Developmental and Comparative Immunology 34 (2010) 1199–1208

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Molecular structure, expression, cell and tissue distribution, immune evolution and cell proliferation of the gene encoding bovine (*Bos taurus*) TNFSF13 (APRIL)

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ARTICLE INFO

Article history:
Received 7 April 2010
Received in revised form 21 June 2010
Accepted 21 June 2010
Available online 6 July 2010

Keywords:
Bovine APRIL
Molecular structure
Gene expression
Immune evolution
EGFP
Cell proliferation

ABSTRACT

A novel bovine cDNA has been isolated by EST assembly and subsequently confirmed by using RT-PCR and designated bovine A Proliferation-Inducing Ligand belonging to TNF family (bAPRIL). The open reading frame (ORF) of this cDNA covers 753 bp, encoding 250 amino acids. The functional soluble part of bAPRIL (bsAPRIL) shows 97% and 92% identity with its pig and human counterparts, respectively, at the level of the primary protein structure. The bAPRIL genomic sequence consists of six exons and five introns, is approximately 1.8 kb in size, and maps to bovine chromosome 19q. Real-time quantitative PCR (qPCR) analysis revealed that bAPRIL is predominantly expressed in bovine lymphoid tissues spleen. The predicted three-dimensional (3D) structure of the bsAPRIL monomer analyzed by "comparative protein modelling" revealed that it is very similar to its mouse counterpart. The bsAPRIL and EGFP/bsAPRIL were efficiently expression in *Escherichia coli* BL21 (DE3) and its expression was confirmed by SDS-PAGE and Western blotting analysis. After purification, the EGFP/bsAPRIL fusion protein obtained similar fluorescence spectrum to those of EGFP. Laser scanning confocal microscopy analysis showed EGFP/bsAPRIL could bind to its receptor. *In vitro*, bsAPRIL could promote the proliferation of bovine or mouse splenic B cells together with/without SAC or anti-mouse IgM. Furthermore, compared to mouse soluble APRIL, the bovine soluble APRIL has the similar proliferation to mouse B cell. Those findings indicated that bsAPRIL plays an important role in proliferation of bovine B cells and has functional cross-reactivity among cow and other mammalians. Therefore, APRIL may be a potential immunologic factor for enhancing immunological efficacy in animals.

1. Introduction

APRIL (A Proliferation-Inducing Ligand, also named TALL-2, TRDL-1 and TNFSF-13a) is a type α membrane 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). Two TNF receptor family members, B-cell maturation antigen (BCMA) and transmembrane activator and cyclophilin ligand interactor (TACI), bind to APRIL with high affinity (Rennert et al., 2000; Yu et al., 2000; Wu et al., 2000; Marsters et al., 2000). Both receptors are shared with BAFF (B-cell activating factor of the TNF family, also named BLyS, THANK, TALL-1, zTNF-4), another important ligand of the TNF family. 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). Recent studies have localized APRIL expression to immune cell subsets that also produce BAFF: monocytes, dendritic cells, macrophages and T cells. In addition, APRIL is expressed in cells outside the immune system, including osteoclasts and tumor tissues. APRIL binds to proteoglycan structures on the cell surface (Hendriks et al., 2005; Ingold et al., 2005). However, the relevance of this binding is unclear, but may serve to accumulate and/or multimerize APRIL in the extracellular matrix or at the surface of syndecan-positive cells. It thus may facilitate access to its receptor TACI, which also interacts with syndecans, or to intracellular BCMA upon syndecan internalization (Dillon et al., 2006; Bossen and Schneider,

Abbreviations: EST, expressed sequence tag; RNA, ribonucleic acid; cDNA, DNA complementary to RNA; IPTG, isopropy-b-D-thiogalactoside; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; bsAPRIL, bovine soluble APRIL; EGFP, Enhanced green fluorescent protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; IgG, immunoglobulin G; IgM, immunoglobulin M; SAC, Staphylococcus aureus Cowan 1; PI, propidine iodide.

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doi:10.1016/j.dci.2010.06.015

2006). The latest research advancements revealed that neutrophil-derived APRIL accumulated on tumor cells via proteoglycan binding correlates with human B-cell lymphoma aggressiveness (Schwaller et al., 2007) and simultaneous binding of TACI and HSPG on B cells with APRIL is crucial for IgA production (Sakurai et al., 2006). APRIL serves an important role in immunological responses, such as the contribution to B-cell survival, Ig secretion, isotype switching and T-independent antibody responses (Dillon et al., 2006; Stein et al., 2002; Litinskiy et al., 2002; Castigli et al., 2004; Mackay and Leung, 2006). These activities indicate that APRIL might have an adjuvant-like effect on the immune system to enhancing antigen-specific humoral immunity.

The cow (*Bos taurus*) is an important model organism for biomedical research and development, in addition to being important agriculturally. In this study, based on EST assembly, the cDNA of bovine APRIL (bAPRIL) was predicted and subsequently confirmed by its cloning using RT-PCR from total RNA of spleen. Furthermore, we reported for the first time the genomic structure, gene copy number, tissue expression, evolution, interaction of ligand/receptor in B cell and cell proliferation activity.

2. Materials and methods

2.1. Animal and cell preparations

Cows were maintained in Red-sun breed cultivation farm, Jiangsu, China. Bovine splenocytes were prepared by disruption of fragments of freshly dissected bovine spleen under sterile conditions followed by filtering of cell suspensions through a 0.45-mm nylon mesh, lysis of erythrocytes, and resuspension in RPMI1640/10% fetal calf serum (FCS). Purified bovine B cells were prepared from erythrocyte-depleted splenocyte suspensions by incubation at room temperature for 30 min in 10-cm tissue culture dishes that were coated with mouse anti-bovine IgG (Sigma, USA), as described by (Kanaan et al., 2003). Non-adherent cells were removed by repeated gentle washing with PBS following which adherent B cells were removed by flushing the plates with culture medium. ICR mice of 20–25 g were obtained from Experiment Animal Center of Nanjing Medical University, Nanjing, China. The mice spleens were collected under sterile conditions. Spleens were minced into pieces with dissecting scissors, followed by further grinding and filtering through sterilized nylon membranes and washed in Hank's solution (pH 7.2). Via twice centrifugations at 1500 rpm for 5 min after erythrocytes were dissolved with 0.85% amchlor solution, the isolated cells are fresh splenic cells. B cells of >97% purity were isolated from the fresh splenic cell suspensions using anti-CD19 magnetic fluorobeads as described (Moore et al., 1999; Schneider et al., 1999), All cells were maintained in RPMI1640 medium with penicillin/streptomycin (Gibco-BRL, USA) supplemented with 10% FCS at 37 °C in a CO_2 incubator.

2.2. EST database searches

The human APRIL cDNA of 1793 bp (GenBank accession no. AY081050) was used as a seed to search the NCBI EST database of bovine (*Bos taurus*) (http://www.ncbi.nlm.nih.gov/blast). Retrieved four homologous ESTs (GenBank accession nos. CO881201, EH207252, DY187801, BF602147) were constructed into a contig by CAP3 (http://pbil.univ-lyon1.fr/cap3.php). The open reading frame of this contig sequence was determined by the program of NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence and putative protein sequence were submitted to NCBI TPA databases (Third Party Annotation), and deposited at GenBank.

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

Tissue samples of bovine were collected, immediately placed in liquid nitrogen, and stored at −85 °C until use. Total RNA was extracted using TRIzol reagent (Gibco-BRL, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μg of RNA isolated from various tissues using Reverse Transcriptase XL (Takara, Japan) according to the standard protocol. A pair of primers B1 (5′-ATGCCGGCCTCATCTCCTTCCTTG-3′) and B2 (5′-TCACAGTTTCACAAGCCCCAGGA-3′) (Table 1) was designed according to the obtained predicted bAPRIL contig. Using these two primers, we performed PCR amplifications from the cDNA of bovine spleen to clone the APRIL cDNA. The PCR products were cloned into pMD18-T vector (Takara, Japan) and sequenced by the ABI Prism automated sequencing method (YingJun, China).

2.4. Bioinformatics analyses

The searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). For the exact localization of the exon–intron boundaries, the mRNA-to-genomic alignment program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used. The putative promoter sequence was analyzed "in silico" with the program PROSCAN Version 1.7 from Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and the protein domain features of bAPRIL were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Isoelectric point and molecular weight prediction was carried out at http://cn.expasy.org/tools/pi_tool.html. Real-time quantitative PCR (qPCR) primer pairs were designed manually based on Primer Express 2.0 software guidelines (Applied Biosystems).

2.5. 3D modelling of the of bAPRIL

A suitable structural template for bAPRIL, mouse APRIL PDB file was identified by a BLAST search as implemented in the SWISS-MODEL Protein Modelling Server. The automatic sequence alignment thus obtained was used for homology modelling with SWISS-MODEL, presenting a global energy of −7304.564 kJ/mol (Arnold et al., 2006). The resulting theoretical model of a protein monomer was displayed and analyzed using RasWin Molecular Graphics Program (RasMol, version 2.7.2), software package (http://www.umass.edu/microbio/rasmol/).

2.6. Real-time quantitative PCR (qPCR) analysis

The mRNA expression of bAPRIL was measured by real-time qPCR. Total RNA from various tissues was prepared as described above. Two bAPRIL primers B3 (5′-CGAGAAGGGAGAAGGGAATC -3′) and B4 (5′-ATGGGAACGAGATGCAGAAC -3′) (Table 1) were used to amplify a product of 99 bp. A constitutive expression gene, the glyceraldehyde phosphate dehydrogenase (GAPDH), was used as internal control to verify the real-time qPCR reaction. Two primers B5 (5′-GCATTGCCCTCAACGACCACTTTGTC-3′) and B6 (5′-CTCCTTGGAGGCCATGTGGACCATG-3′) (Table 1) were used to amplify a 111 bp fragment of bovine GAPDH cDNA. DEPC-water for the replacement of cDNA template was used as negative control. The SYBR Green RT-PCR assay was carried out as previously described in detail (Guan et al., 2007a).