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Cloning and characterization of a homologue of the alpha inhibitor of NF-κB in Rainbow trout (*Oncorhynchus mykiss*)

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Abstract

A homologue of IκBα, the alpha member of the IκB family of NF-κB inhibitors, was identified in a Rainbow trout suppression subtractive hybridization library enriched in sequences up-regulated in cultured leukocytes after lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNFα) stimulation. The full-length cDNA was isolated and sequenced. The predicted amino acid sequence is 61.5% similar and 54% identical to human IκBα, while only 42% similar and 35% identical to IκBβ, and 38% similar and 32% identical to IκBε. Rainbow trout IκBα contains a central ankyrin repeat domain required for its interaction with NF-κB and a putative PEST-like sequence in the C-terminus. Expression of IκBα is up-regulated by LPS and TNFα treatment, two known activators of NF-κB, suggesting the existence of an autoregulatory loop in fish, as is the case for mammals. These results confirm the existence of the NF-κB signalling pathway in fish and suggest a similar functional interaction between IκBα and NF-κB.

Keywords: *Oncorhynchus mykiss*; Suppression subtractive hybridization; Immuno-stimulation; Innate immunity; IκBα; NF-κB inhibitor

1. Introduction

NF-κB is an inducible transcription factor that plays a key role in the regulation of the immune response, cell proliferation and apoptosis (Ghosh et al., 1998; May and Ghosh, 1998). Potent inducers of NF-κB include inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), bacterial and viral products, and pro-apoptotic and necrotic stimuli. NF-κB consists of homodimers or heterodimers of a family of structurally related proteins. Each member of this family contains a conserved N-terminal region called the Rel-homology domain (RHD), which comprises the DNA-binding and dimerization domains, together with the nuclear localization signal (NLS) (Baldwin, 1996).

Abbreviations: NF-κB, Nuclear factor kappa B; IκBα, Inhibitor kappa B alpha; LPS, Lipopolysaccharide; RHD, Rel-homology domain; NLS, Nuclear organization signal; SSH, Suppression subtractive hybridization

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In most cells NF-κB exists in the cytoplasm in an inactive form associated with inhibitory proteins. These inhibitor proteins are members of the IκB family that contains multiple copies of a 30–33 amino acid sequence referred to as ankyrin repeats. The ankyrin repeats interact with the Rel-homology domain and mask the NLS of NF-κB, preventing its nuclear translocation. In response to a large variety of extracellular stimuli, the IκB proteins are phosphorylated by the IκB kinases, IKKα and IKKβ, resulting in their ubiquitination and degradation by the 26S proteasome (May and Ghosh, 1999; Perkins, 2000). NF-κB is subsequently free to translocate to the nucleus where it activates gene transcription.

The IκB family in mammals consists of three main members, IκBα, IκBβ, IκBε, and also the nuclear protein Bcl-3 and the NF-κB precursors p105 and p100. Despite their structural similarities, the members of the IκB family appear to play different roles in vivo. Individual IκB family members show binding specificities for particular Rel protein dimers, which are believed to activate different genes in the nucleus (Perkins, 1997). IκBε functions mainly in the cytoplasm whereas IκBα and IκBβ share the additional function of entering the nucleus to inhibit NF-κB (Simeonidis et al., 1997). IκBα and IκBβ are closely related (Budde and Ghosh, 2000) but also exhibit significant functional differences. While all the known inducers of NF-κB cause the degradation of IκBα, IκBβ responds only to certain inducers. For example, TNFα causes a transient activation of NF-κB and loss of IκBα, with no effect on IκBβ (Beg et al., 1993). This transient response is due to the positive regulation of the IκBα gene by NF-κB, causing IκBα to reappear rapidly following its destruction (Brown et al., 1993; Sun et al., 1993). On the other hand, lipopolysaccharide (LPS) and interleukin-1 (IL-1) cause persistent activation of NF-κB. It appears that the newly synthesised, basally unphosphorylated IκBβ binds to NF-κB, but cannot mask the NLS, and instead acts by protecting these complexes from inactivation by IκBα (Suyang et al., 1996).

Evolutionarily, the IκB protein family is quite old, as members have been found in insects, birds and mammals (Ghosh et al., 1998). Here, we describe the Rainbow trout IκBα homologue and we compare it to other organisms.

2. Materials and methods

2.1. Primary culture and stimulation

Head kidney leukocytes from Rainbow trout (*Oncorhynchus mykiss*) were prepared by density gradient centrifugation over a 51% percoll gradient. Leukocytes were cultured in L15 growth media (2% foetal calf serum, 0.1% heparin) supplemented with penicillin and streptomycin with and without 25 μg/ml LPS (Sigma, Ireland) plus 12.5 units/ml of recombinant human TNFα (R&D systems, UK). Recombinant human TNFα has been shown to have biological cross reactivity in trout leukocytes (Hardie et al., 1994). Plates were incubated at 15 °C for different periods of time prior to RNA isolation. RNA was isolated using TRIzol reagent (Life Technologies, UK) following the manufacturer's protocol.

2.2. Construction and screening of the suppression subtractive hybridization (SSH) library

PolyA$^+$ RNA was purified by oligo dT cellulose chromatography (Pharmacia, UK) from the total RNA of both cells treated with LPS and TNFα for 24 h and untreated 24 h control cells. Two μg of this polyA$^+$ RNA isolated from treated and untreated cultures were used as tester and driver respectively. SSH was performed using the PCR-Select cDNA subtraction kit (Clontech, UK). The amplified cDNA fragments were subcloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen, UK).

2.3. Full-length IκBα cDNA isolation

The full-length IκBα cDNA was isolated from a Rainbow trout cDNA library made in λ ZAP Express (Stratagene, UK) from 4h phytohemagglutinin (PHA)-stimulated head kidney leukocytes. Plaques from the cDNA library were transferred in duplicate to Hybond-N nylon membranes (Amersham, UK), and probed with the IκBα fragment isolated from the SSH library, amplified and labelled with α^{32}P dCTP (High Prime, Roche Diagnostics Gmbh, Ger).

Positive plaques were re-plated at low density and purity was confirmed by hybridisation with the same probe. The recombinant cDNA was rescued in vector pBK-CMV by in vivo excision using helper phage