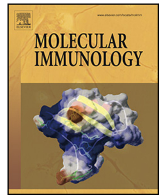
Molecular Immunology 76 (2016) 1–6

Contents lists available at ScienceDirect

Molecular Immunology

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



Short communication

Promoter structures and differential responses to viral and non-viral inducers of chicken melanoma differentiation-associated gene 5

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 28 May 2016
Accepted 7 June 2016
Available online 18 June 2016

Keywords:
MDA5
Promoter
Chicken
Innate immunology
RIG-I-like receptor (RLR)
Virus

ABSTRACT

Melanoma differentiation-associated gene 5 (MDA5) is a member of the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) family and plays a pivotal role in the anti-viral innate immune response. As RIG-I is absent in chickens, MDA5 is hypothesized to be important in detecting viral nucleic acids in the cytoplasm. However, the molecular mechanism of the regulation of chicken MDA5 (chMDA5) expression has yet to be fully elucidated. With this in mind, a ~2.5 kb chMDA5 gene promoter region was examined and PCR amplified to assess its role in immune response. A chMDA5 promoter reporter plasmid (piggybac-MDA5-DsRed) was constructed and transfected into DF-1 cells to establish a Piggybac-MDA5-DsRed cell line. The MDA5 promoter activity was extremely low under basal condition, but was dramatically increased when cells were stimulated with polyinosinic: polycytidylic acid (poly I:C), interferon beta (IFN-β) or Infectious Bursal Disease Virus (IBDV). The DsRed mRNA level represented the promoter activity and was remarkably increased, which matched the expression of endogenous MDA5. However, Infectious Bronchitis Virus (IBV) and Newcastle disease virus (NDV) failed to increase the MDA5 promoter activity and the expression of endogenous MDA5. The results indicated that the promoter and the Piggybac-MDA5-DsRed cell line could be utilized to determine whether a ligand regulates MDA5 expression. For the first time, this study provides a tool for testing chMDA5 expression and regulation.

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1. Introduction

The innate immune system acts as the first defensive barrier against invading pathogens, using pattern recognition receptors to recognize viral pathogen-associated molecular patterns (PAMPs). Pattern recognition receptors consist of toll-like receptor, nucleotide-binding oligomerization domain protein-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Janeway and Medzhitov, 2002; Meylan et al., 2006). The RLR family is composed of RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2, all of which play important roles in recognizing viral RNA in the cytoplasm (Yoneyama and Fujita, 2010). RIG-I and MDA5 both harbor a central DExD/H-box RNA helicase domain, two N-terminal caspase activation and recruitment domains (CARDs), and a C-terminal regulatory domain. The RNA helicase domain is responsible for ATP hydrolysis (Yoneyama et al., 2005, 2004). The C-terminal regulatory domain is essential for binding viral RNA (Cui et al., 2008; Takahasi et al., 2009). The two CARDs function via CARD–CARD interactions with interferon promoter-stimulating factor 1, leading to the activation of interferon regulatory factor 3 and nuclear factor kappa-light chain-enhancer of activated B cells (Belgnaoui et al., 2011), which finally induces type I interferon (IFN-I) production and triggers a series of initial antiviral activities (Stark et al., 1998). During viral infection, swift upregulation of IFN-I is critical to the host antiviral responses (Bracci et al., 2005). Specifically, the expression of RIG-I and MDA5 are greatly induced by IFN-I as a positive-feedback mechanism, which results in signaling cascade to promote antiviral response (Gack et al., 2008).

In mammals, RIG-I and MDA5 recognize distinct, but overlapping, families of RNA viruses (Kawai and Akira, 2007; Ramos and Gale, 2011). However, chickens lack RIG-I and this may provide

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http://dx.doi.org/10.1016/j.molimm.2016.06.006
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a rationale for increased susceptibility to certain pathogens in comparison to ducks and mammals, such as Highly Pathogenic Avian Influenza (Barber et al., 2010; Karpala et al., 2011). This suggests that chicken MDA5 (chMDA5) may have an important role in chicken during viral invasion. Unlike ducks and mammals, chMDA5 is able to recognize both long and short poly (I:C) and induce IFN-I beta (IFN-β) expression (Hayashi et al., 2014; Karpala et al., 2011) and the expression of chMDA5 is upregulated and plays a role during infection with influenza. Interestingly, the expression of chMDA5 does not seem to be sufficient to prevent influenza proliferation (Karpala et al., 2011). A plethora of research exists pertaining to MDA5 function in mammals, little is known about the role of chMDA5 and the ligands that interact with chMDA5. Similar to mammalian MDA5, the expression of chMDA5 is enhanced by IFN-β (Kang et al., 2002; Lee et al., 2012), but the molecular mechanism is not yet known. A greater understanding of the transcriptional regulation of chMDA5 is essential to elucidating the role of MDA5 in chicken antiviral response.

In the present study, we cloned and characterized a 2.5 kb promoter of chMDA5. This promoter was inserted into a reporter plasmid and transfected into chicken cells to generate a cell model which could be used to investigate the promoter activity. The activity of the promoter reflected the expression of endogenous MDA5, indicating that the system could be used to determine potential MDA5 ligands.

2. Materials and methods

2.1. Amplification of the 5′- upstream region of chMDA5 gene

An about 2500 bp of the 5′-flanking region of MDA5 gene was amplified using White Leghorn chicken genomic DNA as the template. Primers (F1 and R1, listed in Table 1) were designed according to the sequence published on NCBI GenBank. PCR amplification was performed using KOD-Plus-Neo polymerase (TOYOBO) according to the manufacturer's instruction. The PCR product was cloned into the pMD19-T vector (Takara) to generate the 19T-MDA5 plasmid and was completely sequenced.

Seven MDA5 promoter sequences from birds, fish and mammals obtained from the NCBI (http://www.ncbi.nlm.nih.gov/), were compared with the chMDA5 promoter sequence. Turkey was chosen to representative for birds that lack RIG-I and duck for birds that have RIG-I. Zebrafish and the mammals all have RIG-I. The tree was generated using MEGA4.0 by the Bootstrap of Test (neighbor-joining method, 1000 replicates; seed = 58150).

2.2. Construction of chMDA5 promoter reporter plasmid

The promoter was amplified using primers designed with a *Pci*I restriction site on the forward primer and a BamHI restriction site on the reverse primer (Table 1). The PCR reaction was carried out as described above. The amplification product was purified and double digested with restriction enzymes *Pci*I and BamHI. The purified MDA5 promoter was ligated into the pDsRed1-N1 Vector (CLONTECH) that was previously digested with the same restriction enzymes to replace the CMV promoter. This recombinant plasmid is referred to as the MDA5-DsRed plasmid. Primers were designed with a *Sfi*I restriction site on the forward primer and a NheI restriction site on the reverse primer (Table 1) and PCR reaction was carried out using the MDA5-DsRed plasmid as a template to amplify the region containing the MDA5 promoter and the coding region of DsRed. The amplification product was purified and digested with restriction enzymes *Sfi*I and NheI and the purified MDA5-DsRed fragment was ligated into piggybacGFP Plasmid (SBI), which was previously digested with the same restriction enzymes to replace the CMV 7 promoter, generating the piggybac-MDA5-DsRed plasmid.

2.3. Cell culture, transfection and puromycin-selection

DF1, a chicken embryonic fibroblast cell line (Himly et al., 1998), was cultured in Dulbecco's modified Eagle's medium (DMEM)(GIBCO) supplemented with 3.7 mg/mL sodium bicarbonate, 1 × MEM Non-Essential Amino Acids (GIBCO), 100 U/mL penicillin, 100 ng/mL streptomycin and 10% fetal bovine serum (FBS) (Hyclone), in a 5% CO_2 incubator at 37 °C. The piggybac-MDA5-DsRed plasmids were transfected into DF1 cells using Lipofectamine® LTX & PLUS™ Reagent (Invitrogen) according to the manufacturer's instructions. 48 h after transfection, 5 μg/mL puromycin was added to the culture media for selection. The continuous cell line was passaged 3–5 times with puromycin selection to obtain a stable cell line containing piggybac-MDA5-DsRed plasmids and that expressed GFP (Piggybac-MDA5-DsRed).

2.4. Poly (I:C), IFN-β and virus challenge

Piggybac-MDA5-DsRed cells were seeded overnight in 12-well plates to 70–80% confluence prior to treatment. For Poly (I:C) challenge, 2.5 μg/mL of long synthetic dsRNA analog poly (I:C) (size:1.5–8 kb) (Invivogen) or 2.5 μg/mL short synthetic dsRNA analog poly(I:C) (size: <1 kb) (Yuanye) was transfected into cells using Lipofectamine® LTX & PLUS™ Reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24 h post-transfection for fluorescence assay, fluorescence-activated cell sorting (FACS) and qPCR analysis. For IFN-β treatment, 8 μg/mL chicken IFN-β was added into the culture medium for 48 h before the cells were collected for fluorescence assay, FACS and qPCR analysis. For viral challenge, live vaccines of Infectious Bursal Disease Virus (IBDV) NF8, Newcastle Disease Virus (NDV) Lasota and Infectious Bronchitis Virus (IBV) H52, obtained from Qianyuanhao, were diluted with DMEM without FBS and used to replace the culture medium. For the mock treatment, DMEM without FBS was used to replace the culture medium. After 2 h incubation, the diluted virus was replaced with DMEM supplemented with 2% FBS. Cells were observed and collected for analysis 84 h post infection (hpi).

2.5. Gene expression analysis by real-time quantitative PCR

RNA was extracted using Total RNA kit II (OMEGA) followed by reverse transcription using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa) according to the manufacturer's instructions. The relative quantitation of gene expression from the Piggybac-MDA5-DsRed cell line following treatment was performed on a LightCycler® 96 (Roche) to determine transcript levels of DsRed, MDA5, IFN-α, IFN-β and β-actin, using the comparative threshold cycle (ΔΔCT) method. 2 μL of cDNA was amplified in a 25 μL reaction using SYBR® Premix Ex Taq II (TliRNaseH Plus; TaKaRa). The corresponding primer sets are listed in Table 1. The relative expression levels of target genes were expressed in terms of CT value normalized to β-actin as described previously (Lee et al., 2014). The fold changes for each gene were normalized to the β-actin expression in each sample. The relative expression of target gene normalized to β-actin is showed as $2^{-\Delta CT}$, where $\Delta CT = CT_{targetgene} - CT_{\beta\text{-actin}}$. The relative expression in the treatment group versus that in the mock group was calculated using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT_{targetgene}$ in the treatment group$-\Delta CT_{targetgene}$ in the mock group. The geometric mean of the fold change in expression is shown as $\log_2$ scale. The mean of ΔΔCT was based on three samples (n = 3).

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