TAK1 activation of the mouse JunB promoter is mediated through a CCAAT box and NF-Y

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Abstract The JunB gene is activated by many stimuli including transforming growth factor β (TGFβ) family members and interleukin-6 (IL-6). Here the effect of TGFβ activated kinase 1 (TAK1), a mitogen activated protein kinase kinase kinase (MAPKKK) implicated in TGFβ, bone morphogenetic protein (BMP) and interleukin-1 (IL-1) signaling, on JunB promoter activity was investigated. Promoter analysis led to the identification of a CCAAT motif in the JunB gene, essential for activation by TAK1. Transfer of this CCAAT element to a heterologous minimal promoter conferred TAK1-responsiveness. The CCAAT-binding transcription factor, nuclear factor Y (NF-Y), activated the JunB promoter and a dominant negative NF-YA construct inhibited TAK1 activation of JunB. Our results demonstrate that JunB gene activation by TAK1 is mediated by the CCAAT-binding factor NF-Y.

Key words: JunB; Transforming growth factor β activated kinase 1; Nuclear factor Y; Promoter; CCAAT motif

1. Introduction

The mitogen activated protein kinase (MAPK) signaling cascades are involved in multiple biological processes and are activated by various stimuli including growth factors, stress-inducing agents and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [1–3]. The MAPK kinase kinase (MAPKKK) transforming growth factor β (TGFβ) activated kinase 1 (TAK1) has been identified as a mediator of TGFβ, bone morphogenetic protein (BMP) and interleukin-1 (IL-1) signaling [4–6]. TAK1 binding protein 1 and 2 (TAB1 and TAB2) are adapter proteins that activated TAK1 in response to TGFβ and IL-1, respectively [7,8].

The JunB gene, a member of the AP-1 family of transcription factors, is induced by TGFβ, IL-6, activators of protein kinase A (PKA) and TPA [9–12]. Previously, we showed that a Smad-binding element can mediate activation of the JunB gene by TGFβ [13]. In this manuscript we have analyzed JunB activation by IL-1, TGFβ and TAK1. The aim of this study was to identify the JunB promoter region responsive to TAK1 and the factor that mediates this activation. We found that JunB promoter activation by TAK1 requires a CCAAT motif and depends on the CCAAT-binding transcription factor, nuclear factor Y (NF-Y).

2. Materials and methods

2.1. Plasmids

The JunB promoter constructs used are described in [13]. The 3× CCAAT Luc, 2×IR Luc and the 2×GC Luc reporters were generated by insertion of concatamerized synthetic oligonucleotides in the *Bgl*II site of pGL3ti (see [13] for pGl3ti). The ΔCCAAT Luc construct was constructed using polymerase chain reaction (PCR). NF-YA and NF-YA29 expression plasmids and αNF-YB antibodies were generous gifts of Dr. R. Mantovani; TAK1, TAB1 and K63W plasmids were generously provided by Dr. K. Matsumoto.

2.2. Reverse transcription (RT)-PCR

Total RNA was isolated from HepG2 cells, using Trizol, according to the supplied protocol (Life Technologies). RNA was reverse transcribed with M-MuLV (Boehringer Mannheim) and subjected to PCR. After 35 cycles, 10 μl of the reaction volume was resolved on agarose gels and photographed. The sequences of the PCR primers used were: JunB: forward: CCAGTCCTTCCACCTCGACGTTTACAAG, reverse: GACTAAGTGCGTGTTTCTTTTCCACAGTAC; GAPDH: forward: ATCACCATCTTCCAGGAG, reverse: GCCATCCACAGTCTTC.

2.3. Cell culture and transient transfection

HepG2 and NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (HepG2) or 10% NCS (NIH-3T3), 100 IU/ml penicillin, 1 mg/ml streptomycin, 2 mM L-glutamine and 1×MEM non-essential amino acids (Life Technologies). For transient transfections, 100 000 NIH-3T3 cells were seeded per 35-mm dish and transfected using the calcium phosphate co-precipitation method. The following day, the media was changed and 48 h after transfection the cells were harvested in reporter lysis buffer (Promega). Luciferase activity was determined using the Promega luciferase assay system. In all transfections, a β-galactosidase expression plasmid (pDM2LacZ, [14]) was included to normalize luciferase activities. β-Galactosidase activity was determined in 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol and 0.67 mg/ml *O*-nitrophenylgalactopyranoside. All transfections were carried out in triplicate and repeated at least twice in independent experiments using different batches of plasmid DNA.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from NIH-3T3 cells as described in [15]. Annealed oligonucleotides (forward: GATCCCGCGTCGGCCAATCGGAGTGCACA; reverse: GATCTGTGCACTCCGATTGGCCGACGCGG) were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and purified with spin columns (Qiagen). Binding reactions were carried out for 30 min at room temperature in 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 2 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and

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Abbreviations: TAK1, transforming growth factor β activated kinase 1; NF-Y, nuclear factor Y; nt, nucleotides; TGFβ, transforming growth factor β; MAPK, mitogen activated protein kinase; EMSA, electrophoretic mobility shift assay

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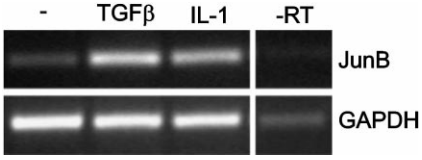

Fig. 1. Activation of JunB gene expression by TGFβ and IL-1. HepG2 cells were either untreated (lane -) or treated with either TGFβ or IL-1 (lanes TGFβ and IL-1). After 1 h, RNA was extracted and JunB and GAPDH mRNA levels were determined using RT-PCR. The lane −RT indicates pooled RNAs from all experimental conditions, processed without reverse transcriptase. GAPDH is used as an internal control.

0.01% NP-40 containing 5 μg extract, 1 μg poly(dI-dC), 50 000 cpm probe and where appropriate 5-, 10- or 100-fold molar excess competitor oligos. Preincubation with NF-YB antibodies (240 ng/sample) was carried out at room temperature, 30 min prior to probe addition. Before loading onto 5% polyacrylamide gels (0.5×TBE), 20% Ficoll was added to the reactions, after electrophoresis gels were dried and autoradiographed.

3. Results

3.1. IL-1 and TGFβ activate the JunB gene

To determine the effect of IL-1 and TGFβ on JunB gene expression, HepG2 cells were stimulated with IL-1 or TGFβ for 1 h and JunB mRNA levels were determined using RT-PCR analysis. Both TGFβ, as was shown earlier [9], and IL-1 induced JunB gene expression (Fig. 1, panel JunB) whereas GAPDH expression levels were not altered (Fig. 1, panel GAPDH). To control for the presence of genomic DNA, total RNA from all experimental conditions was pooled and subjected to PCR. No significant amplification of either JunB or GAPDH was observed (Fig. 1, lane −RT).

3.2. TAK1/TAB1 transactivates the proximal JunB promoter

Since TAK1 has been implicated in both TGFβ and IL-1 signaling [4,6], we next determined the effect of TAK1 on the JunB promoter. A minimal promoter fragment (−87 Luc), still responsive to TAK1, was identified by progressive deletion of 5′-JunB promoter sequences. Although a decrease in basal promoter activity was observed, the fold-activation by TAK1 was largely unaffected by removal of upstream sequences (Fig. 2A). A catalytically inactive TAK1 mutant, K63W, did not result in reporter activation and promoter stimulation by TAK1 was TAB1-dependent (Fig. 2B).

3.3. TAK1 activates the −87 Luc reporter via a CCAAT box

The smallest TAK1-responsive construct, −87 Luc, contained 47 nucleotides (nt) of JunB sequence, including a GC-rich sequence, an inverted repeat and a CCAAT box (see Fig. 3). Reporters containing multiple copies of these elements were generated and tested for TAK1-responsiveness. Reporters either containing the inverted repeat or the GC-rich motif were not activated by TAK1 (data not shown) whereas a reporter containing three CCAAT boxes (3×CCAAT Luc) was stimulated by TAK1 (4.2-fold induction, Fig. 3). Next, a point mutation was introduced in the CCAAT sequence (CCAAT→CCGAT) which resulted in a complete loss of TAK1-responsiveness. These results indicated that a CCAAT motif can confer TAK1-sensitivity and that activation of the proximal JunB promoter by TAK1 is dependent on the presence of an intact CCAAT motif (Fig. 3).

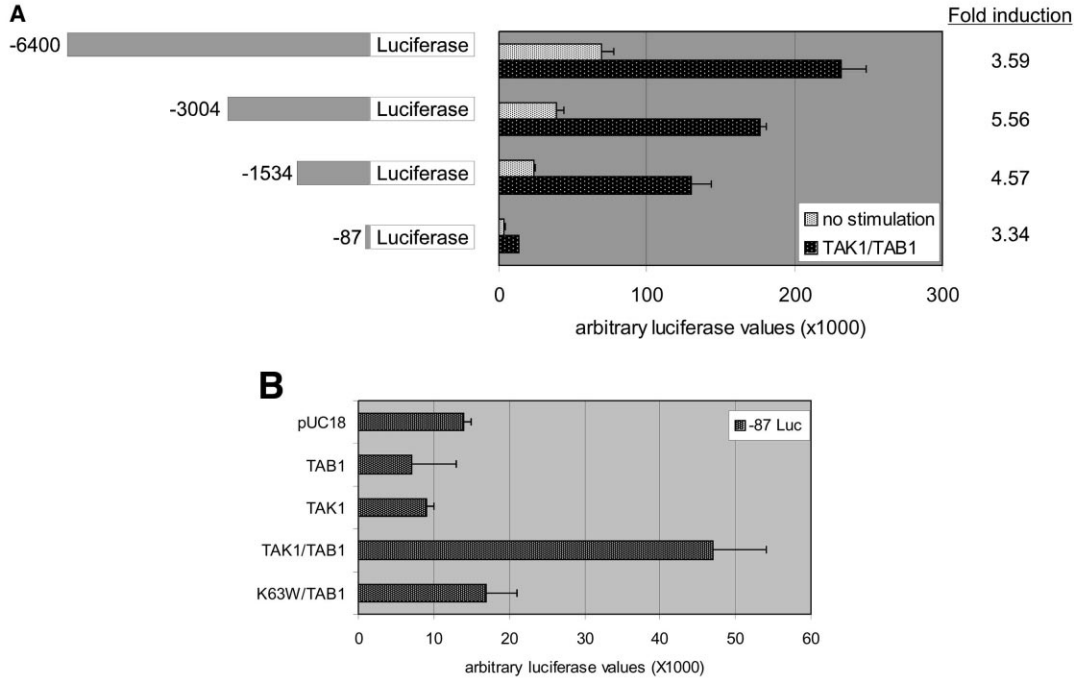

Fig. 2. A: Analysis of the mouse JunB promoter in NIH-3T3 cells. The figure to the left indicates the 5′-end of the tested promoter constructs, in nt, relative to the ATG. The normalized activity of the reporter constructs is given as the average of at least two triplicates with the standard error of the mean ($n \geq 6$). The 'fold induction' column to the right of the figure gives the ratio of promoter activity between unstimulated and TAK1/TAB1 stimulated reporters. B: Activation of the −87 Luc reporter by TAK1 is TAB1-dependent. The −87 Luc reporter was transfected into NIH-3T3 cells with the plasmids indicated to the left of the figure. The normalized luciferase activity is given as the average of at least two triplicates with the standard error of the mean ($n \geq 6$).

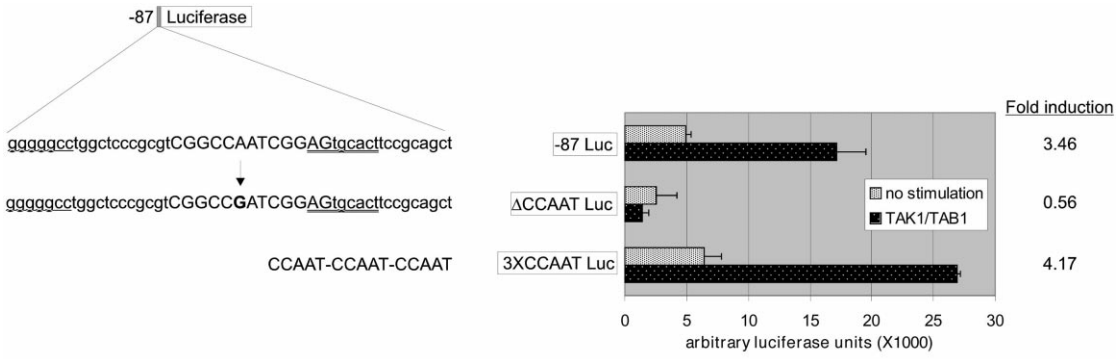

Fig. 3. Identification of a CCAAT sequence in the JunB promoter as a response element required and sufficient for TAK1 activation. The JunB promoter sequence present in the −87 Luc construct is depicted with the consensus NF-Y-binding sequence in capitals, the GC-rich box is underlined and the inverted repeat is double underlined. The arrow points to the point mutation introduced in the ΔCCAAT Luc reporter. The 'fold induction' column to the right of the figure gives the ratio of promoter activity between unstimulated and TAK1/TAB1 stimulated reporters. The normalized luciferase activity is given as the average of at least two triplicates with the standard error of the mean ($n \geq 6$).

3.4. NF-Y mediates activation of the JunB promoter by TAK1

The CCAAT box present in the −87 Luc construct, including flanking sequences, matched a consensus NF-Y site [16]. Moreover, Bowden et al. recently showed that NF-Y mediates JunB promoter activation in response to okadaic acid [17]. NF-Y is a ubiquitous heterotrimeric transcription factor consisting of three subunits, NF-YA, NF-YB and NF-YC, of which NF-YA contains a DNA-binding domain and a subunit interaction domain and all three subunits are required for DNA interaction [18]. We determined whether NF-Y activated the JunB promoter and mediated activation by TAK1. The −87 Luc reporter was activated by both TAK1/TAB1 (3.5-fold induction) and NF-YA (4.3-fold induction, Fig. 4). Simultaneous transfection of NF-YA, TAK1 and TAB1 with the −87 Luc reporter further increased promoter activity (a 6.6-fold induction). Where a dominant negative NF-YA, NF-YA29 [19], by itself had no significant effect on promoter activity, TAK1-mediated transactivation of the −87 Luc reporter was significantly inhibited by NF-YA29 (Fig. 4, compare TAK1/TAB1 with TAK1/TAB1/NF-YA29) indicating that TAK1 activation of JunB is dependent on NF-Y. As expected, a catalytically inactive TAK1 construct (K63W) had no effect on promoter activation by NF-YA (Fig. 4).

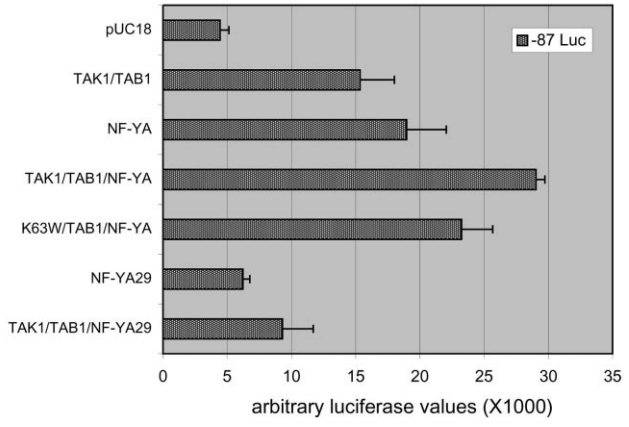

Fig. 4. NF-Y mediates TAK1 activation of the JunB promoter. NIH-3T3 cells were transfected with the −87 Luc reporter and the plasmids indicated to the left of the figure. The normalized luciferase activity is given as the average of at least two triplicates with the standard error of the mean ($n \geq 6$).

3.5. NF-Y binds the JunB CCAAT sequence

To determine whether NF-Y binds the proximal JunB promoter, EMSAs were performed using oligonucleotides containing the JunB CCAAT element and flanking sequences. To determine the specificity of the observed shifted complexes, increasing amounts of unlabeled oligonucleotides were added as competitor. Concentration-dependent competition for one shifted complex was observed, indicating this complex specifically bound to the probe (Fig. 5, arrow). Since Mantovani et al. previously showed that αNF-YB polyclonal antibodies prevented interaction between NF-Y and DNA [20], we used these antibodies to determine whether the observed, specific complex contained NF-Y. Preincubation with NF-YB antibodies (lane 7) deprived NIH-3T3 nuclear extracts of the

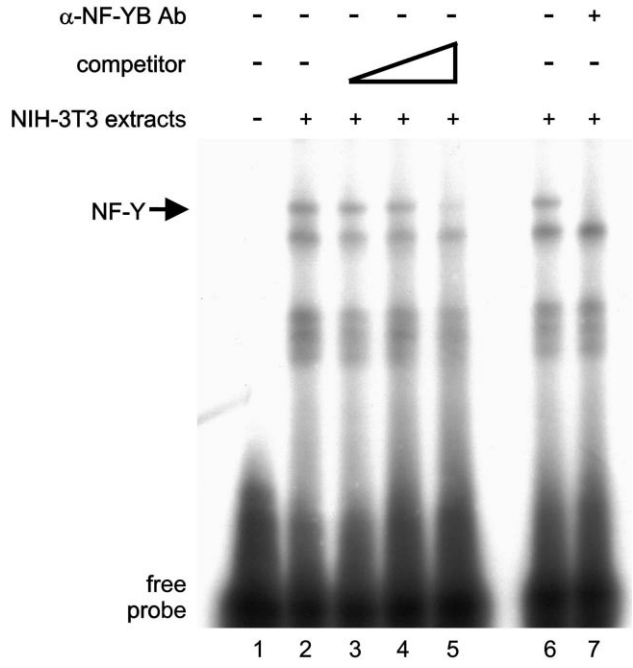

Fig. 5. NF-Y binds the JunB CCAAT sequence. An end-labeled oligonucleotide containing the JunB CCAAT sequence was incubated with NIH-3T3 nuclear extracts. In lanes 3–5, respectively, 5-, 10- and 100-fold molar excess unlabeled competitor oligonucleotide was added. In lanes 6–7, nuclear extracts were preincubated with pre-immune serum and NF-YB antibodies, respectively. The arrow indicates the NF-Y containing complex.

same DNA-binding activity that was competed for with excess unlabeled oligos and preincubation with preimmune serum (lane 6) had no effect. These results indicated that the protein complex specifically binding to the JunB CCAAT sequence element contained NF-Y.

4. Discussion

In this report we describe that activation of the JunB promoter by the IL-1- and TGFβ-activated MAPKKK TAK1 is mediated through a CCAAT motif and NF-Y. Mutation analysis revealed that a CCAAT sequence element, localized 65 nt 5′ of the ATG, was required for TAK1-mediated activation of the JunB promoter. Through transfections and EMSAs we demonstrated that NF-Y acted downstream of TAK1 and showed NF-Y-binding to the JunB CCAAT motif.

4.1. TAK1 activation is mediated via a CCAAT box in the proximal promoter

Analysis of a series of JunB promoter constructs indicated the presence of a TAK1-responsive element in the −87 Luc reporter. Activation of the JunB promoter constructs by TAK1, depicted as 'fold induction', was not significantly altered by deleting 5′-sequences. The observed decrease in basal promoter activity is probably due to deletion of upstream enhancer sequences. These results showed that the −87 Luc JunB reporter construct contained a TAK1-responsive region (Fig. 2A) but the presence of additional TAK1-responsive sequence elements in upstream promoter sequences cannot be excluded. Activation of the −87 Luc reporter by TAK1 is TAB1-dependent, suggesting TAK1 kinase activity is required for JunB activation (Fig. 1B) which is further illustrated by the observation that a catalytically inactive TAK1 mutant, K63W, failed to activate the −87 Luc reporter (Fig. 1B).

Sequence analysis of the −87 Luc reporter pointed to a CCAAT box as a potential sequence element mediating TAK1 activation of the JunB promoter. The JunB CCAAT sequence element, including flanking sequences, completely matched a consensus NF-Y-binding site defined by Mantovani [16]. Besides a preference for these flanking sequences, all five nucleotides of the CCAAT sequence are required for NF-Y-binding which is in agreement with the observation that a single point mutation in the JunB CCAAT sequence abolishes responsiveness of the JunB promoter to TAK1 [16].

4.2. NF-Y mediates TAK1 activation of the JunB promoter

Transient transfections with the −87 Luc reporter showed that NF-YA can activate this reporter (Fig. 3). Overexpression of the CCAAT-binding transcription factors C/EBP and CTF/NF-I did not result in −87 Luc transactivation showing that the JunB promoter is specifically activated by NF-Y (Eggen and Kruijer, unpublished results). Parallel overexpression of either an inactive TAK1 mutant, K63W, or TAK1 had little effect on NF-YA induced activation of the JunB promoter, indicating that when NF-YA is overexpressed, TAK1 kinase activity is not required for promoter transactivation (Fig. 3).

To show that NF-Y mediates TAK1 activation of the JunB promoter, transient cotransfections of TAK1 with a NF-YA29 mutant were performed. The NF-YA29 construct carries a mutation that destroys the DNA-binding ability of NF-YA; the domain that is required for subunit interaction is not changed [19]. Since all three subunits of NF-Y are required for DNA binding, overexpression of NF-YA29 results in the formation of NF-Y complexes that lack DNA-binding ability. Since the CCAAT sequence was not occupied under these conditions and overexpression of NF-YA29 strongly inhibited activation of the JunB promoter by TAK1 we concluded that NF-Y is the CCAAT-binding transcription factor that mediated TAK1 activation of the JunB promoter.

In addition to these findings, EMSAs verified that NF-Y binds the CCAAT sequence element in the JunB promoter. Competition analysis indicated the presence of a specific complex and preincubation of nuclear extracts with αNF-YB antibodies completely eliminated this complex.

At present it remains unclear how NF-Y activity is regulated by TAK1. NF-Y consists of three subunits (NF-YA, -B and -C) and each subunit is able to bind co-factors prior to subunit association and DNA binding of the NF-Y complex. TAK1 is a MAPKKK that has been shown to activate several MAPKK–MAPK cascades. It is possible that phosphorylation of NF-Y subunits or NF-Y cofactors by (one of) these TAK1-activated MAPK cascades modulates subunit–subunit or subunit–cofactor interaction, complex stability or DNA-binding properties of the complex, resulting in an increased JunB transactivation. Further experiments however will be required to delineate the exact mechanism of NF-Y activation by TAK1.

In summary, our results indicate that TAK1, a MAPKKK homolog positioned downstream of TGFβ, BMPs and IL-1 activates the JunB gene through the CCAAT-binding transcription factor NF-Y. The JunB gene is an immediate early gene whose expression is rapidly upregulated in response to multiple stimuli. Since the CCAAT motif is present in many eukaryotic promoters and NF-Y is a common transcription factor, it is an unlikely factor to mediate stimulus-specific gene activation. We hypothesize that the role of NF-Y in JunB promoter activation is to enhance JunB promoter activation by stimulus-specific transcription factors like the Smad proteins. Further experiments will have to determine whether and how these pathways interact to modulate JunB promoter activity in response to particular stimuli.

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