Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

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

Duck stimulator of interferon genes plays an important role in host anti-duck plague virus infection through an IFN-dependent signalling pathway

Shun Chen[a,b,c,*,1], Zhen Wu[a,1], Jinyue Zhang[a], Mingshu Wang[a,b,c], Renyong Jia[a,b,c], Dekang Zhu[b,c], Mafeng Liu[a,b,c], Kunfeng Sun[a,b,c], Qiao Yang[a,b,c], Ying Wu[a,b,c], Xinxin Zhao[a,b,c], Anchun Cheng[a,b,c,*]

[a] Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
[b] Research Center of Avian Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
[c] Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

ARTICLE INFO

Keywords:
Duck
STING
IFN-dependent signal pathway
DPV
Antiviral immune response

ABSTRACT

The human stimulator of interferon gene (STING) is an important molecule in innate immunity that stimulates type I interferon (IFN) production. However, the role of duck STING (duSTING) in innate immunity has yet to be explained. In this study, the full length of the duSTING cDNA sequence (1149 bp), which encodes 382 amino acid (aa) residues, was reported and showed the highest sequence similarity with chicken STINGs. The phylogenetic analysis based on STING aa showed that duSTING was grouped onto the birds clade. According to the tissue distribution spectrum analysis, duSTING was highly present in the bursa of Fabricius, glandular stomach, liver, pancreas, and small intestine of ducklings, as well as in the blood and pancreas of the adult duck. DuSTING mainly colocalized with the endoplasmic reticulum (ER) and mitochondria in transfected Baby Hamster Syrian Kidney (BHK21) and duck embryo fibroblasts (DEF) cells by an indirect immunofluorescence assay. The transfection of the DEFs with duSTING activated NF-κB, which induced the transcription of IFN-β, and the activated IFN induced the interferon-stimulated response element (ISRE). Furthermore, the overexpression of duSTING significantly upregulated the mRNA level of duck IFN-β and IFN-stimulated genes (ISGs), such as duMx and duOASL and inhibited the replication of the double-stranded DNA duck plague virus (DPV) *in vitro*. In addition, the knockdown of endogenous duSTING by shRNA significantly reduced the poly (I:C) (pIC), poly (dA:dT), and Tembusu virus (TMUV), induced IFN-β production and significantly promoted DPV replication *in vitro*. In general, these data demonstrate that duSTING is vital for duck type I interferon induction and plays an important role in the host defence of DPV infection.

1. Introduction

Innate immunity is the first line of host defence against pathogen invasion. Type I interferon (IFN) plays an important role in innate immunity. Type I IFN activates a series of ISG transcripts by paracrine and autocrine pathways during viral infection and establishes the antiviral status to limit viral replication. One of the important factors for the production of type I IFN is the recognition of viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [1]. Retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs) are cytosolic PRRs relevant to RNA virus infection that are well-characterized [2]. Viral double stranded RNA (dsRNA) receptors, RIG-I/melanoma differentiation-associated gene 5 (MDA5), are activated by dsRNA virus, leading to the activation of NF-κB and interferon regulatory factor (IRF) through the mitochondrial antiviral signalling protein (MAVS) (also known as the CARD-adapter, which induces IFN-β, Cardif, IFN-β promoter stimulator 1, IPS-1 and virus-induced signalling adapter, VISA) [2,3].

Stimulator of the IFN gene (STING) is a newly discovered key type I IFN positive regulatory factor and is also known as MPYS, mediator of IRF-3 activation (MITA), endoplasmic reticulum IFN stimulator (ERIS) or transmembrane protein 173 (TMEM173) [4–6]. STING is a membrane-related protein that contains four or five transmembrane regions, which are mainly located in the endoplasmic reticulum and partially located in mitochondria and mitochondria-associated membranes (MAMs) [4–7]. When STING is overexpressed in cells, IRF3 is activated

* Corresponding authors at: Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, No. 211 Huimin Road, Wenjiang District, Chengdu city, Sichuan province 611130, China.
E-mail addresses: shunchen@sicau.edu.cn (S. Chen), chenganchun@vip.163.com (A. Cheng).
[1] These authors contributed equally as co-first authors of this work.

http://dx.doi.org/10.1016/j.cyto.2017.09.008
Received 17 June 2017; Received in revised form 7 September 2017; Accepted 7 September 2017

and viral replication is suppressed [4,5]. RNA interference assays demonstrate that by reducing the expression of STING, the activation of IRF3 is inhibited and viral replication increases [4,5]. Bacteria and DNA viruses activate STING, but recent studies show that STING also plays an important role in inhibiting positive strand RNA viral replication [8–10]. STING functions downstream of RIG-I and MAVS and upstream of TBK1 and is considered a scaffold protein for the MAVS/tank binding kinase 1 (TBK1)/IRF3 complex when an RNA virus is infected and is capable of binding to RIG-I (rather than MDA5), MAVS, TBK1 and IκB kinase ε [7,11]. STING is a newly discovered regulatory molecule in the type I interferon pathway, and its immunological properties and antiviral properties require further study.

Aquatic birds play a critical role in the transmission and dissemination of many important pathogens. However, there is less known about the molecular mechanism of the IFN pathway of aquatic birds, and only chicken STING is identified in birds [12]. The overexpression of chicken STING (chSTING) inhibits the viral replication of Newcastle disease virus (NDV) and avian influenza virus (AIV) *in vitro*. However, whether the duck STING (duSTING) activates the type I IFN pathway and causes an antiviral statue or not is still unknown. In this study, we cloned duSTING and studied its distribution in ducklings and adult ducks. We demonstrated that duSTING was an important molecule in the IFN-β signalling pathway, which activates NF-κB to induce IFNβ and ISGs, resulting in a dramatic antiviral state in DEFs.

2. Materials and methods

2.1. Cells, tissues, virus, and reagents

Duck plague virus (BAC-DPV-EGFP) and Baby Hamster Kidney (BHK21) were obtained from the Institute of Preventive Veterinary Medicine, Sichuan Agricultural University. The BAC-DPV-EGFP virus was constructed by our research centre and was employed in this study [13]. Furthermore, the viral titre of BAC-DPV-EGFP was determined by the tissue culture infective dose ($TCID_{50}$ was $10^{-6.125}$/0.1 mL) in duck embryo fibroblasts (DEFs). The DEFs were Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% new-born calf serum (NBCS, Gibco, Gaithersburg, MD, USA). Various tissues obtained from 5- and 60-day-old healthy ducks, including the brain (B), bursa of Fabricius (BF), Harderian gland (HG), blood (BL), heart (H), muscle stomach (MS), kidney (K), spleen (Sp), lymph node (LN), liver (Li), lung (Lu), muscle (M), pancreas (P), glandular stomach (GS), small intestine (SI), large intestine (LI), thymus (T), and trachea (Tr), were immediately snap-frozen in liquid nitrogen before being stored at −80 °C for further use. poly(I:C) (pIC) was purchased from Sigma (St Louis, MO, USA), and poly (dA:dT) was purchased from InvivoGen (San, Diego, USA).

2.2. Cloning of duSTING

Consistent with the predicted STING coding sequence of Anas platyrhynchos (GenBank accession number XM_013100766.1), the duSTING-pMD-F and duSTING-pMD-R primers (Table 1) were designed and used to amplify the duSTING cDNA fragment by RT-PCR from the total RNA extracted from duck spleen by using RNAiso plus (TaKaRa). The PCR product was cloned into the pMD19-T plasmids (TaKaRa Bio Inc., Otsu, Shiga, Japan), resulting in pMD-duSTING.

2.3. Sequence alignment and phylogenetic analysis

The amino acid sequences were aligned with DNAMAN software, and a phylogenetic tree was generated using the Geneious program. Comparisons of the transmembrane protein of STING from duck, pig, mouse and human were performed on the Phobius (http://phobius.sbc.su.se).

2.4. duSTING tissue expression analysis

Total cellular RNA was prepared from the different tissues and was homogeni chicken zed by using the RNAiso plus reagent (Takara, Dalian, China) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using random hexamer primers (ABM, Richmond, BC, Canada). A quantitative Real-time RT-PCR analysis was conducted to assess the abundance of the duSTING mRNA transcripts using SYBR Green real-time PCR assay (CFX96 Bio-Rad, Hercules, CA, USA). The expression of β-actin was used as an internal control. The primer sequences are listed in Table 1.

2.5. Plasmid construction

The DNA fragment containing the entire open reading frame (ORF) of duSTING was excised from pMD-duSTING and was inserted into the corresponding pCAGGS vector site, resulting in the posting expression constructs pCAGGS-duSTING-Flag. IFN-β-Luc expresses firefly luciferase under the control of the duck IFN-β promoter (−96 to +1). pDsRed2-ER was kindly provided by Sun [12], and pDsRed2-Mito was kindly provided by Luo [14]. pGL4.45 was purchased from Promega.

2.6. Indirect immunofluorescence analysis

BHK21 cells or DEFs were seeded on coverslips that were placed in 6-well dishes and were co-transfected with the pCAGGS-duSTING-Flag plasmid and pDsRed2-ER or pDsRed2-Mito until they reached approximately 70–90% confluence. The cells were fixed in 4% paraformaldehyde for 1 h at 24 h post-transfection and were then permeabilized with 0.25% Triton X-100 for 1 h at 4 °C. After three washes with PBS, the cells were blocked with 5% bovine serum albumin in PBS for 1 h and were then incubated with mouse anti-Flag antibodies (Ruiying Biological, Suzhou, China) for 24 h at 4 °C. The cells were treated with goat anti-mouse Alexa 568 (Life Technologies) for 1 h and then with 4′, 6-diamidino-2-phenylindole (Invitrogen) for 15 min at room temperature. The cells were always washed three times with PBS before each step. The fluorescence was examined using a microscope (80i, Nikon, Japan).

2.7. Luciferase reporter assays

Transient transfection was performed using Lipofectamine 3000 (Invitrogen). The DEFs were seeded in 24-well plates and were incubated until they were 70–80% confluent. The cells were co-transfected with 400 ng/well of the specific expression plasmids or an empty vector, together with 400 ng/well reporter plasmid IFN-β-Luc, NF-κB-Luc or the pGL4.45 vector (Promega), as well as 4 ng/well of the pRL-TK as an internal control vector (Promega) using Lipofectamine 3000 (Invitrogen) as specified by the manufacturer. The cells were harvested at the indicated time, and firefly luciferase activity was measured by the dual-luciferase assay system (Promega) according to the manufacturer's directions.

2.8. Western blotting analysis

Briefly, the DEFs were cultured in 6-well plates and were transfected with the expression plasmids or an empty control plasmid. The cells were harvested 24 h post-transfection by three rounds of freeze-thaw. The cell lysates were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat dry milk and was then incubated for 1 h with mouse anti-Flag antibodies (ProteinTech, Shenzhen, China) or mouse anti-β-actin monoclonal antibodies (Ruiying Biological, Suzhou, China) at a 1:2000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG