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SCIENCE DIRECT

Gene 353 (2005) 118 – 133

GENE
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Genomic structure and functional characterization of the promoter region of human IκB kinase-related kinase IKKi/IKKε gene☆

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Received 28 September 2004; received in revised form 18 March 2005; accepted 7 April 2005

Received by J.A. Engler

Abstract

The inducible IκB kinase (IKKi/IKKε) is a recently described serine–threonine kinase that activates the transcription factors NFκB, interferon regulatory factor-3 (IRF3) and CCAAA/enhancer-binding protein (C/EBPδ). Several inflammatory agents have been shown to induce the expression of the IKKi gene in macrophages and other cell types but the mechanism is unknown. We have found that the IKKi expression was constitutive in human chondrocytes from OA cartilage and a human chondrocytic cell line C28/I2 but was up-regulated by the inflammatory cytokines TNFα or IL-1β in an NFκB-dependent manner. To understand the constitutive and inducible expression of the IKKi gene we localized the transcription start site (TSS), cloned and sequenced a 2 kb genomic DNA fragment 5′ of the TSS and characterized the putative promoter region (PPR), and identified the motifs therein that are required for basal and cytokine-induced IKKi gene promoter activity. We found that IKKi core promoter was TATA-less and by using PCR generated deletion mutants of the PPR we found that the cis-elements responsible for basal transcriptional activity were located between −51 and −100 bp upstream of the TSS while the cytokine response elements were located distally between −501 and −1000 bp upstream of the TSS. The DNA region containing the cytokine response elements had two κB sites as the most relevant regulatory motifs. The results of site-directed mutagenesis revealed that the κB site located between −833 and −847 bp upstream of the TSS was biologically functional and required for cytokine-induced IKKi promoter activity in human chondrocytes and HeLa cells. The silence of the other κB site (−816/−802) was positional, rather than sequence-specific. Over-expression of NFκB p65 mimics the TNFα-induced activation of the IKKi promoter. Also the gel shift assay suggested that NFκB p65 is responsible for activation of the IKKi promoter. These data for the first time characterize the promoter region and provide further insights into the transcriptional regulation of IKKi in human chondrocytes and other cell types.

Keywords: IKKi/IKKε promoter; Cis-acting element; Transcriptional regulation; NFκB; Chondrocyte

1. Introduction

Transcription factor NFκB is a strong transcriptional activator that plays an important role in the expression of genes involved in diverse cellular functions including growth, survival, immune and inflammatory responses (Karin and Ben-Neriah, 2000). In resting cells NFκB is sequestered in the cytoplasm in association with proteins of the IκB family of inhibitory proteins (IκBα, IκBβ, or IκBε). Stimulation of cells by cytokines or other agonists results in phosphorylation, ubiquitination, and degradation of IκBs thus exposing the nuclear localizing signals (NLS) on NFκB, leading to its nuclear translocation where it binds to

Abbreviations: 5′-RACE, 5′-rapid amplification of cDNA ends; ActD, actinomycin D; bp, base pair(s); BRE, TFIIB responsive element; CHX, cycloheximide; DPE, downstream promoter element; EMSA, electrophoretic mobility shift assay; EMSSA, electrophoretic mobility supershift assay; IκB, inhibitor of NFκB; IKKi/IKKε, inducible IκB kinase; IL, interleukin; Inr, initiator; NFκB, nuclear factor kappa B; nt, nucleotide(s); PPR, putative promoter region; RT-PCR, reverse transcriptase-polymerase chain reaction; SEAP, secreted form of human placental alkaline phosphatase; TBP, TATA-binding protein; TNF, tumor necrosis factor; TSS, transcription start site.

☆ The nucleotide sequences reported in this paper have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession numbers AY660028 and AY660029.

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doi:10.1016/j.gene.2005.04.013

and activate the promoters of NFκB responsive genes (Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). The two IκB kinases (IKKs) which specifically phosphorylate the critical serine residues in IκBs (IKKα or IKK-1 and IKKβ or IKK-2) have been cloned and characterized (DiDonato et al., 1997; Zandi et al., 1997). Both IKK-1 (85-kDa) and IKK-2 (87-kDa) are activated by phosphorylation and contains an N-terminal serine/threonine kinase catalytic domain, a leucine zipper-like amphipathic helix, and a C-terminal helix–loop–helix domain (DiDonato et al., 1997; Mercurio et al., 1997; Connelly and Marcu, 1995). A third member of this group called NEMO (NF-κB essential modulator or IKKγ) has no kinase activity but is essential for IKK phosphorylation and activation by upstream kinases (Yamaoka et al., 1998; Mercurio et al., 1999). The IKKs are ubiquitously expressed in most human tissues and serve as the converging point in the activation of NFκB by a wide variety of agents (Zandi et al., 1997).

Recently, a new homolog of the IKK-1 and IKK-2 kinases was found to be induced in response to stimulation with LPS and designated inducible IκB Kinase (IKKi/IKKε) and was shown to activate NFκB in macrophages (Shimada et al., 1999; Peters et al., 2000). The IKKi protein shares 31% amino acid identity with IKK-2 in the highly conserved N-terminal kinase domain but differs from IKK-2 in several important aspects. For example, it is now known that recombinant human IKKi phosphorylates IκBα at S36 but not at S32 in vitro while IKK-2 phosphorylates both of these residues. Also, IKKi is predominantly expressed in cells and tissues of the immune system such as peripheral blood leukocytes, thymus, and spleen (Shimada et al., 1999; Peters et al., 2000), while the expression of IKK-2 is ubiquitous. Third, IKKi shows significant kinase activity when over-expressed and isolated from un-stimulated cells and was found to be associated with I-TRAF/TANK proteins within the cells (Pomerantz and Baltimore, 1999, 2002; Nomura et al., 2000), while activation of IKK-2 is stimulus-dependent. Perhaps most importantly, IKKi mRNA, but not IKK-2 mRNA or protein, can be induced in multiple cell types in response to cytokines and LPS indicating that pro-inflammatory agents-mediated stimuli are required for its expression (Shimada et al., 1999; Kravchenko et al., 2003). Although initially identified as an inducible gene, more recent studies found that the expression of IKKi was constitutive in human fibroblast-like synoviocytes from RA patients (RASF), and in agreement with previous studies, stimulation with TNFα or IL-1β also increased the IKKi mRNA and protein expression in RASF, suggesting a role for IKKi in the chronic activation of the inflamed synovium in an arthritic joint (Aupperle et al., 2001).

Despite rapid advances in exploring the expression and biological functions of IKKi (Kishore et al., 2002; Huynh et al., 2002; Sharma et al., 2003; Kravchenko et al., 2003; Mori et al., 2004; Perry et al., 2004; Ehrhardt et al., 2004), the genomic structure of the promoter region and the identity of motifs present therein that regulate the inflammatory cytokine-induced IKKi gene expression have not been reported. In results reported here we identified the transcription start site (TSS), characterized the DNA regions responsible for the basal transcriptional activity and cytokine-induced expression, and demonstrated the functional and silent κB sites on the IKKi promoter. Furthermore, we show in the present study that the NFκB p65 protein is the transcription factor that interacts with the κB site on the promoter region of the human IKKi gene suggesting that IKKi is the target gene of NFκB activation. Altogether, these data extend the previous findings substantially and also provide a foundation for exploring further the biological role and regulation of IKKi in the cell type of interest.

2. Materials and methods

2.1. Cell culture, transfection, treatment, and reporter activity assay

Human OA cartilage samples were procured through the Cooperative Human Tissue Network/Human Tissue Procurement Facility of the University Hospitals of Cleveland and Case Western Reserve University, with prior approval of the Institutional Review Board of University Hospitals of Cleveland. Chondrocytes were prepared by the enzymatic digestion of knee cartilage as previously described (Ahmed et al., 2004) and maintained in DMEM/F12 (Mediatech) supplemented with 10% FBS. HeLa cells were purchased from American Type Culture Collection and maintained in MEM supplemented with 10% FBS. Immortalized human chondrocyte cell line C28/I2 cells (courtesy of Dr. Mary B. Goldring, Harvard medical School) were maintained in DMEM/F12 supplemented with 10% FBS. Cells (1.5×10^5 HeLa or 2×10^5 C28/I2 cells) were cultured in humidified air with 5% CO_2 at 37 °C for 24 h in 12-well culture plates and transiently transfected with 0.5 μg of IKKi promoter constructs or the empty vectors using the FuGENE 6 reagent (Roche). A 1/50 ratio (0.01 μg) of phRL-TK (Promega) containing a Renilla luciferase reporter gene was also co-transfected with luciferase reporter constructs as an internal control to normalize the transfection efficiency. Twenty-four hours after the transfection, cells were treated with 50 ng/ml of TNFα (Roche) or 10 ng/ml of IL-1β (eBiosciences). Twenty-four hours after the cytokine treatment, either conditioned media were collected for measurement of SEAP activity (BD Biosciences Clontech), or the cells were lysed for measurement of dual luciferase activity (Promega).

2.2. RNA isolation, reverse transcription, and TaqMan real-time PCR

Total RNA was prepared using the RNeasy Mini kit (Qiagen) and the first-strand cDNA was generated using