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ScienceDirect
Molecular Immunology 44 (2007) 3137–3146
Molecular Immunology
www.elsevier.com/locate/molimm

Cloning and characterisation of p38 MAP kinase from Atlantic salmon A kinase important for regulating salmon TNF-2 and IL-1β expression

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Received 16 January 2007; received in revised form 31 January 2007; accepted 5 February 2007
Available online 27 March 2007

Abstract

p38 mitogen-activated protein kinase is activated by environmental stress and cytokines and plays a role in transcriptional regulation and inflammatory responses. In this study, three distinct Atlantic salmon p38 (As-p38) cDNAs were cloned, which all translated into 361 amino acid proteins. The As-p38 protein sequences possessed showed >85% identity to the mammalian homolog, p38α. All three contained the conserved phosphorylation motif TGY located in the activation loop of the kinase. Salmon p38 showed ubiquitous tissue distribution, including expression in the immune organs head kidney and spleen. A higher p38 mRNA expression was detected in the ovary compared to other organs suggesting that p38 may perform specific functions within this organ. Western blot analysis with an antibody specific for phosphorylated p38 showed that ectopically expressed As-p38 variants were activated in CHSE-214 cells in response to chemical stress. Furthermore, lipopolysaccharide, CpG oligonucleotides and recombinant trout IL-1β induced endogenous phosphorylation of p38 in salmon head kidney macrophages in a dose-dependent manner. The importance of p38 for regulation of salmon innate immunity was further demonstrated by the ability of the p38 specific inhibitor SB203580 to completely abolish LPS-stimulated TNF-2 and IL-1β mRNA expression in the macrophages.

Keywords: Atlantic salmon; p38 MAPK; CpG; LPS; IL-1β; TNF-2; Cytokine regulation; Macrophages; Stress; SB203580 inhibitor

1. Introduction

The p38 group of mitogen-activated protein kinases (MAPKs) is activated by a wide variety of extracellular signals including pro-inflammatory cytokines and exposure to environmental stress (Raingeaud et al., 1995). There are three major groups of MAPKs in mammalian cells, the extracellular signal-regulated kinase (ERK), the c-Jun NH terminal kinase (JNK), and the p38 MAP kinase. In response to stimuli the MAPKs are activated by dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues at their Thr-Xaa-Tyr (TXY) motif found in a highly conserved activation loop near the active site (Hanks and Hunter, 1995). The sequence of the TXY motif is different for each group of MAPK. In the case of p38 MAPK, it is phosphorylated at the Thr-Gly-Tyr (TGY) motif (Raingeaud et al., 1995). A variety of extracellular stimuli are able to trigger the activation of MAPKs. The ERK pathway is activated mainly by mitogens such as growth factors (Chen et al., 2001). The JNK and p38 pathways, in contrast, are activated by cellular stress such as UV irradiation and osmotic shock, and by pro-inflammatory cytokines (Raingeaud et al., 1995). In addition, studies of zebrafish, *Xenopus, Drosophila*, and mouse have revealed important roles of p38 MAPK during early development (Adams et al., 2000; Fujii et al., 2000; Keren et al., 2005; Nebreda and Porras, 2000).

Four p38 isoforms have been identified in human and mice: p38α, p38β, p38γ and p38δ (Goedert et al., 1997; Han et al., 1994; Jiang et al., 1996; Kumar et al., 1997; Mertens et al., 1996). Both p38α and p38β genes are expressed ubiquitously (Jiang et al., 1996), while p38γ is mainly expressed in skeletal muscle (Li et al., 1996), and p38δ is expressed in lung, kidney testis, pancreas, prostate and small intestine (Kumar et al., 1997). Phosphorylation and activation of the p38 members are mediated by the upstream MAPK kinases (MKKs) named MKK3 (Derijard et al., 1995; Han et al., 1997) and MKK6 (Han et al., 1996; Moriguchi et al., 1996). MKK6 activates all four p38

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doi:10.1016/j.molimm.2007.02.006

members, while MKK3 activates all except p38β (Cuenda et al., 1997; Derijard et al., 1995; Enslen et al., 1998; Goedert et al., 1997; Han et al., 1996, 1997; Jiang et al., 1996, 1997; Moriguchi et al., 1996). MKK3 and MKK6 are activated by MAPKK kinases (MKKKs) by dual phosphorylation of serine and threonine (Derijard et al., 1995; Raingeaud et al., 1996). There are several MKKKs that can activate MKK3 and MKK6 (reviewed in Symons et al., 2006), which may explain why the p38 MAPK can be activated by such a variety of stimuli. Activation of p38 results in phosphorylation of transcription factors like myocyte enhancer factor 2 (MEF2) (Zhao et al., 1999) and activating transcription factor 2 (ATF-2) (Raingeaud et al., 1995), and also downstream kinases as MAPK-activated protein kinase 2 and 3 (MK2 and 3) (Ludwig et al., 1996; McLaughlin et al., 1996; Rouse et al., 1994).

Inflammatory mediators, including leukotrienes and pro-inflammatory cytokines, are produced in macrophages during bacterial infections. These substances play key roles in mounting the immune responses needed to fight infections. One important function of the p38 signalling pathway appears to be the regulation of cytokine expression (Frevel et al., 2003; Holtmann et al., 1999; Kotlyarov et al., 1999; Neininger et al., 2002), and this pathway was first defined by the anti-inflammatory effect of the p38 inhibitor SB 203580, a pyridyl imidazole compound (Lee et al., 1994). Several cytokines, like IL-6, IL-8 and TNF-α, contain adenylate/uridine-rich elements (AREs) in their 3′ untranslated region (3′ UTR) that destabilizes their mRNA (Kontoyiannis et al., 1999; Miyazawa et al., 1998; Winzen et al., 2004). MK2 is a downstream target of p38 shown to be essential for LPS-induced up-regulation of cytokine mRNA stability and translation. Spleen cells of MK2-deficient mice produced levels of TNF-α, IL-6 and IFN-γ that were only 10–20% of those of wild-type after LPS stimulation. The effect of MK2 on TNF-α biosynthesis was shown to be directly dependent on the ARE in the 3′UTR of TNF mRNA (Kotlyarov et al., 1999; Neininger et al., 2002).

The MAPK signalling pathway is highly conserved through evolution and p38 homologues have been identified in both higher and lower vertebrates (Fujii et al., 2000; Goedert et al., 1997; Han et al., 1994; Hashimoto et al., 2000; Jiang et al., 1996; Kumar et al., 1997; Mertens et al., 1996) and also in the invertebrate species (Han et al., 1998) and in yeast (Brewster et al., 1993). Although p38 MAP kinases are described for several fish species (Fujii et al., 2000; Hashimoto et al., 2000), their role in piscine immune responses are unexplored. In the present study, we have cloned three Atlantic salmon p38 MAP kinase cDNAs named As-p38a, As-p38b1 and As-p38b2. They were 85–89% identical to the human p38α kinase primary amino acid sequence and all three showed wide tissue distribution. Bacterial LPS, synthetic CpG oligonucleotides (ODNs) and recombinant rainbow trout IL-1 were shown to activate the p38 kinase pathway in salmon head kidney (HK) macrophages. In LPS-stimulated macrophages a high expression of TNF-2 and IL-1β transcripts was detected. Pre-treatment of these cells with the p38 specific inhibitor SB203580 reduced TNF2 and IL-1β expression to nearly the levels found in non-stimulated macrophages. The SB203580 inhibitor also blocked endogenously MK2 phosphorylation in stimulated CHSE-214, indicating that MK2 exist as a downstream target of p38 in salmonids.

2. Materials and methods

2.1. Fish

Two-year-old non-vaccinated Atlantic salmon, *Salmo salar* L., strain Aquagen standard (Aquagen, Kyrksæterøra, Norway), 350–600 g, was obtained from Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept at natural temperature in tanks supplied with running filtered sea water and fed commercial dry feed.

2.2. Reagents and antibodies

Phosphothiorate-modified CpG ODN1681 (5′-ACCGATGT-CGTTGCCG GTGACG-3′) was purchased from Eurogentec. The ODN was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The lipopolysaccharide (LPS; from *Escherichia coli* O111:B4), sodium arsenite and sorbitol used, were obtained from Sigma. Recombinant rainbow trout (*Oncorhynchus mykiss*) IL-1β (rIL-1β) (Hong et al., 2001) was kindly provided by Dr. C.J. Secombes, University of Aberdeen. The p38 inhibitor SB203580 was purchased from Alexis. The rabbit antibodies to phospho-p38 MAPK, phospho-MK2 and the eukaryotic elongation factor 2 (eEF2) were obtained from Cell Signalling, rabbit anti-actin antibody was purchased from Sigma and mouse anti-Myc antibody was purchased from Invitrogen. Horseradish peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

2.3. Cell cultures and transfection

Chinook salmon embryonic cells (CHSE-214) were cultured in Eagle minimal essential medium (EMEM, Invitrogen), supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 7.5% fetal bovine serum (FBS, Euroclone). Cells were grown at 20 °C in a 5% humified CO_2 incubator.

CHSE-214 cells were seeded in 6 wells culture plates and transfected the next day at 80–90% confluence. Transfection was performed by using Lipofectamine 2000 (Invitrogen) transfection reagent, according to the manufacturer's instruction. The total amount of plasmids used for transfection was 4 μg/well. The cells were harvested 20–24 h post transfection in 200 μl Tris lysis buffer (20 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 0,137 M NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 0.5 mM DTT, 1mM sodium orthovanadate, 2 mM NaPPi, 5 mM sodium fluoride), with addition of complete protease inhibitor cocktail (Roche). The lysate was harvested and centrifuged for 15 min at 15,000 × *g*. NuPAGE LDS sample buffer (Invitrogen) was added to the lysate and the sample was heated for 10 min at 70 °C.