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Immune-modulation of two BATF3 paralogues in rainbow trout *Oncorhynchus mykiss*

Jun Wang[a,b], Min Liu[c], Yang Wu[c], Sohye Yoon[a], Abdo Alnabulsi[d], Fuguo Liu[a], Clara Fernández-Álvarez[e], Tiehui Wang[a], Jason W. Holland[a], Chris J. Secombes[a], Jun Zou[a,f,*]

[a] Scottish Fish Immunology Research Centre, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, AB24 2TZ, UK
[b] College of Life Science, Neijiang Normal University, Key Laboratory of Sichuan Province for Fishes Conservation and Utilization in the Upper Reaches of the Yangtze River, Neijiang, 641100, China
[c] College of Animal Science and Technology, Northeast Agriculture University, 59 Mucai Street, Harbin, Heilongjiang Province, China
[d] Division of Applied Medicine, School of Medicine and Dentistry, University of Aberdeen, Aberdeen, UK
[e] Departamento de Microbiología y Parasitología, Edificio CIBUS-Facultad de Biología and Instituto de Investigación y Análisis Alimentarios. Universidade de Santiago de Compostela, Santiago de Compostela, 15782, Spain
[f] International Research Center for Marine Biosciences, College of Aquaculture and Life Science, Shanghai Ocean University, Ministry of Science and Technology, Shanghai 201306, China

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ABSTRACT

Basic leucine zipper transcription factor ATF-like (BATF) -3 is a member of the activator protein 1 (AP-1) family of transcription factors and is known to play a vital role in regulating differentiation of antigen-presenting cells in mammals. In this study, two BATF3 homologues (termed BATF3a and BATF3b) have been identified in rainbow trout (*Oncorhynchus mykiss*). Both genes were constitutively expressed in tissues, with particularly high levels of BATF3a in spleen, liver, pyloric caecae and head kidney. BATF3a was also more highly induced by PAMPs and cytokines in cultured cells, with type II IFN a particularly potent inducer. In rIL-4/13 pre-stimulated cells, the viral PAMPS polyI:C and R848 had the most pronounced effect on BATF3 expression. BATF3 expression could also be modulated in vivo, following infection with *Yersinia ruckeri*, a bacterial pathogen causing redmouth disease in salmonids, or with the rhabdovirus IHNV. The results suggest that BATF3 may be functionally conserved in regulating the differentiation and activation of immune cells in lower vertebrates and could be explored as a potential marker for comparative investigation of leucocyte lineage commitment across the vertebrate phyla.

1. Introduction

Basic leucine zipper transcription factor ATF-like (BATF) proteins are a group of small transcription factors belonging to the activation protein 1 (AP-1) superfamily which consist of several basic leucine zipper (bZIP) transcription factors including FOS, JUN and ATF (Landschulz et al., 1988; Murphy et al., 2013). Three BATF proteins (BATF1-3) have been characterised and all comprise an α-helical bZIP domain which can be further divided into a DNA-binding motif and a leucine zipper motif. The leucine zipper motif is known to be essential for the interaction with bZIP proteins or non-bZIP transcription factors such as interferon regulatory factors (IRFs) to regulate target genes.

The BATF3 gene has been described in mice and humans. It exists as a single copy in chromosome 1 in both species, upstream of another AP-1 family member ATF3 (Murphy et al., 2013). The genes encode a protein of 127 aa and 118 aa respectively, that share high homology (80% similarity), lack a signal peptide and bind to short nucleotide motifs in the promoter region of target genes. The BATF3 gene is expressed mainly in immune cells of hematopoietic organs (Williams et al., 2001), and in particular in dendritic cells (DCs). For example, it can be detected in common DC precursors such as monocytes (which also mature into macrophages) and is induced when DCs differentiate into fully developed conventional DCs (cDCs) (Hildner et al., 2008). In mice, BATF3 is found in both lymphoid-resident CD8α+ cDCs and non-lymphoid CD103+ cDCs which are speculated to share a common origin (Ginhoux et al., 2009; Edelson et al., 2010). T helper cells such as

Abbreviations: AP-1, activator protein 1; BATF3, basic leucine zipper transcription factor ATF-like (BATF) -3; bZIP, basic leucine zipper; CD, cluster of differentiation; DB, DNA binding domain; DC, dendritic cell; HK, head kidney; IFN, interferon; IL, interleukin; IRF, interferon regulated factor; LPS, lipopolysaccharide; LZ, leucine zipper; PAMP, pathogen-associated molecular pattern; PHA, phytohaemagglutinin; polyI:C, polyinosinic:polycytidylic acid; TLR, Toll-like receptor

* Corresponding author.
E-mail address: jzou@shou.edu.cn (J. Zou).

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Th1 and Th17 cells also express BATF3 (Hildner et al., 2008).

The central roles of BATF3 in orchestrating leucocyte lineage commitment have drawn significant attention in recent years. Emerging evidence indicates that BATF3 together with other members of the BATF family play critical roles in regulating leucocyte differentiation, especially in directing the commitment of DC precursors into specific lineages. Gene-knockout studies in mice demonstrate that BATF3 is indispensable for the development of cDCs. The Batf3−/− mice do not develop CD8α+ cDCs which are required for cytotoxic T cell immunity and antiviral defence (Hildner et al., 2008; Sun et al., 2017). In adult mice, the intestinal BATF3-dependent cDCs are required for homeostasis and antiviral T-cell immunity (Edelson et al., 2010; Sun et al., 2017). Further, tissue-resident BATF3-dependent CD103+ DCs once activated can produce large amounts of interleukin (IL)-12, promoting a local Th1 response to combat *Leishmania major* infection (Martinez-Lopez et al., 2015). However, other members of the BATF3 family may also be involved in regulation of immune responses. Recent studies indicate that the roles of BATF3 in promoting expansion of functional CD8+ cDCs to control infection of intracellular pathogens may be compensated by other members of the BATF family via the interaction of the conserved LZ domain with IRF4 or IRF8 (Tussiwand et al., 2012).

A recent study has shown that in rainbow trout (*Oncorhynchus mykiss*) skin CD8a+MHC II+ DC-like cells constitutively express BATF3. This finding is interesting and implies that BATF3 may have conserved roles during vertebrate evolution (Granja et al., 2015). Since no further analysis of BATF3 has been undertaken to date, in this study we determined initially whether other BATF3 paralogues exist in teleost/salmonid fish, as a consequence of the 3rd or 4th whole genome duplication events seen in these species, and analysed the phylogeny of BATF3 in the context of vertebrate phyla. Two BATF3 homologues were identified in rainbow trout and their expression was studied in vivo after bacterial and viral infection and in vitro in cultured monocytes/macrophages after stimulation with TLR ligands, a lectin and interferons (IFNs). The results provide a first insight into the evolution of BATF3 in lower vertebrates and will help develop potential comparative markers to study leucocyte differentiation between fish and higher vertebrates.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) weighing ~100 g were maintained in 1 m diameter tanks supplied with a continuous flow of recirculating freshwater at 15 ± 1 °C in the aquarium facilities in the Zoology building, University of Aberdeen. Fish were fed with commercial trout pellets (EWOS) and acclimated to aquarium conditions for at least 2 weeks before use. Fish were anaesthetised using 2-phenoxyethanol (0.05%, Sigma Aldrich) and killed by subsequent destruction of the brain prior to tissue harvest. All experiments at Aberdeen were carried out under the UK Home Office project license PPL 60/4013. For the infectious hematopoietic necrosis virus (IHNV) challenge experiment, rainbow trout weighing ~3 g were obtained from the cold-water fish experiment station (Mudanjiang, China) and maintained in 120 cm × 50 cm × 60 cm tanks with aeration at 16°C. The fish were fed daily with a dry pellet food and were also acclimated to aquarium conditions for at least 2 weeks before use. The experiment was undertaken according to the guidance of the local animal ethics committee.

2.2. RNA extraction, cDNA synthesis and gene cloning

The trout tissues and cells were collected for extraction of total RNA using TRI Reagent® (Sigma-Aldrich, UK) according to the manufacturer's instructions. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). The cDNA samples were kept at -20 °C before use.

The human BATF3 sequence (GenBank Acc. No., NP_061134) was used as the bait sequence to undertake BLAST (tBLASTn) analysis of the Whole-genome shotgun (WGS) database, transcriptome shotgun assembly (TSA) database and expressed sequence tags (ESTs) database, to obtain the trout BATF3 sequences. The WGS contigs were retrieved and analyzed for prediction of coding sequences using the GenScan program (Burge and Karlin, 1997). Predicted potential coding DNA sequence (CDS) were confirmed for sequence similarity by the BLASTp analysis in the non-redundant protein sequence database. Two WGS contigs (Accession Nos., CCAF010027628.1 and CCAF010060656.1) were identified to contain homologues of BATF3 genes and contained complete coding sequences (CDS) and untranslated regions (UTRs). Primers (supplementary Table 1) located in the 5' and 3' UTRs were designed for amplification of full length cDNA using trout head kidney cDNA as template. The PCR reaction volume was 25 μL including 2 μL of each of the primers (10 μM), 2 μL of cDNA, 5 μL of 5 × MyFi Reaction Buffer, 13 μL of PCR water and 1 μL MyFi DNA Polymerase (Bioline, UK). The PCR reaction conditions were performed using the following program: 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, 62°C for 30 s, 72°C for 1-2.5 min, and a final extension at 72°C for 5 min. The purified PCR products were cloned into the pGEM®-T Easy cloning vector (Promega, UK) and transformed into RapidTrans™ TAM1 competent *Escherichia coli* cells (Active Motif, Belgium). The transformed cells were cultured on LB agar plates (Sigma-Aldrich, UK) with ampicillin (100 μg/mL) overnight at 37°C and colonies were screened by colony PCR using the vector specific primer M13F and a gene specific primer (supplementary Table 1). Plasmid DNA was purified using a QIAprep® spin DNA miniprep kit (QIAGEN, UK) according to the manufacturer's instructions and the size of the inserts was verified by digestion with the restriction enzyme, *Eco*RI (New England Biolabs, UK). Plasmids were sequenced by Eurofins MWG Operon.

2.3. Bioinformatics analyses

The CDS regions and deduced amino acid sequences of BATF3 were analyzed using the ExPASy Translate tool (http://web.expasy.org/translate/) and the homology was analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) against the proteins in the National Center for Biotechnology Information (NCBI). The gene structure was predicted using the Spidey program at NCBI (http://www.ncbi.nlm.nih.gov/spidey/). Genome synteny data were obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html) for Mammalia (human and mouse), Aves (chicken), Amphibia (*Xenopus tropicalis*) and Teleostei. Alignment of protein sequences between *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Chrysemys picta bellii*, *Xenopus tropicalis*, *Danio rerio*, *Oreochromis niloticus*, *Salmo salar* and *O. mykiss* was conducted using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). Protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The tertiary structure of domains was predicted using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels/). Domain identity/similarity was analyzed using Pairwise sequence alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Protein sequences of selected vertebrate BATF1, BATF2 and BATF3 homologues were aligned with the ClustalW program and a phylogenetic tree was constructed using the Mega 6.0 software (Tamura et al., 2013). The neighbour-joining algorithm was used as the clustering method and the distances matrix calculated using the Poisson correction method (Saitou and Nei, 1987). The bootstrap values of tree nodes were obtained by 10,000 bootstrap repetitions using the Poisson model for amino acid substitution.

2.4. Tissue distribution of BATF3

Multiple tissues including brain, intestine, pyloric caeca, gill, thymus, muscle, spleen, liver and head kidney were collected from