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Characterization of the molecular structure, expression and bioactivity of the TNFSF13B (BAFF) gene of the South African clawed frog, *Xenopus laevis*

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ABSTRACT

B cell activating factor (BAFF), a member of the tumor necrosis factor family, is critical to B cell survival, proliferation, maturation, and immunoglobulin secretion and to T cell activation. In the present study, the full-length cDNA of BAFF from the South African clawed frog (*Xenopus laevis*, designated xlBAFF) was cloned using rapid amplification of cDNA ends (RACE) techniques and RT-PCR. The full-length cDNA of xlBAFF consists of 1204 bases including an open reading frame (ORF) of 801 nucleotides that are translated into a predicted 266 amino acid protein. Sequence comparison indicated that the amino acids of xlBAFF possessed the TNF signature, including a transmembrane domain, a putative furin protease cleavage site and three cysteine residues. The predicted three-dimensional (3D) structure of the xlBAFF monomer revealed that it was very similar to its counterparts. Real-time quantitative PCR analysis revealed that xlBAFF could be detected in various tissues and predominantly expressed in the spleen and other lymphoid tissue. The soluble xlBAFF had been cloned into a pET28a vector to express the recombinant protein. The His_6-xlBAFF was efficiently expressed in *Escherichia coli*. BL21 (DE3) and its expressions were confirmed by SDS-PAGE and Western blotting analysis. After purification, laser scanning confocal microscopy analysis showed that xlBAFF could bind to its receptors on B cells. CCK-8 assays revealed that xlBAFF is not only able to promote survival/proliferation of South African clawed frog lymphocytes but also able to stimulate survival/proliferation of mouse B cells. These results will allow for further investigation the use of *X. laevis* as an *in vivo* model for related studies.

1. Introduction

Members of the tumor necrosis factor (TNF) superfamily and their receptors have been shown to play important roles in the immune system [1]. B-cell activating factor (BAFF, also known as BLyS, TALL-1, THANK, zTNF4, or TNFSF13b) [2–5], is considered an important member of the TNF family of cytokines that plays a major role in B-cell survival, proliferation and differentiation [6–8]. BAFF is a type II single transmembrane protein that can function in the membrane-bound form or as a soluble trimeric ligand upon proteolytic processing at a furin consensus site in the C-terminal extracellular domain [9]. It is mainly produced by innate immune cells such as monocytes, dendritic cells (DCs), follicular DCs (FDCs) and some T cells [2,10,11]. The production of BAFF by innate immune cells and some lymphocytes plays a central role in immune responses. The crystal structure of soluble BAFF shows that it has an unusually long D–E loop (also known as the "flap") relative to other TNF family members, which forms a region that may be important for receptor binding and virus-like assembly [12,13].

Released soluble BAFF binds to three cell-surface receptors: BAFF-receptor (BAFF-R or BR3), B cell maturation Ag (BCMA), transmembrane activator and calcium-modulating/cyclophilin ligand-interacting protein (TACI) [14–16]. BAFF-R and BCMA are expressed exclusively in B cells, whereas TACI is expressed in B cells and activated T cells [17–20]. BAFF is the unique ligand for BAFF-R [18], while it shares receptor specificity for TACI and BCMA with a proliferation-inducing ligand (APRIL) [21]. Recent data reveals that BAFF-R may be the primary BAFF binding receptor responsible for B-cell development and survival [18]. Emerging reports on the role of TACI in B-cell survival suggest an important negative regulatory role for this receptor in B-cell homeostasis and autoimmunity because TACI-deficient mice have an accumulation of splenic B cells and increased levels of serum immunoglobulin levels [22,23]. Recent data shows that BCMA function is likely restricted to germinal center B cells or terminally different plasma cells [17,24–27].

In vitro, soluble BAFF was found to promote B-cell survival and costimulate B-cell proliferation with anti-IgM or *Staphylococcus aureus* Cowan I (SAC I) [2,4]. *In vivo* administration of recombinant BAFF in mice promotes B-cell survival, expansion and differentiation, whereas BAFF transgenic mice develop hypergammaglobulinemia, proteinuria,

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vasculitis and autoimmune diseases such as systemic lupus erythematosus (SLE) and, at a later stage, SjoÈgren's syndrome (SS), suggesting that BAFF has a key role in autoimmune disease [28,29]. In contrast, BAFF-deficient mice exhibit dramatically reduced B cell numbers peripherally and impaired development of germinal center responses [30,31]. Indeed, BAFF is important for maintaining peripheral B-lymphocyte homeostasis and enhancing antigen-specific humoral immunity and also acts as a T-cell co-stimulatory factor *in vitro* [32]. Current information demonstrates that BAFF is a very important factor controlling several aspects of B cell biology with the potential to break immune tolerance when over expressed. These activities indicate that BAFF might have an adjuvant-like effect on the immune system to boost immunity.

Human BAFF was first identified by sequence homology as a possible novel member of the TNF ligand family [4]. With the development of the technique of gene cloning, molecular techniques have recently enabled the identification of other cytokine genes. Till now, BAFF genes have been identified and characterized in mammals, birds, reptiles, and fishes. However, still little is known about the characterization and function of amphibian BAFF *in vitro* and *in vivo*. Whether the function of amphibian BAFF is the same as mammalian BAFF in the immune response remains unknown. South African clawed frog (*Xenopus laevis*) is an amphibian that last shared a common ancestor with mammals 350 million years ago, and now it is one of the most important model animals in life science. In this study, we cloned the full-length cDNA of the BAFF gene (here designated xlBAFF) from the *X. laevis*, a species representative of amphibians. To our knowledge, this is the first BAFF gene to be cloned from amphibians. Phylogenetic analysis, comparisons of 3D structure, and analyses of expression and bioactivity of the BAFF from the *X. laevis* were conducted. This study may help to elucidate the character of immunity in the *X. laevis* and the evolutionary history of amphibian immunity.

2. Materials and methods

2.1. Animal and cell preparations

Adult South African clawed frogs (50–80 g) were obtained from the Animal Research Center of Nanjing University, China. Splenic lymphocytes were separated from spleen using Lymphocyte Separation Medium (BD Pharmingen, USA) according to the manual. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (P/S) at 28 °C. ICR mice were obtained from the Experimental Animal Center of Nanjing Medical University, Nanjing, China. Mouse B cells were isolated from spleen using B cell specific antibodies B220 (CD45R) coupled to magnetic beads (Miltenyi Biotech). The mouse B cells were maintained in RPMI1640 medium with penicillin/streptomycin (Gibco-BRL, USA) supplemented with 10% FCS at 37 °C in an atmosphere of 5% CO_2. The use of all South African clawed frogs and mice in this study has been approved by the scientific ethical committee of the Nanjing Normal University.

2.2. RNA isolation and reverse transcription PCR (RT-PCR)

Spleens from South African clawed frog were collected, immediately snap frozen by liquid nitrogen and stored at −80 °C until total RNA extraction. The total RNA was extracted using the RNA prep pure Tissue Kit (Tiangen Biotech Co. Ltd) according to the manufacturer's protocol. A first-strand cDNA was synthesized from 1 μg of RNA isolated from spleen using Reverse Transcriptase XL (Takara, Japan) according to the manufacturer's protocol. Two pairs of degenerate primers, F1/R1 and F2/R2 (Table 1), were designed based on multiple alignments of the highly conserved TNF domains of Chinese alligator (GU126733), chicken (AY263378), dove (EU334145), and duck (DQ445092). Nested PCR was used and PCR conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, and then 72 °C for 10 min. The RT-PCR product, the expected size 330 bp, was gel-purified, cloned into the pMD19-T vector (Takara, Japan), and sequenced.

2.3. Rapid amplification of cDNA ends (RACE)

The RACE technique was used to obtain the full-length cDNA of xlBAFF, complete with 5′ and 3′ untranslated regions (UTRs). Primers were further designed based on the obtained partial cDNA sequences for 5′ and 3′ rapid amplification of cDNA ends. Nested PCR was used for RACE PCR. Two sets of gene-specific primers (GSP), 5′GSP/5′NGSP and 3′GSP/3′NGSP (Table 1), were designed to clone the 5′ and 3′ ends of the xlBAFF cDNA, respectively. For each 5′ and 3′ RACE, the cDNA was synthesized using the SMART™ RACE cDNA Amplification Kit (Clontech, Japan) according to the manufacturer protocol. For RACE-PCR, SMARTer II A oligonucleotide, UPM, NUP, and four gene-specific primers (3′GSP and 3′NGSP for 3′RACE, 5′GSP and 5'NGSP for

Table 1
Primers used for cloning and expression analysis of the *Xenopus laevis* BAFF gene. (F forward primer and R reverse primer).

Primer	Sequence(5′-3′)	Amplification
F1	AAGAACAAGTCTTCCATTCTTGCTT	Homologous fragment
R1	ACTCAGTGATATCTTTGTTTTCCTT	Homologous fragment
F2	TGCTTGCAATTGATTGCTGAT	Homologous fragment
R2	CCTTCTTCTAATTTTGCAATGCCA	Homologous fragment
5′GSP	GGTGGCATATTCTGAATGCACTTAAA	5′RACE
5′NGSP	GGATCATCACCCACTTTCTGGGCCTTT	5′RACE
3′GSP	GGGCAGGTTTGGTTCACAGATAAAGTGTT	3′RACE
3′NGSP	GAAAGTGGGTGATGATCCCAGTTTAGTGA	3′RACE
F3	ATGACTTCTAAAAACTATTTTCCAA	ORF of xlBAFF gene
R3	TCACAAAATTTTAATTGCTCCAAAA	ORF of xlBAFF gene
F4	GGGAATTCCATATGTTTGTTTCTGGAACTCAAGAACAAGTCTT	Expression of xlBAFF
R4	CCGCTCGAGTCACAAAATTTTAATTGCTCCAAAAAATGT	Expression of xlBAFF
A1	CGCCATATGGTGAGCAAGGGCGAGGA	Expression of GFP/xlBAFF
A2	GCTGCCACCTCCACCCTTGTACAGCTCGTC	Expression of GFP/xlBAFF
B1	GGTGGAGGTGGCAGCTTTGTTTCTGGAACTCA	Expression of GFP/xlBAFF
B2	CCGCTCGAGTCACAAAATTTTAATTGCTCCAAAAAATGT	Expression of GFP/xlBAFF
F5	TGATGGCTCTGATGGAATGA	Real time-PCR
R5	AAAGCTTAGCAGCCACGGTA	Real time-PCR
β-actin-F	CATGGACTCAGGTGATGGTG	Real time-PCR control
β-actin-R	GCTGTGGTGGTGAAGCTGTA	Real time-PCR control
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′RACE and 3′RACE in contech
UPM (short)	CTAATACGACTCACTATAGGGC	5′RACE and 3′RACE in contech
NUP	AAGCAGTGGTATCAACGCAGAGT	5 RACE and 3′RACE in contech