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Molecular cloning and functional analysis of duck ubiquitin-specific protease 18 (USP18) gene

Wei Qian [a, b, 1], Xiaoqin Wei [a, b, c, 1], Hongbo Zhou [a, b, d], Meilin Jin [a, b, d, *]

[a] State Key Laboratory of Agriculture Microbiology, Huazhong Agricultural University, Wuhan, 430070, PR China
[b] Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, PR China
[c] College of Agricultural and Animal Husbandry, Tibet University, Linzhi, 860000, PR China
[d] Key Laboratory of Development of Veterinary Diagnostic Products, Ministry of Agriculture, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, PR China

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ABSTRACT

In mammals, ubiquitin-specific protease 18 (USP18) is an interferon (IFN)-inducible gene and is a negative regulator of Toll-like receptor-mediated nuclear factor kappa B (NF-κB) activation. The role of USP18 in ducks (duUSP18) remains poorly understood. In the present study, we cloned and characterized the full-length coding sequence of duUSP18 from duck embryo fibroblasts (DEFs). In healthy ducks, duUSP18 transcripts were broadly expressed in different tissues, with higher expression levels in the spleen, lung and kidney. Quantitative real-time PCR (qRT-PCR) analysis revealed that duUSP18 could be induced by treatment with Poly(I:C) or LPS. Overexpression of duUSP18 inhibited NF-κB and IFN-β expression. Furthermore, deletion mutant analysis revealed that the duUSP18 region between aa 75 and 304 was essential for inhibiting NF-κB. In addition, overexpression of duUSP18 also suppressed the secretion of NF-κB-dependent proinflammatory cytokines. Taken together, these results suggest that duUSP18 regulates duck innate immune responses.

1. Introduction

USP18 (also known as UBP43) is a member of the ubiquitin-specific protease (UBP) family (Liu et al., 1999; Schwer et al., 2000). The UBP family is composed of deubiquitinating enzymes that regulate the balance of protein ubiquitination in combination with the action of ubiquitin-conjugating enzymes in a variety of cellular events (Chung and Baek, 1999; D'Andrea and Pellman, 1998; Guo et al., 2014; Wing, 2003). The family members differ in protein size and amino acid sequence; however, all share several consensus sequences surrounding the cysteine residues (Cys domain) and histidine residues (His domain) that are required for catalytic activity of the enzyme (Hochstrasser, 1996). USP18 was originally identified as a type I interferon-responsive gene that is rapidly upregulated by IFN-β treatment through the JAK/STAT kinase pathway (Kang et al., 2001). USP18 efficiently cleaves ISG15 conjugates, maintaining cellular homeostasis of ISG15-conjugated proteins (Malakhov et al., 2002). USP18 also negatively regulates type I IFN signaling independent of its ISG15 isopeptidase activity (Kim et al., 2006; Malakhova et al., 2006).

NF-κB is clearly one of the most important regulators of the expression of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, which, in turn, promote inflammation and induce subsequent adaptive immune responses (Iwai, 2012; Karin and Delhase, 2000). Activation of NF-κB is also required for the transcription of IFN-β in the MyD88-and TRIF-dependent signaling pathways (Kawai and Akira, 2006; Takeuchi and Akira, 2009). Increasing evidence indicates that prolonged activation of NF-κB may result in inflammatory diseases (Tak and Firestein, 2001; Takeuchi and Akira, 2010). Moreover, proinflammatory cytokines produced by innate immune cells under chronic inflammatory conditions have been shown to play decisive roles in tumor development (Grivennikov et al., 2010; Karin, 2006). Therefore, NF-κB is tightly regulated through multiple negative regulators to avoid persistent inflammatory responses. Recently, USP18 was reported to be a negative regulator of NF-κB, which is upregulated by

Abbreviations: USP18, ubiquitin-specific protease 18; duUSP18, duck USP18; MyD88, myeloid differentiation primary response gene 88; Poly(I:C), polyinosinic-polycytidylic acid; LPS, lipopolysaccharides; qRT-PCR, quantitative real-time PCR.

* Corresponding author. State Key Laboratory of Agriculture Microbiology, Huazhong Agricultural University, Wuhan, 430070, PR China.
E-mail address: jinmeilin@mail.hzau.edu.cn (M. Jin).

[1] These authors contributed equally to this work.

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various TLR ligands, and inhibited IκB degradation as well as NF-κB activation in a negative feedback loop. USP18 can control NF-κB signaling by inhibiting k63-linked polyubiquitination of TAK1 and NEMO (Yang et al., 2015). Furthermore, USP18 inhibited NEMO ubiquitination, independent of its protease activity (Yang et al., 2015).

Recently, there has been increasing interest in the duck immune system. Previous studies have demonstrated that MDA5 (Wei et al., 2014), RIG-I (Barber et al., 2010; Chen et al., 2013), TLRs (Cheng et al., 2015a; Huang et al., 2011; Jia et al., 2012; Jiao et al., 2012; MacDonald et al., 2008), MAVS (Li et al., 2016), MyD88 (Cheng et al., 2015b), TRAF6 (Zhai et al., 2015), IFIT5 (Wang et al., 2015) and IFITM3 (Blyth et al., 2015) play important roles in duck innate immunity. In this study, we first cloned the duUSP18 gene introduce DEFs and determined the duUSP18 expression profile in different tissues. We also examined the response of DEFs to treatment with Poly(I:C) or LPS, and found that duUSP18 was upregulated. Furthermore, we identified duUSP18 as a negative regulator of NF-κB activation.

2. Materials and methods

2.1. Animals and sample collection

Healthy cherry valley ducks were purchased from Wuhan Chunjiang Waterfowl Limited Liability Company. Various tissues of ducks, including the heart, liver, spleen, lung, kidney, brain, pancreas, intestine, duodenum, trachea, muscle and stomach were collected from healthy adult ducks and frozen in liquid nitrogen for further study.

Forty three-day-old ducks were divided randomly into two groups of twenty. Group 1 was injected with 0.4 mL of Poly(I:C) (0.5 mg/mL). Group 2 was injected with PBS only as a control. Samples from ducks with no symptoms were collected at 0, 4, 8, 12, and 24 h after injection. All ducks were euthanized after anesthesia with an intraperitoneal injection of sodium pentobarbital.

2.2. Cells and reagents

Duck embryo fibroblasts (DEFs) were prepared from 10-day-old embryonated eggs (purchased from Wuhan Chunjiang Waterfowl Limited Liability Company) and maintained in complete Dulbecco's Modified Eagle Medium (HyClone, Thermo Scientific, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Human embryonic kidney 293T cells and Hela cells were purchased from ATCC (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (with 10% FBS) (HyClone, China). The cells were transfected with the indicated constructs using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Poly(I:C) and LPS were purchased from Sigma (St Louis, MO, USA). Mouse monoclonal antibodies (mAbs) against the HA-tag (PMKbio, China) were used for western blot, while a Cy3-conjugated goat anti-mouse IgG (Invitrogen) was used for indirect immunofluorescence analysis.

2.3. duUSP18 cloning, sequence alignment, and homology analysis

Based on the duck genomic sequence and published predicted sequence of TRAF3 from *Anas platyrhynchos* (GenBank accession number XM_005009931), gene-specific primers (Table 1) for PCR amplification of the complete coding region were designed using the Primer Premier 5 software. The software package DNAman 6.0 was used to deduce the amino acid sequence. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and ESPript3.0 (http://espript.ibcp.fr/ESPript/ESPript/) were used to construct multiple sequence alignments of the amino acid sequences of USP18 proteins, and a phylogenetic tree was constructed using the MEGA 5.2 program. The GenBank accession numbers used in the comparison are human (NP_059110.2), mouse (NP_036039.2), rat (NP_001014080.1), macaque (XP_005568067.1), cattle (NP_001017940.1), pig (NP_998991.1), duck (XP_005009988.1), chicken (XP_416398.3), turkey (XP_010712227.1), rock pigeon (XP_005500407.1), owl (XP_009973776.1), zebra fish (XP_005164589.1), crucian carp (ABC86864.1). chimpanzee (XP_001164261.1), egret (XP_009646205.1), red-throated loon (XP_009810201.1), swift (XP_010001300.1), African elephant (XP_003410800.1) and ferret (XP_004773838.1). The duUSP18 domain and motif analysis were performed using the Sample Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from DEFs or different tissues of cherry valley ducks using TRIzol (Invitrogen) following standard instructions, and 1 μg of RNA was treated with DNase (Promega) before reverse transcription. The cDNA was synthesized from 1 μg RNA samples using the avian myeloblastosis virus (AMV) reverse transcriptase and an oligo(dT-)18-adaptor primer (TaKaRa Biotechnology, DaLian, China). The reaction mixtures were incubated at 42 °C for 1 h and terminated by heating at 95 °C for 5 min, and the cDNAs were stored at −80 °C for later analysis by quantitative real-time PCR (qRT-PCR). The primers used in qRT-PCR are listed in Table 1. The assay was performed on an ABI ViiA 7 PCR system (Applied Biosystems, USA) in a total volume of 10 μl per sample, containing 5 μl of 2 × SYBR Green Master Mix (Rox), 1 μl of the diluted cDNA, 0.25 μl of each primer (10 mM), and 3.5 μl of DEPC-treated water, with a holding step at 95 °C for 15 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. To detect and validate the specific amplification of PCR products, dissociation curve analysis of the products was conducted at the end of each PCR reaction. The transcript level of each gene was normalized to the expression of GAPDH, and the $2^{-\triangle\triangle Ct}$ method was used to analyze the gene expression of the samples(Livak and Schmittgen, 2001).

2.5. Plasmids

The complete coding sequence (CDS) of duUSP18 was amplified from duck cDNA (prepared from total RNA extracted from DEFs after Poly(I:C) treatment) by PCR using TransTaq-T DNA Polymerase (TransGen Biotech, China). The CDS was cloned into the pMD-18T vector (TaKaRa, China), and the insert was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China) and named T-duUSP18. pCAGGS-HA (pCA-HA) expression plasmids with an N-terminal HA tag encoding duUSP18 and truncated duUSP18 fragments, including 75N, 304N, 304C, 75C and ID(intermediary domain), were obtained by PCR amplification of T-duUSP18 using PrimeSTAR® HS DNA Polymerase (TaKaRa, China). The amplification products were digested with KpnI/XhoI (TaKaRa, China) and subsequently cloned into the pCA-HA expression vector digested previously with the same restriction enzymes. A plasmid encoding pCA-duMyD88 was made by inserting the CDS of duMyD88 (GenBank accession number KP729184) into the EcoRI/XhoI sites of the pCA-HA expression vector. DNA fragments of duMyD88 were amplified from duck cDNA. PCR products were digested with EcoRI/XhoI enzymes (TaKaRa, China) and ligated into the same sites of the pCA-HA vector. Duck luciferase reporter plasmids (IFN-β-Luc and 4 × NF-κB-Luc) were constructed as previously described (Zhai et al., 2015). The primers used are listed in Table 1. All of the plasmids in this