Gene 646 (2018) 47–55

Contents lists available at ScienceDirect

Gene

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

Research paper

Porcine enterocyte protein Btnl5 negatively regulates NF-kappa B pathway by interfering p65 nuclear translocation

Qi Bao[a,b,c,e], Chong Li[d], Chongfeng Xu[c], Ruiying Zhang[c], Kai Zhao[a,b,*], Ziyuan Duan[c,**]

[a] Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China
[b] Laboratory of Plateau Fish Evolutionary and Functional Genomics, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China
[c] Genetic Resource Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[d] Core Facility for Protein Research, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[e] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Keywords:
Btnl5
IECs
NF-κB
Immune regulation
Interacting proteins

ABSTRACT

Porcine butyrophilin-like 5 (Btnl5) is a novel member of the butyrophilin family, which consists of immune regulators. The expression pattern and the function of this gene remain unclear. In this study, Btnl5 is identified as a negative regulator of the NF-κB pathway. Our study indicates that Btnl5 is mainly expressed in intestinal epithelial cells (IECs) and expressed in membrane systems. Btnl5 inhibits MyD88-mediated activation of the NF-κB pathway. Btnl5 interacts with TNF receptor-associated factor 2 (TRAF2) and transcription factor p65. Besides, Btnl5 inhibits p65-mediated activation of the NF-κB pathway and inhibits nuclear translocation of p65. These results suggest that Btnl5 may inhibit NF-κB pathway through binding and interfere nuclear translocation of p65.

1. Introduction

The butyrophilin family composes butyrophilin (Btn), butyrophilin-like (Btnl) and Skint (selection and upkeep of intraepithelial T cells), belongs to immunoglobulin superfamily (Afrache et al., 2012). Most of the Btn/Btnl proteins have two extracellular immunoglobulin domains, a transmembrane region and an intracellular B30.2 domain (Abeler-Dorner et al., 2012; Rhodes et al., 2016). It is considered to be an immune regulation family as the extracellular domain of Btn/Btnl proteins share strong homology with the B7 co-stimulators and many of them located within the MHC region (Linsley et al., 1994; Arnett and Viney, 2014; Rhodes et al., 2016).

Btn/Btnl play crucial roles in the immune regulation of the gut. Previous studies revealed that the expression of human and mouse Btn/Btnl are altered in intestinal inflammation and colon cancer (Lebrero-Fernández et al., 2016). Btnl2 negatively regulates intestinal inflammation (Arnett et al., 2007) and is associated with ulcerative colitis (Pathan et al., 2009). Mouse Btnl1 impairs cytokine induction of intestinal epithelial cells (IECs) and is speculated to inhibit NF-κB pathway (Bas et al., 2011). As known, NF-κB is critical for the inflammatory reaction in IECs (Swamy et al., 2010; Pasparakis, 2012), as its activation is one of the most efficient ways of host immune defense. NF-κB transcription family consist of five members: p65/RelA, c-Rel, RelB, p50/p105 and p52/p100, these subunits form homodimers or heterodimers as exerting unit. In most cells, NF-κB dimer (commonly the p50:p65 heterodimer) is sequestered in the cytosol by inhibitory κB (IκB), and stimuli like some microbial products or pro-inflammatory cytokines will lead to the nuclear translocation of p50:p65 heterodimer and the activation NF-κB pathway (Peterson and Artis, 2014). The negative control of NF-κB pathway is also significant, otherwise, the persistent activation will result in tissue damage (Ruland, 2011; Marusawa and Jenkins, 2014).

Porcine *Btnl5* on chromosome 7 (28093234-28108144) is a novel gene predicted by Ensembl (http://asia.ensembl.org/index.html). Its expressed sequence tags were mainly detected in small intestine (Gorodkin et al., 2007). We supposed that porcine Btnl5 might play a role in immune regulation since it located within the MHC region according to Ensembl. In this study, we find that Btnl5 is mainly expressed in IECs and plays a role as a negative regulator of the NF-κB pathway. Therefore, Btnl5 plays an important role in immune regulation in IECs.

Abbreviations: Btnl5, butyrophilin-like 5; IECs, intestinal epithelial cells; TRAF2, TNF receptor-associated factor 2; Btn, Butyrophilin; Btnl, butyrophilin-like; Skint, selection and upkeep of intraepithelial T cells; IκB, inhibitory κB; IPTG, isopropy-β-D-thiogalactoside; PVDF, polyvinylidene fluoride; EDTA, ethylene diamine tetraacetic acid; DAPI, 4′,6-diamidino-2-phenylindole; βGal, β-galactosidase; RACE, rapid-amplification of cDNA ends; RIP1, receptor interacting protein kinase 1; TRIM25, tripartite motif containing 25

* Correspondence to: K. Zhao, Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, 810001, China.
** Corresponding author.
E-mail addresses: zhaokai@neopb.ac.cn (K. Zhao), zyduan@genetics.ac.cn (Z. Duan).

https://doi.org/10.1016/j.gene.2017.11.070
Received 2 July 2017; Received in revised form 12 November 2017; Accepted 28 November 2017
Available online 29 November 2017
0378-1119/

Table 1
PCR primers.

Primers	Sequences (5′-3′) (underlined sequences are restriction enzyme sites)
For cloning aa 276–559 of Btnl5 (ENSSSCT00000001595.3)	
Btnl5P F	GAAGAATTCATCAAGTTGCGGGA
Btnl5P R	GTGACTCGAGCTATGGGGACACAG
For cloning full length or truncated Btnl5 (Myc tagged)	
Btnl5 F	TGAACACTCGAGGGAAATGGAGGAT
Ig F	GCCCCTGAATTCCGCATCACAG
TM F	GCCGAATTCATTCCAGAGCCCTT
Signal R	GAGAATTCAAGCACCGAGAACTCCT
TM R	CCGGTACCCGCAACTTGATAGAATG
Ig like R	CCTGTGAATTCCACCTCAGGGG
Btnl5 R1	GGGAATTCTGGGGACACAGCCTC
Btnl5 R2	GGGGTACCGGGGACACAGCCTC
For cloning Flag tagged Btnl5	
Btnl5-Flag-F	GGGGGTACCCTAGAGGAGTTCTCGG
Btnl5-Flag-R	CAGTCTCGAGACCTATGGGGACACA
For cloning Flag tagged p65 (NM_021975.3)	
p65-Flag-F	GGGGTACCATGGACGACCTCTTCCCCCTCATCT
p65-Flag-R	GGGGCTCGAGTTAGGAGCTGATCTGACTCAGAAGG
For cloning Flag tagged TRAF2 (NM_021138.3)	
TRAF2-Flag-F	CAGGTACCATGGCTGCAGCTAGCGTG
TRAF2-Flag-R	AGACACCAGTAGGCGGCCGTTAGAGCCCTG

2. Materials and methods

2.1. Animal and tissue collection

Bama Miniature Pigs were provided by experimental pig farm of Institute of Zoology, Chinese Academy of Sciences, Beijing. Brain and small intestine of one-month-old Bama Miniature Pigs were collected and stored in liquid nitrogen or fixed in paraformaldehyde immediately after slaughtering.

2.2. Plasmids

To construct NF-κB promoter luciferase reporter gene, promoter sequence contain three κB site was cloned into pGL3-basic plasmid (E1751, Promega). The 276–559 amino acids of Btnl5 was amplified and cloned into pET30a (69909-3, NOVAGEN) with restriction sites *Eco*RI/*Xho*I to generate prokaryotic expression construct pET30a-Btnl5. Full length or truncated Btnl5 were cloned into pcDNA3.1-Myc-His (V855-20, Invitrogen). For construction of Flag-tagged Btnl5 expression plasmid (pcDNA3-Btnl5-Flag), the coding sequence of Btnl5 was amplified and inserted into the restriction sites *Kpn*I/*Xho*I of pcDNA3-Flag. Similar strategy was applied to construct pcDNA3-TRAF2-Flag and pcDNA3-p65-Flag vectors. Primers used are listed in Table 1.

2.3. Cell culture and transient transfection

293T and porcine intestinal epithelial cell line IPI-2I (93100622, ECACC) were cultured in DMEM (Gibco). Porcine intestinal epithelial cell line IPEC-1 (ACC 705, DSMZ) was cultured in DMEM/F12 (Gibco). The mediums were supplemented with 10% fetal bovine serum (Gibco), 100 mg/mL streptomycin and 100 U/mL penicillin. Cell lines were maintained at 37 °C with 5% CO_2. 293T cells were transfected using polyethylenimine as described previously (Boussif et al., 1995) and harvested 24 h post transfection. IPI-2I and IPEC-1 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and harvested 24 h post transfection.

2.4. Production of Btnl5 polyclonal antibody

Plasmid expressing Btnl5 (pET30a-Btnl5) was transformed into *Escherichia coli* strain Rosetta. Inclusion body contain high content of recombinant protein was collected by sonication and centrifugation. Then, recombinant protein was dissolved in 8 M urea and renatured by dialyzing in the phosphate-buffered saline solution overnight at 4 °C. Mouse was immunized by 50 μg of Btnl5 protein every two weeks. The specificity of anti-Btnl5 antibody was determined by Western blotting. Pre-immune serum was collected as a negative control.

2.5. Western blotting

Samples were mixed with 5 × loading buffer and boiled at 100 °C for 10 min before separating by 12% SDS-PAGE. The separated proteins were then transferred to PVDF membranes. Non-specific bindings were blocked in 1 × TBST (20 mM Tris, 150 mM NaCl, 50 mM KCl, 0.1% Tween-20, pH 7.5) containing 5% fat-free dry milk (232100, BD) at room temperature for 1 h. The membranes were incubated with corresponding primary antibodies overnight at 4 °C. After three washes with TBST, horseradish peroxidase (HRP)-conjugated secondary antibody was incubated with the membrane at room temperature for 1 h. Signals were measured with BeyoECL Plus (Beyotime) and autoradiography. The following antibodies were used for Western blotting: anti-Btnl5, Pre-immune serum, anti-β-actin (CW0096M, CWBIO), anti-Flag (F3165, Sigma), anti-Myc (TA150121, OriGene), anti-HSP90 (AH732, Beyotime), anti-PARP (AP102, Beyotime), anti-p65 (sc-372, Santa Cruz Biotechnology), HRP-conjugated goat anti-mouse secondary antibody (115-035-003, Jackson ImmunoResearch) and HRP-conjugated goat anti-rabbit secondary antibody (111-035-003, Jackson ImmunoResearch). DyLight 488 AffiniPure Goat Anti-Mouse IgG (A23210-2, Abbkine).

2.6. Immunohistochemistry

The samples of small intestine fixed in paraformaldehyde were embedded in paraffin and sliced into 5 μm sections. Antigen was retrieved in EDTA buffer (CW0129, CWBIO). Endogenous peroxidase activity was blocked by 3% H_2O_2. After blocking with normal goat serum (CW0130, CWBIO) for 2 h at room temperature, the sections were incubated with anti-Btnl5 antibody or pre-immune serum at 4 °C overnight. Then, secondary HRP-conjugated goat anti-mouse IgG (CW0102A, CWBIO) was incubated with the sections for 3 h at room temperature. Peroxidase stained sections were developed using DAB kit (ZLI-9019, ZSGB-BIO). After counterstaining with hematoxylin, the sections were mounted in automatic mounting machine (ST5020, Leica). Images were captured by Nikon Eclipse Ci-L microscope.

2.7. Cellular localization

293T cells were seeded on coverslips in 6-well plates. 12 h later, 2 μg pcDNA3.1-Myc-His-Btnl5 plasmid or empty pcDNA3.1-Myc-His plasmid were transfected into 293 T cells. 24 h post transfection, cells were fixed by 4% paraformaldehyde solution for 20 min at room temperature. Expression of Btnl5-Myc was visualized and photographed under fluorescence confocal microscope (Observer Z1, Carl Zeiss) after staining with anti-Myc antibody and 4′,6-diamidino-2-phenylindole (DAPI). Subcellular localization of Btnl5 protein (ENSSSCP00000001553.3) was also predicted using Psort II (http://psort.hgc.jp/form2.html).

2.8. Luciferase reporter assays

24 h post transfection, luciferase activity was measured using the