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Molecular structure, expression analysis and functional characterization of APRIL (TNFSF13) gene in bat (*Vespertilio superans* Thomas)

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ABSTRACT

A proliferation-inducing ligand (APRIL) is a novel member of the tumor necrosis factor (TNF) superfamily, which is involved in immune regulation. In the present study, the full-length cDNA of APRIL (designated bAPRIL) from bat was cloned using RT-PCR and its biological activities have been characterized. The open reading frame (ORF) of this cDNA consists of 753 bases, encoding a protein of 250 amino acids. This protein was found to contain a predicted transmembrane domain, a putative furin protease cleavage site, and a typical TNF homology domain corresponding to other, known APRIL homologs. Real-time quantitative PCR (qPCR) analysis indicated that bAPRIL mRNA was predominantly expressed in bat lymphoid tissue spleen. The SUMO–bsAPRIL was efficiently expressed in *Escherichia coli* BL21 (DE3) and confirmed by SDS-PAGE and Western blot analysis. Laser scanning confocal microscopy analysis showed that bsAPRIL could bind to its receptors on B cells. In vitro, MTT assays indicated that bsAPRIL could promote the survival/proliferation of mouse splenic B cells grown with anti-mouse IgM. These findings indicate that bsAPRIL plays an important role in the survival and proliferation of B cells and has functional cross-reactivity among mammalians. The present findings may provide valuable information for research into the immune system of the bat.

1. Introduction

Members of the tumor necrosis factor (TNF) family and their receptors are important regulators of the immune system (Locksley et al., 2001). APRIL (A proliferation-inducing ligand, also named TALL-2, TRDL-1 and TNFSF-13a) is a type II transmembrane protein which belongs to the TNF family (Hahne et al., 1998). It does not exist as a membrane-bound form, but is processed intracellularly within the Golgi apparatus by a furin pro-protein convertase prior to secretion of the biologically active form (Lopez-Fraga et al., 2001). It shares ~30% sequence identity in the TNF domain with BAFF (B cell activating factor, also named BLyS, THANK, TALL-1, and zTNF-4), another important ligand of the TNF family, and they share two receptors, BCMA (B cell maturation antigen) and TACI (transmembrane activator and cyclophilin ligand interactor) (Bossen and Schneider, 2006; Mackay et al., 2003). APRIL and BAFF and their receptors, also termed "the BAFF/APRIL system", play important immunological roles, especially in the B-cell arm of the immune system (Mackay et al., 2003, 2007).

Although both APRIL and BAFF can be expressed in monocytes, dendritic cells, macrophages and T cells, APRIL is abundantly expressed in cells outside the immune system, including osteoclasts and tumor tissues (Hahne et al., 1998; Moreaux et al., 2005). These results suggest that the physiological function of APRIL is distinct from other members of the TNF family, and that APRIL may be implicated in the regulation of tumor cell growth (Hahne et al., 1998). However, neither TACI nor BCMA appears crucial to the tumor-promoting effects of APRIL, because these tumor cells, such as lung carcinoma A549 cell and Jurkat T leukemia cell, lack both of them, indicating the existence of a third receptor that is specific for APRIL (Rennert et al., 2000). Recently, reporters demonstrated that heparin sulfate proteoglycans (HSPGs) are the novel 'binding partner' for APRIL, and a basic sequence (QKQKKQ) seems to be responsible for binding HSPG (Guan et al., 2006; Hendriks et al., 2005; Ingold et al., 2005). APRIL serves an important role in immunological responses, such as B-cell survival, Ig secretion, isotype switching, and T-independent antibody responses (Castigli et al., 2004; Dillon et al., 2006; Litinskiy et al., 2002; Mackay and Leung, 2006; Stein et al., 2002). These activities indicate that APRIL might have an adjuvant-like effect on the immune system to enhance antigen-specific humoral immunity.

Bat (*Vespertilio superans* Thomas) is not only the second largest mammalian order but also the most gregarious of all mammals,

Abbreviations: APRIL, A proliferation-inducing ligand; bp, base pair(s); BAFF, B cell activating factor; BCMA, B cell maturation antigen; cDNA, DNA complementary; DMSO, dimethylsulfoxide; dNTP, deoxyribonucleoside triphosphate; HSPG, heparin sulfate proteoglycan; IPTG, final isopropyl-beta-D-thiogalactopyranoside; kDa, kilodalton(s); LB, Luria-Bertani (medium); FBS, fetal bovine serum; FITC, fluoresceine isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PI, isoelectric point; qPCR, real-time quantitative PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; SUMO, small ubiquitin-like modifier; TACI, transmembrane activator and cyclophilin ligand interactor; TMD, transmembrane domain; TNF, tumor necrosis factor.

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which is the only flying mammal. The immune study is essential for the protection of bat. In this study, we cloned the full-length cDNA of the APRIL gene (designated as bAPRIL) from the bat. Phylogenetic analysis, analyses of expression and bioactivity of the APRIL gene from the bat were conducted. This work may provide the basis for investigations on the character of bat immunity.

2. Materials and methods

2.1. Animal and cell preparations

Adult bat (*Myotis lucifugus*) was obtained from the Jiangsu Key Laboratory for Biodiversity and Biotechnology, Nanjing Normal University, China. ICR mice (20–25 g) were obtained from the Experimental Animal Center of Nanjing Medical University, Nanjing, China. Mouse splenocytes were prepared by disruption of fragments of freshly dissected mouse spleen under sterile conditions followed by filtering of the cell suspensions through sterilized nylon mesh. B-cell samples of >97% purity were isolated from the fresh splenic cell suspensions using anti-CD19 magnetic fluorobeads as described (Schneider et al., 1999; Shu et al., 1999). The B cells were maintained in RPMI 1640 medium with penicillin and streptomycin (Gibco-BRL, U.S.) supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol at 37 °C in an atmosphere of 5% CO_2.

2.2. Tissue sampling, RNA isolation, and RT-PCR

Various tissues including spleen, kidney, liver, heart, lung and intestine from bat were collected, immediately snap-frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. The total RNA was extracted using the RNAprep pure Tissue Kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instruction. First-strand cDNA was synthesized from 1 μg RNA isolated from spleen using a TaKaRa PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacturer's instruction. A pair of degenerate primers, bAPRIL1 (5′-ATGCCRGCYTCATCTCCTTCCTTGC-3′, Y = C/T) and bAPRIL2 (5′-TCACAGTTTCACAAVYCCCAGGAAG-3′, Y = C/T, V = A/C/G), was designed based on multiple alignments of the highly conserved TNF domains of dog, horse, cattle, and pig (GenBank accession nos. EU909456.1, GU982935.1, FJ539091.1, and NM_001112690.1, respectively). PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s, and then 72 °C for 10 min. The RT-PCR product was gel-purified, cloned into the pMD19-T vector (TaKaRa, Japan), and sequenced.

2.3. Bioinformatics analyses

The searches for nucleotide and protein sequence similarities were conducted using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/), while the putative signal peptide was predicted using SignalP (www.cbs.dtu.dk/services/SignalP). The protein domain features of bAPRIL were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and the predicted transmembrane domain was based on SOUSI software (http://bp.nuap.nagoya-u.ac.jp/sosui/). Molecular weight and theoretical isoelectric point were predicted using compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Multiple sequence alignment was performed using the ClustalW (version 1.83) multiple alignment program (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994). Percentage identity was calculated using the FASTA program. The phylogenetic tree was constructed from the ClustalW-generated alignments using the neighbor-joining method and MEGA4.0 program (Tamura et al., 2007). The reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

2.4. Quantifications of bAPRIL mRNA expression by real-time PCR

To evaluate the tissue expression profile of bAPRIL mRNA, SYBR® Green I based quantitative PCR (qPCR) was carried out using SYBR® Premix Ex Taq™ (Perfect Real Time, Takara, Japan). Total RNA from various tissues was extracted as described above. Briefly, first-strand cDNAs were synthesized using PrimeScript™ RT Reagent Kit (Takara, Japan). A pair of primers, bAPRIL3 (5′-CAGCAAACAGAGCTGCAGAG-3′) and bAPRIL4 (5′-ATGTTAACGGGGACGAGATG-3′), was used to amplify a product of 224 bp. A constitutive expression gene, the glyceraldehyde phosphate dehydrogenase (GAPDH), was used as an internal control. Two primers G1 (5′-GAGCTGAATGGGAAGCTCAC-3′) and G2 (5′-GGAGGAGTGGGTGTCACTGT-3′) were used to amplify a 213 bp fragment of bat GAPDH cDNA. The SYBR Green real-time PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) as described (Guan et al., 2007). Real-time qPCR for each tissue was repeated three times with three parallel reactions each. Values are expressed as mean counts ± standard error, estimated using Student's *t*-test in the software STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, U.S.).

2.5. Construction of expressing vector pSUMO–bsAPRIL

To enhance the soluble expression, we fused the extracellular region of the bAPRIL gene with SUMO (The effect that SUMO has on enhancing protein solubility can be explained in part by its structure. SUMO has a hydrophilic external surface and hydrophobic inner core. These exert a detergent-like effect on otherwise insoluble proteins.). A fragment of 453 bp, predicted to encode the bsAPRIL domain, was generated using PCR with the forward primer bsAPRIL1 (5′-TCGGAG-GAGGAGAGCAGTGCTCGCCC-3′) and reverse primer bsAPRIL2 (5′-CCCAAGCTTTCACAGTTTCACAAACCCCAGGAAG-3′). These introduced unique *StuI* and *HindIII* restriction sites, respectively. Following digestion with *StuI* and *HindIII*, the PCR product was then cloned into the pE-SUMO expression vector (LifeSensors, U.S.), forming a sequence encoding a fusion protein of bsAPRIL and an NH_2-terminal His_6-tag. This recombinant plasmid was sequenced on both strands and termed pSUMO–bsAPRIL.

2.6. Expression, purification, and Western blotting of recombinant bsAPRIL

The constructed recombinant plasmid, pSUMO–bsAPRIL, was transformed into competent *Escherichia coli* BL21 (DE3) cells (Novagen, U.S.), cultured in Luria-Bertani (LB) medium for kanamycin resistance with vigorous shaking (200 rpm) at 37 °C to the density of $OD_{600} \approx$ 0.6. Then, the induction scheme was established as follows: final isopropyl-beta-D-thiogalactopyranoside (IPTG) concentration, 0.2 mM; induction temperature, 16 °C; duration of induction, 24 h; and shaking speed, 100 rpm. After induction, cells were harvested by centrifugation at 6000 *g* for 10 min at 4 °C. The soluble protein SUMO–bsAPRIL in the supernatant was collected by refrigerated centrifugation at 12,000 *g* for 15 min at 4 °C after sonication. Finally, the target protein was purified with His-Bind Columns (Qiagen, Germany) according to the manufacturer's instruction. The expression of His_6-tagged SUMO–bsAPRIL was analyzed by SDS-PAGE and Western blotting with an anti-His_6-tag mouse antibody (Invitrogen, U.S.). The SUMO proteins from the BL21 (DE3) strain transformed with empty pE-SUMO plasmids were prepared and used as control proteins.

2.7. Laser scanning confocal microscopy

The mouse B cells (1×10^5) were separated from the spleen using anti-CD19 magnetic fluorobeads. They were cultured in RPMI 1640