. When Myc-tagged Smads were co-transfected with Flag-tagged CBF-Cb, immunoprecipitation with an anti-Myc antibody was able to co-purify CBF-Cb, as determined by anti-Flag Western blotting analysis (Figure 2, lanes 5–8). This *in vivo* interaction between Smads and CBF-Cb appears to be specific, as the expression of Smads or CBF-Cb alone did not give rise to any detectable presence of Flag-tagged CBF-Cb (Figure 2, lanes 2–4). In addition, the interaction appeared not to be dependent on the activation of Smad2/Smad3 by TGF-β signalling: both Smad2 and Smad3 were able to associate with CBF-Cb in the absence of TGF-β receptor activation (Figure 2, lanes 5 and 6). Interestingly, activation of the TGF-β pathway by a constitutively active TGF-β type I receptor (CA-ALK5) seemed to slightly decrease the interaction of CBF-Cb with the Smad proteins, as similar amounts of Smads and CBF-Cb were expressed in the experiment (Figure 2, lanes 7 and 8).

Ectopic expression of CBF-Cb represses the transcriptional activity of Smad2/Smad3

To determine the biological significance of the interaction of CBF-Cb with Smad2 and Smad3, we analysed the effect of ectopically expressed CBF-Cb on the transcriptional activation mediated by these Smad proteins. Smads regulate gene expression mainly by two mechanisms: via direct binding to an SBE in the promoter region, and by indirectly modulating transcription due to association with other transcription factors that bind DNA receptors [1]. To explore the first possibility, Smad2 and Smad3 were expressed in HEK293 cells together with a constitutively active TGF-β type I receptor (CA-ALK5). As shown in Figure 3(A), Smad3 alone, or either Smad2 or Smad3 together with CA-ALK5, were able to significantly activate a TGF-β-responsive luciferase reporter that contained two tandem repeats of SBE found in the mouse Smad7 promoter [26]. Co-expression of CBF-Cb, however, was able to markedly inhibit this Smad-

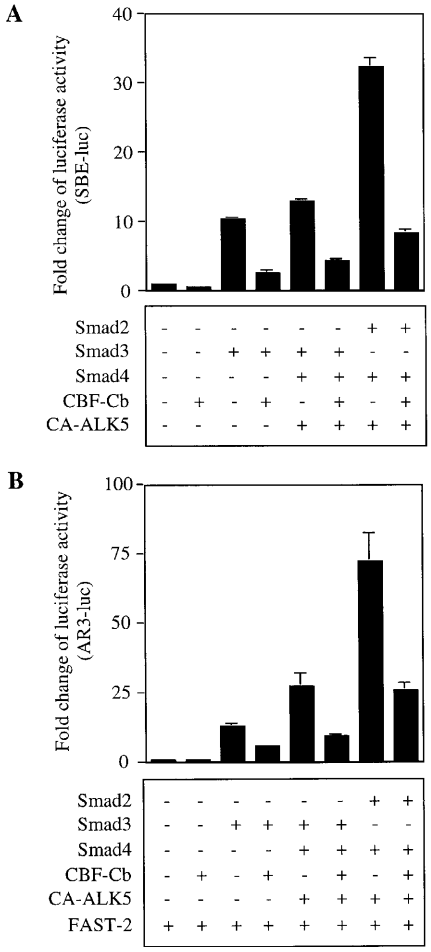

Figure 3 Repression of Smad2- and Smad3-mediated transcriptional activation by CBF-Cb

HEK293 cells were transiently transfected with 0.25 μg of pCMV-β-gal and a promoter containing SBE (**A**) or ARE (**B**). In addition, Smad2, Smad3, Smad4, CA-ALK5 and mouse FAST-2 were co-transfected as indicated (each 0.25 μg). Luciferase activities were measured 24 h after transfection. The fold changes in luciferase activity as compared with that in the vector-transfected cells (set to 1) are shown as means $\pm$ S.D. after being normalized by β-galactosidase activity. At least three independent experiments were performed with similar results.

mediated transcriptional activation to approx. 3–4-fold (Figure 3A). When different amounts of CBF-Cb were expressed in this experiment, it was able to repress the Smad-activated luciferase activity in a dose-dependent manner (results not shown). Taken together, these experiments indicated that CBF-Cb is able to significantly repress the transcriptional activation mediated by TGF-β/activin-specific Smad proteins through binding to the SBE.

We also examined the effects of CBF-Cb on the other mode of Smad-mediated transcription, i.e. via the interaction with another DNA-binding transcription factor, mouse FAST-2. FAST-2 is a mammalian homologue of frog FAST-1, and this group of forkhead transcription factors binds a specific DNA sequence and regulates genes involved in the early development of the animal [7,8,32]. FAST-2 is dependent on its interaction with Smad proteins to activate target gene transcription [8,33]. In the presence of FAST-2, expression of either Smad3 alone, or Smad2 or Smad3 in combination with CA-ALK5, was able to activate a luciferase reporter that bears three tandem repeats of an activin-responsive element (ARE) [34] which is bound specifically by FAST-2 (Figure 3B). Co-expression of CBF-Cb in this system was also able to inhibit FAST-2-mediated and Smad-activated transcriptional regulation (Figure 3B).

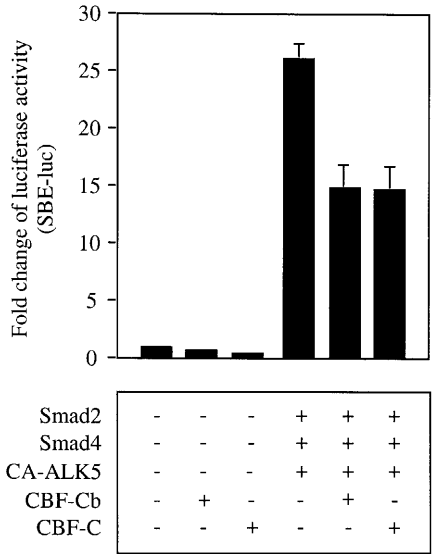

Figure 4 Inhibition of Smad2-mediated transcriptional activation by unspliced CBF-C

HEK293 cells were transiently transfected with 0.25 μg of pCMV-β-gal and a promoter containing the SBE of the Smad7 promoter (0.25 μg). In addition, Smad2, Smad4 and CA-ALK5 were co-transfected as indicated (each 0.25 μg). Luciferase and β-galactosidase activities were determined 24 h after transfection. The fold change in luciferase activity as compared with that in the vector-transfected cells is shown, as described for Figure 3.

Unspliced CBF-C is also able to inhibit the transactivating activity of TGF-β Smads

Our experiments have suggested that CBF-Cb is able to interact with Smad2 and Smad3 and functionally inhibit the transcriptional stimulation by these Smads following TGF-β receptor activation. Does this inhibitory effect also occur with unspliced CBF-C, in addition to the splice variant CBF-Cb? To address this issue, we isolated full-length CBF-C by RT-PCR and used it in a transcriptional assay, as described for CBF-Cb in Figure 3(A). In this experiment, CA-ALK5 markedly stimulated the SBE-containing luciferase reporter in the presence of Smad2 and Smad4 (Figure 4). Both unspliced CBF-C and the splice variant CBF-Cb significantly inhibited CA-ALK5-stimulated transcriptional regulation. In addition, the inhibitory effect of CBF-C appeared to be similar to that of CBF-Cb, indicating that the two forms of CBF-C are able to modulate the transactivating activity of Smad proteins downstream of TGF-β/activin receptors by a similar mechanism.

Overexpression of p300 abrogates the repression by CBF-Cb

The transactivating activity of Smads is achieved via interaction with the general transcription co-activator p300/CBP that links specific transcription factors with the basal transcriptional machinery. Smads interact with p300/CBP through their MH2 domain [9–11]. We hypothesized that CBF-Cb may inhibit the transcriptional activity of Smads by 'masking' their MH2 domain, thereby preventing the recruitment of p300/CBP. If this hypothesis holds true, overexpression of p300/CBP would compete for the binding of CBF-Cb to the MH2 domain of Smads,

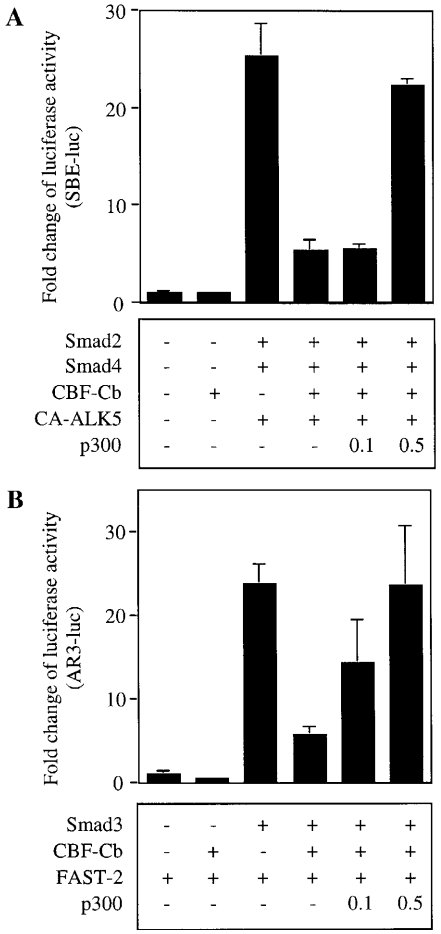

Figure 5 Abrogation of the repressive activity of CBF-Cb by overexpression of p300

HEK293 cells were transiently transfected with 0.25 μg of pCMV-β-gal and a promoter containing SBE (**A**) or ARE (**B**), along with Smad2, Smad3, Smad4, CA-ALK5 and mouse FAST-2, as indicated (each 0.25 μg). In addition, different amounts of p300 (0.1 or 0.5 μg) were co-transfected. Luciferase activity was measured 24 h after transfection, and the fold change after normalization to β-galactosidase activity is shown (mean $\pm$ S.D.; that of vector-transfected cells was set to 1).

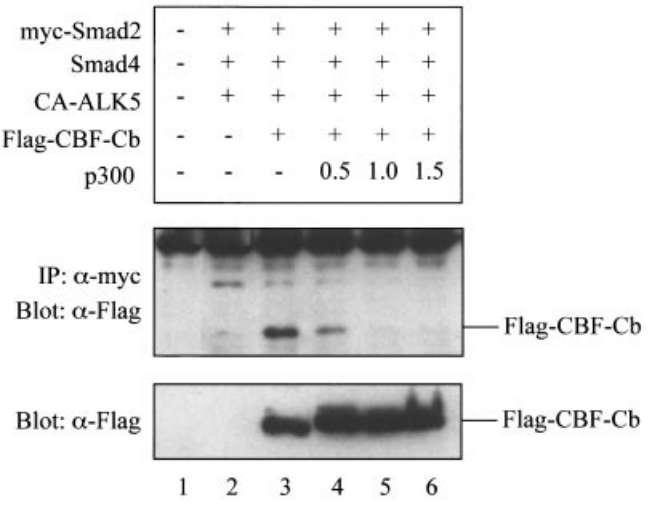

Figure 6 Inhibition by p300 of the *in vivo* association of CBF-Cb with Smad2

Different amounts of p300 (0.5–1.5 μg), along with Flag-tagged CBF-Cb, Myc-tagged Smad2, Smad4 and CA-ALK5 as indicated (each 0.25 μg), were expressed in HEK293 cells. The cell lysate was immunoprecipitated (IP) with an anti-Myc antibody, followed by Western blotting with an anti-Flag antibody to detect Flag-tagged CBF-Cb. The lysate was also used in straight Western blot analysis with an anti-Flag antibody to determine the level of expression of CBF-Cb.

and alleviate the repressive activity of CBF-Cb. To test this hypothesis, we analysed the effect of overexpression of p300 on the inhibitory activity of CBF-Cb. As shown in Figure 5(A), Smad2-mediated transcriptional stimulation after TGF-β receptor activation was inhibited by CBF-Cb, and this repression was completely abrogated when a higher amount of p300 was co-expressed. We also examined the transcriptional regulation of Smads through FAST-2. As shown in Figure 5(B), CBF-Cb was able to repress Smad3-mediated transcriptional activation. However, the repressive activity of CBF-Cb on Smad3-mediated transactivation through FAST-2 was reversed by increasing amounts of co-expressed p300. Taken together, these experiments provide evidence that CBF-Cb may repress Smad-mediated gene transcription by preventing the association of the MH2 domain of Smads with the general transcriptional co-activator p300.

To further invesitgate whether the association of p300 with Smad and CBF-Cb may play a role in the modulatory activity of CBF-Cb on TGF-β signalling, we analysed the association of CBF-Cb with Smad2 in the presence of overexpressed p300 by a co-immunoprecipitation assay. If our hypothesis holds true, overexpression of p300 would compete with the association of Smad2 with CBF-Cb. As shown in Figure 6, Myc-tagged Smad2 was co-expressed with Flag-tagged CBF-Cb in HEK293 cells and immunoprecipitated with an anti-Myc antibody. Western blotting analysis with an anti-Flag antibody indicated that CBF-Cb was expressed at a similar level (Figure 6, lower panels). In this experiment, CBF-Cb was able to associate with Smad2 (lane 3), similar to what was described in Figure 2.When an increasing amount of p300 was expressed in these cells, however, the association of CBF-Cb with Smad2 was diminished in a dose-dependent manner (Figure 6, lanes 4–6). These results provide further evidence that the inhibitory effect of CBF-Cb on Smad-mediated transcription is probably caused by interference with the association of Smad proteins with the general transcriptional co-activator p300.

DISCUSSION

We have isolated a novel splice variant of CBF-C that interacts with the MH2 domains of Smad2 and Smad3, which transduce the signalling of TGF-β/activin. This splice variant, CBF-Cb, was ubiquitously expressed in various mouse tissues. In transient transfection experiments, CBF-Cb was able to inhibit the transcriptional activity of Smad2 and Smad3. Furthermore, the interaction of CBF-Cb with the MH2 domain of Smads may prevent the association of Smads with the general transcriptional co-activators, therefore repressing the transactivating activities of Smad proteins. Transcriptional repression by Smad proteins is mediated by different mechanisms, linked in part to the HDAC activity that is associated with Smad-binding partners such as TGIF and SnoN/Ski, or with the Smad proteins themselves [12,15,16]. Our findings here reveal a different mode of modulating the transcriptional activity of Smad proteins, i.e. via masking of the MH2 domain and preventing its association with the general transcriptional co-activators. Therefore the transcrip-

tional activity of Smad proteins in a particular cell could be fine-tuned by the balance between three groups of factors: the general transcription co-activators such as p300/CBP, the transcription repressors such as TGIF, and the transcriptional modulators such as CBF-Cb. This cell-context-dependent action by Smad proteins would partly explain the diverse biological effects of TGF-β in different type of cells.

CBF (NF-Y) is a heterotrimeric protein complex that binds the CAATT motif found in the promoter or activator of many eukaryotic genes. CBF is composed of three subunits, CBF-A, CBF-B and CBF-C. The B and C subunits associate through a subdomain that binds DNA and resembles an α-helical structure found in the core histone proteins [22]. The A subunit associates with the B/C heterodimer to form a functional CAATT-binding complex. The transactivating activity of CBF is mediated by the association of the B/C heterodimer with the histone acetyltransferase P/CAF [23] and the association of the B subunit with p300 that has an intrinsic histone acetyltransferase activity [25]. In the complex, CBF-C is involved in DNA binding and interaction with the A subunit. A structure/function relationship study with CBF-C indicated that the amino acid residues comprising positions 34–119 are required for DNA binding, and the region between positions 59 and 108 is necessary for the interaction with CBF-A [22]. The splice variant we found here lacks a region spanning positions 36–97. Therefore CBF-Cb is unlikely to form a functional complex with the A and B subunits of CBF to bind and transactivate the CAATT motif. Thus CBF-Cb may not play an active role in CBF-mediated transcriptional regulation. In addition, we found that the unspliced form of CBF-C is able to inhibit the transactivating activity of Smad proteins downstream of TGF-β/activin receptors. Furthermore, this inhibitory activity of CBF-C is similar to that of CBF-Cb. These data suggest that CBF-C may modulate Smad activity via a mechanism similar to that employed by CBF-Cb.

In summary, we have found that Smad2 and Smad3 are able to associate with CBF-Cb via their MH2 domain. The MH2 domains of different Smad proteins share high sequence identity with each other [1,2]. Therefore the possibility cannot be ruled out at present that CBF-Cb may associate with the MH2 domains of other pathway-specific Smads involved mainly in BMP signalling pathways, i.e. Smad1, Smad5 and Smad8. If such an interaction is found, CBF-Cb would be seen to act as a general transcriptional modulator of the Smad family, not only limited to the regulation of the TGF-β/activin pathways. Nevertheless, our finding that CBF-Cb regulates the transcriptional activity of Smad proteins through interaction with their MH2 domain has revealed a novel mode of modulation of TGF-β/activin signalling, which justifies further investigation.

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