Developmental and Comparative Immunology 65 (2016) 369–376

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Molecular cloning and functional analysis of the duck TIR domain-containing adaptor inducing IFN-β (TRIF) gene

Xiaoqin Wei [a, b], Wei Qian [a], Suolang Sizhu [b], Lijuan Shi [a], Meilin Jin [a, c, d], Hongbo Zhou [a, c, d, *]

[a] State Key Laboratory of Agriculture Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, PR China
[b] College of Agricultural and Animal Husbandry, Tibet University, Linzhi, 860000, PR China
[c] The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan, Hubei, 430070, PR China
[d] Key Laboratory of Development of Veterinary Diagnostic Products, Ministry of Agriculture, Huazhong Agricultural University, Wuhan, 430070, PR China

ARTICLE INFO

Article history:
Received 1 July 2016
Received in revised form
12 August 2016
Accepted 12 August 2016
Available online 15 August 2016

Keywords:
Duck
TRIF
IFN-β

ABSTRACT

Toll-like receptors (TLRs) trigger the innate immune response by responding to specific components of microorganisms. The TIR domain-containing adaptor inducing IFN-β (TRIF) plays an essential role in mammalian TLR-mediated signaling. The role of TRIF in ducks (duTRIF) remains poorly understood. In this study, we cloned and characterized the full-length coding sequence of duTRIF from duck embryo fibroblasts (DEFs). In healthy ducks, duTRIF transcripts were broadly expressed in different tissues, with higher expression levels in the spleen and liver. Using quantitative real-time PCR (qRT-PCR), we demonstrated the upregulation of duTRIF in DEFs infected with AIV or DTMUV, and DEFs treated with Poly I:C or LPS. Overexpression of duTRIF was able to induce the NF-κB and IFN-β expression. Furthermore, the IFN induction function of duTRIF was impaired when Ala517 was mutated to Pro or His. Taken together, these results suggested that duTRIF regulated duck innate immune responses.

1. Introduction

In immune systems of mammals, the TLRs play a crucial role in recognition of pathogen-associated molecular patterns and are the first lines of defense against bacterial and viral infection (Aderem and Ulevitch, 2000). TLRs are expressed either as membrane-bound or intracellular proteins (Kumar et al., 2009). Individual TLRs interact with different combinations of adapter proteins (MyD88, TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP), TRAM, and SARM) and lead to activation of the nuclear factor kappa B (NF-κB) and IFN-regulatory factor (IRF) pathways (Kawai and Akira, 2006b). Activation of these pathways triggers the expression of inflammatory cytokines and IFN-β (Medzhitov et al., 1998; Moynagh, 2005; Takeda and Akira, 2005). TIRAP is involved specifically in TLR2- and TLR4-mediated activation of the MyD88-dependent pathway and TRAM is a specific adaptor in the TLR4-mediated, TRIF-dependent pathway (Akira and Takeda, 2004). MyD88 is responsible for inflammatory cytokine production to all TLR ligands, except for the TLR3 ligand (Muzio et al., 1997; Kawai and Akira, 2006b). TRIF is essential for TLR3- and TLR4-mediated signaling pathways leading to IFN regulatory factors (IRFs)-3/7 activation and IFN-β production (Yamamoto et al., 2003). The induction of Type I interferons and interferon-inducible genes are important for antiviral and anti-bacterial responses (Bowie and Haga, 2005; Perry et al., 2005) and can link innate immunity with adaptive immunity (Le Bon and Tough, 2002). Depending on the TLR3 ligand (Poly I:C) stimuli, TLR3 recruits the TIR adaptor molecule TRIF, which induces signaling pathways leading to the activation of IRF3/7 (Doyle et al., 2002; Honda et al., 2005) and the production of IFN-β (Oshiumi et al., 2003). In the TLR4-mediated pathway, TRIF induces IFN-β production via the TLR4 signaling in response to LPS (Fitzgerald et al., 2003).

Recently, there has been increasing interest in the duck immune system. Previous studies have demonstrated that MDA5 (Wei et al.,

Abbreviations: TRIF, TIR domain-containing adaptor inducing IFN-β; duTRIF, duck TRIF; Poly I:C, Polyinosinic-polycytidylic acid; LPS, Lipopolysaccharides; AIV, avian influenza virus; DTMUV, duck Tembusu virus; qRT-PCR, Quantitative real-time PCR.

* Corresponding author. State Key Laboratory of Agriculture Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, PR China.
E-mail address: hbzhou@mail.hzau.edu.cn (H. Zhou).

http://dx.doi.org/10.1016/j.dci.2016.08.006
0145-305X/

2014), RIG-I (Chen et al., 2013; Barber et al., 2010), MAVS(Li et al., 2016), TRAF6 (Zhai et al., 2015), IFIT5 (Wang et al., 2015), IFITM3 (Blyth et al., 2015) and USP18 (Qian et al., 2016) played important roles in duck innate immunity. Duck TLRs also have been identified and characterized (Huang et al., 2011; Jia et al., 2012; Jiao et al., 2012; MacDonald et al., 2008; Cheng et al., 2015a). The repertoire of TLR molecules in the duck is somewhat similar to the repertoire of mammalian TLRs. Previous studies have identified that the MyD88 is important in the duck defense against viral infection (Cheng et al., 2015b). Due to the vital function of TRIF-dependent pathway in IFN-β induction in mammals, it is of particular interest to characterize this pathway in duck to further understand the duck TLR-signaling pathways. In this study, we first cloned the duTRIF gene introduce Duck embryo fibroblasts (DEFs) and determined the duTRIF expression profile in different tissues. We examined the response of DEFs to treatment with Poly I:C or LPS, and found that duTRIF was upregulated. We also demonstrated the upregulation of duTRIF in DEFs in response to different virus. Furthermore, the duTRIF was able to induce the promoter of IFN-β and activate NF-κB promoter.

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 and muscle were collected from healthy adult ducks and frozen in liquid nitrogen for further study.

2.2. Cells, reagents and viruses

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) (Adams et al., 2013; Martin et al., 1971). Human embryonic kidney 293T cells were purchased from ATCC (Manassas, VA, USA) and 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 Flag-tag (Sigma, St Louis, MO, USA) and GAPDH (Coachella, CA, USA) were used for western blot, while a FITC-conjugated goat anti-mouse IgG (Invitrogen) was used for indirect immunofluorescence analysis. The Avian Influenza virus (AIV) strain, A/duck/Hubei/hangmei01/2006(H5N1/HM), and a virulent duck Tembusu virus (DTMUV) strain (GenBank ID: KJ489355) were conserved by the State Key Laboratory of Agricultural Microbiology of China.

2.3. duTRIF cloning, sequence alignment, and homology analysis

Based on the duck genomic sequence and published predicted sequence of TRIF from *Anas platyrhynchos* (GenBank accession number XM_005027700.2), 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 ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/) were used to construct multiple sequence alignments of the amino acid sequences of TRIF proteins, and a phylogenetic tree was constructed using the MEGA 5.2 program. The GenBank accession numbers used in the comparison are *Homo sapiens* (NM_182919.3), *Mus musculus* (NM_174989.4), *Bos taurus* (NM_001030301.1), *Macaca mulatta* (NM_001130428.1), *Pan troglodytes* (NM_001130132.1), *Anas platyrhynchos* (KJ466051.1), *Gallus gallus* (EF025853.1), *Egretta garzetta* (XM_009636689.1), *Gavia stellata* (XM_009809799.1), *Columba livia* (XM_005503964.1), *Meleagris gallopavo* (XM_010725088.1), *Danio rerio* (NM_001044759.1) and *Ictalurus punctatus* (ABD93874.1). The duTRIF domain and motif analysis were performed using the Sample Modular Architecture Research Tool (SMART) (http://smart.embl-heidelbergde/).

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 DNase-treated before reverse transcription (Promega). 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 duTRIF 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-duTRIF. p3XFLAG-CMV-14 expression plasmids with an C-terminal Flag tag encoding duTRIF and single point mutants including A517P and A517H, were obtained by primer extension and fusion PCR using PrimeSTAR® HS DNA Polymerase (TaKaRa, China). The amplification products were digested with *Hin*d III/*Bam*H I (TaKaRa, China) and subsequently cloned into the p3XFLAG-CMV-14 expression vector digested previously with the same restriction enzymes. Duck luciferase reporter plasmids (IFN-β-Luc and 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 study were verified by DNA sequencing at Sangon Biotech Co., Ltd. (Shanghai, China).

2.6. Cell culture and virus infection

DEFs were trypsinized and plated onto coverslips in 12-well plates. When they reached approximately 80% confluence, they were challenged with 1 multiplicity of infection (MOI) of AIV or DIMUV. At 6, 12, 24, 36 h post-infection, cells were collected for real-time PCR analysis.