Biochemical and Biophysical Research Communications 422 (2012) 602–606

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rnf11-like is a novel component of NF-κB signaling, governing the posterior patterning in the zebrafish embryos

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ARTICLE INFO

Article history:
Received 6 May 2012
Available online 15 May 2012

Keywords:
RNF11
NF-κB
Morpholino
Ikk1
Somite
Zebrafish

ABSTRACT

RING finger protein 11 (RNF11) is a novel regulator of immunity and cell survival via ubiquitination process in mammalian cells whereas its vertebrate embryonic roles are undefined. Here, we are reporting the isolation, expression and functional roles of an RNF11 orthologue, Rnf11-like in zebrafish embryos. Zebrafish Rnf11-like is composed of 154 amino acids containing RING-H2-finger domain in the C-terminal region and PY-motif. Spatiotemporal expression patterns of *rnf11-like* indicate that *rnf11-like* is expressed maternally and zygotically throughout embryogenesis. However, *rnf11-like* transcripts are present specifically in the presomatic mesoderm (PSM), and later in the brain and retina. Knock-down of Rnf11-like using *rnf11-like*-specific morpholino causes cell death and developmental defects in the posterior somites, elevating transcripts of NF-κB target gene, *ikk1*, a negative regulator of NF-κB signaling. All these findings indicate that Rnf11-like is an essential component of NF-κB signaling pathway for specification of the posterior somites in zebrafish embryos.

1. Introduction

The transcription factor NF-κB and transforming growth factor-β (TGF-β) family member proteins are pivotal in regulation of cellular mechanisms, such as cellular differentiation, cell death, innate immunity and inflammatory responses [1–3]. TGF-β1 proteins are present in variety of immune cells including dendritic cells and macrophages and involved in autoimmune diseases and susceptible to infections [4]. NF-κB signaling is largely involved in immunity via Nuclear Factor κB (NF-κB), which is activated by various external factors, such as pathogenic infections, inflammation and sepsis [5,6].

Regulation of TGF-β family member proteins and NF-κB is critical to development of immunity, in particular to inflammation and tolerance. A recent study shows that NF-κB signaling synergistically regulates TGF-β signaling in the lung fibroblasts [7]. Smads, elements of TGF-β signaling regulates the immunity in cells via controlling the production of interleukin [8].

Ubiquitin–proteasome system regulates NF-κB signaling by involving IκB, the NF-κB inhibitor [9]. Deubiquinating enzyme TNFaip3 (A20) has been reported as negative regulator of NF-κB signaling pathway. A20 knockout mice have robust inflammation in all organs with constitutive activation of NF-κB signaling [10,11]. A20 performs its regulatory roles in association with other ubiquitin ligases AIP4 (Itch), TAX1BP1 and RNF11 [12,13].

RNF11 is also involved in the regulation of TGF-β and NF-κB signaling [14]. RNF11 modulates TGF-β signaling by direct interaction with Smad4, Smurf1 and 2 [15,16]. RNF11 negatively regulates NF-κB signaling by stabilizing the A20 interaction with RIP1 [13]. Human RNF11 containing N-terminal RING domain and PY domain was originally identified in the tumor cells [15]. RNF11 is expressed in the vulnerable neurons in Parkinson's disease brain [17]. Cell based functional studies of RNF11 indicate that it is an E3 enzyme involved in inflammation and immunity [13]. However, in vivo functions and regulatory roles of RNF11 remains to be further investigated. Here we studied functional roles of Rnf11-like using zebrafish (*Danio rerio*) embryos [18].

In this report we identified and isolated *rnf11-like* gene from zebrafish. We examined spatiotemporal expression and in vivo functions of *rnf11-like* in the zebrafish embryos.

2. Materials and methods

2.1. Sequence analysis

Rnf11-like protein sequence homology and functional motifs were analyzed with ClustalX and Motifscn (http://scansite.mit.edu/), respectively. Phylogentic relationship was analyzed with MEGA5 [19]. Snytenic analysis of *rnf11-like* synteny was performed at

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.034

http://teleost.cs.uoregon.edu/synteny_db with the sliding window size of 25 genes.

2.2. RNA isolation, cDNA preparation and RT-PCR

Total RNA was extracted from various embryonic stages with TRI reagent (SIGMA). cDNA synthesis and RT-PCR were performed as previously described [20].

2.3. Cloning and in vitro transcriptions

rnf11-like cDNA was amplified from the total RNA of the embryos at 24 h post fertilization (hpf) with specific forward primer 5′-ATGGGGAATTGTCTGAAATCT-3′ and reverse primer 5′-AGTT AGTTTCGTAGGATGA-3′. DNA sequences of the amplified cDNA were cloned into pGEM-T Easy (Promega, USA). Partial DNA sequences of zebrafish *Ikk1* (Gene bank number: AY735397) were amplified with forward primer 5′-ACGGACGCAAGATGGAGAAACC-3′ and reverse primer 5′-GAGATCCCTGTGTATGATCTTAT-3′ and cloned into pGEM-T Easy vector. Antisense digoxigenin (DIG) probes of *rnf11-like* and *ikk1* for WISH were synthesized according to the instructions of TranscriptAid™ T7/SP6 High Yield Transcription Kit (Fermentas).

2.4. Animal and embryo care

Zebrafish provided by the Korea Zebrafish Organogenesis Mutant Bank (ZOMB) was maintained as described [18]. Embryos were obtained by natural breeding process and incubated in the egg water at 28.5 °C. Embryos were staged as described [18] and treated with 0.002% phenylthiourea (PTU) to inhibit pigmentation process from early onset of somitogenesis.

2.5. Morpholinos, whole-mount in situ hybridization (WISH) and acridine orange staining

rnf11-like-specific morpholinos (*rnf11-like* MO) were purchased from Gene-Tools (Corvallis, OR). To block translation of *rnf11-like* mRNAs, we injected *rnf11-like* MO (ATG targeting) and control morpholino containing 5 miss-match into embryos at one cell stage. 10 ng of *rnf11-like* MO is sufficient to eliminate the endogenous *rnf11-like* transcripts (Supplementary Fig. 1). 2 ng of *p53*-specific morpholino (*p53* MO, a generous gift from Dr. Cheol-Hee Kim, Chungnam National University, South Korea) was coinjected with *rnf11-like* MO. *rnf11-like* MO sequence: 5′-CCTTTCCAAACTCCTTGTT GTCTAA-3′, *rnf11-like* control MO sequence: 5′-CCTaTgCAAAgTCCT TcTTcTCTAA-3′, *p53* MO sequence: 5′-TTGATTTTGCCGACCTCCTC TCCAC-3′. WISH was performed as previously described [21,22]. Injected embryos were subjected to acridine orange staining for detection of cell death as described [23].

3. Results and discussions

3.1. Identification, isolation and structural characterization of zebrafish Rnf11-like

In order to identify human RNF11 orthologue in zebrafish genome we used human RNF11 (Gene number: NP_055187.1) composed of 154 amino acids. Two orthologues found in the zebrafish genome encode 154 and 146 amino acids on chromosomes 8 and 1, and named as *rnf11-like* (gene accession numbers: NM_0011 28408) and *rnf11* (gene accession numbers: NM_201021) in NCBI database, respectively.

Rnf11 shares 80% amino acid sequence homology with Rnf11-like (Supplementary Fig. 2). Human RNF11 shares 82% and 93% amino acid sequence homology with Rnf11 and Rnf11-like, respectively (Supplementary Fig. 3). We also confirmed the amino acid sequences of Rnf11-like in ENSEMBL database as ID ENSDART00000098691. Both NCBI GenBank and ENSEMBL databases demonstrated its genomic organization made of 1698 base pairs containing 3 exons, generating the 464 nt-long open reading frame. Rnf11-like contains the PY-motif (P-Proline and Y-Tyrosine), myristoylation site in the N-terminal region, and RING domain in the C-terminal region (Supplementary Fig. 3) as human RNF11 does [16]. We accordingly isolated cDNA encoding Rnf11-like from zebrafish embryos and confirmed that DNA sequences of zebrafish *rnf11-like* are well-matched to those of the *in silico* identified *rnf11-like*.

3.2. Zebrafish Rnf11-like is orthologue to human RNF11

We analyzed molecular phylogenetic relationship of Rnf11-like with other vertebrate homologues using Maximum Likehood Algorithm method. It turned out that zebrafish Rnf11-like and Rnf11 are branched from human RNF11, and Rnf11 is sub-branched from Rnf11-like (Fig. 1B). This suggests that zebrafish Rnf11-like is paralogous to Rnf11, and orthologous to human RNF11.

Synteny-based analysis of zebrafish *rnf11-like* shows strong syntenic conservation between human chromosome 1 and zebrafish chromosome 8. In particular, the region around *rnf11-like* shows high degree of syntenic relation with the 8 conserved human genes (CDKN2C, FAF1, DMRTA2, ELAVL4, BEND5, AGBL4, SPATA6 and SLC5A9) in the left flanking region and 3 conserved human genes (TTC39A, EPS15 and OSBPL9) on right side of zebrafish chromosome 8 (Fig. 1C). This syntenic conservation supports that *rnf11-like* is highly conserved in the evolution and orthologous to human RNF11.

3.3. Spatiotemporal expression of zebrafish rnf11-like

Analysis of the temporal distribution of *rnf11-like* transcripts along the developmental process shows that they are abundantly present as maternal and zygotic transcripts throughout the embryogenesis (Fig. 2A). Spatiotemporal expression pattern of *rnf11-like* transcripts using WISH confirmed that maternal transcripts of *rnf11-like* are evenly distributed from one cell to sphere stage (Fig. 2B, C). Zygotic transcripts of *rnf11-like* lessen at dome stage (Fig. 2D). *rnf11-like* transcripts are evenly distributed along the anteroposterior axis at early somitogensis (Fig. 2E), but gradually increased at mid-somitogenesis (Fig. 2F, G). At 22 h post-fertilization (hpf), *rnf11-like* is expressed in the margin of the neural tube, developing retina, anterior somites, and presomatic mesoderm (PSM) in the posterior somites (Fig. 2H, K). At 48 hpf, *rnf11-like* transcripts are restricted to the brain region but prominently concentrated in the eye lens (Fig. 2L, M). It is worthwhile to note that spatiotemporal expression pattern of *rnf11-like* is tightly regulated throughout the embryogenesis while *rnf11* transcripts are ubiquitously expressed [24]. PSM has been implicated as a critical element to the segmental clock to produce somites from the mesodermal progenitor cells (MPCs), and also associated with skeletal myogenesis [25–27]. It thus become of great interest to examine regulatory roles of Rnf11-like in somite formation.

3.4. Rnf11-like is required for cell survival and somite formation in the posterior region

Upon the knock-down of *rnf11-like* expression using *rnf11-like* morpholino (*rnf11-like* MO) we observed severe morphological alteration in the forming somites of the posterior region (Fig. 3A, F). As an initial approach to investigate biological cause for the morphological changes, we examined cellular death in *rnf11-like*