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Research paper

Molecular characterization and expression analysis of the duck viperin gene

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ABSTRACT

Viperin is well known as one of the interferon-stimulated genes involved in innate immunity. Recent studies showed that this gene is mainly responsible for antiviral response to a large variety of viral infections. In this study, we successfully cloned and characterized the complete coding sequence of duck viperin gene. The duck viperin gene encodes 363 amino acids (aa) and is highly similar to viperins from other species. Moreover, secondary and 3D structures were predicted, and these structures showed two main domains, one signal peptide, and one radical S-adenosyl methionine (SAM) domain. Additionally, the duck viperin expression was analyzed in vitro and in vivo, and analysis results indicated that the duck viperin can be strongly up-regulated by poly(I:C) and Newcastle disease virus in primary duck embryo fibroblast cells. Results also demonstrated that Newcastle disease virus significantly induced duck viperin expression in the spleen, kidneys, liver, brain, and blood. Our findings will contribute to future studies on the detailed functions and potential underlying mechanisms of this novel protein in innate immunity.

1. Introduction

Viperin, also known as cig5 and RSAD2, is a highly conserved antiviral protein and is one of the members of over 300 interferon (IFN)-stimulated genes (ISGs) involved in innate immunity (Biron, 2001; Dixit et al., 2010). Viperin was first discovered from a human cytomegalovirus (HCMV)-inducible gene in fibroblasts as an IFN-inducible antiviral protein directly induced by HCMV; this virus was named viperin for virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible (Chin and Cresswell, 2001). Despite more than hundreds of members among the ISGs super family, the functions of most ISGs still remain elusive and only very limited antiviral host factors such as viperin have been described in the literature.

Human viperin has 361 aa with a molecular mass of 42 kDa. Viperin from many species is characterized as the innate immune-mediated protein with direct antiviral activity (Chin and Cresswell, 2001; Helbig and Beard, 2014), which could be induced to express by stimuli, such as IFNs and multiple kinds of viruses. Viperin also plays key roles in defeating and combating the invasion of microbes, including a broad range of viruses ranging from DNA and RNA viruses, germs, and parasites. Furthermore, emerging roles of viperin have been reported, such as regulation of host cell metabolism and modulation of innate immune signaling (Chin and Cresswell, 2001; Fitzgerald, 2011; Helbig and Beard, 2014; Seo et al., 2011).

Newcastle disease virus (NDV), an *Avulavirus* of the family *Paramyxoviridae*, is the causative agent of Newcastle disease (ND) (Diel et al., 2012; Kapczynski et al., 2013). NDV is one of the most important causative agents of poultry because it causes high death rate and huge economic losses in the poultry industry (Aldous and Alexander, 2008; Diel et al., 2012). Over 236 species of birds including pigeons, double crested cormorants, and even human could be infected by NDV, thereby suggesting the extremely wide host range of NDV (Alexander, 2000; Diel et al., 2012; Kaleta and Kummerfeld, 2012; Kapczynski et al., 2013; Kim et al., 2008). The peripheral blood mononuclear cells or lymphocyte cells infected by NDV could induce the expression of many factors like interferon alpha (IFN-α), beta (IFN-β) and gamma (IFN-γ), which are critical components in the innate immune system (Ahmed et al., 2007; Sick et al., 1998).

Although viperin in many different species, such as human and fish, is characterized as a virus-induced protein, whether viperin expression could be induced by NDV in duck cells remains unknown. In this study, we successfully cloned the duck viperin (dViperin) gene from duck peripheral blood mononuclear cells and analyzed its homology with other species in terms of amino acid residues. The relationship between

Abbreviations: CDD, Conserved Domains; CDS, coding sequence; DEF, duck embryo fibroblast; dViperin, duck viperin; HA, Hemagglutination; hpi, hours post-infection; IFN, interferon; ISG, interferon stimulated gene; LDA, limiting dilution analysis; MOI, multiple of infection; NDV, Newcastle disease virus; ORF, open reading frame; poly(I:C), polyinosinic–polycytidylic; 5′UTR, 5′-terminus untranslated regions; SAM, S-adenosylmethionine; SPF, specific pathogen free.

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dViperin and NDV infection was also investigated. Our results would facilitate further insights into understanding the roles of dViperin, as well as its underlying mechanisms.

2. Materials and methods

2.1. Virus, cell and animal

Newcastle disease virus, A/Chicken/Guangdong/2008/G7, designated as G7, used in this study was isolated and identified from chicken in Guangdong province, China in 2008 through a hemagglutination (HA) inhibition assay. Ten-day-old specific pathogen free (SPF) embryonated hen eggs were used to purify G7 by using limiting dilution analysis (LDA), and the allantoic fluid with the highest dilution and hemagglutination titer was harvested and stored at −70 °C for future use. The abovementioned steps were repeated thrice according to LDA principle, and the EID_{50} of G7 was calculated using the formula method of Reed and Muench (1938). The inactivation of G7 was achieved using 0.02% formaldehyde at 37 °C for 20 h and was confirmed through a hemagglutination assay of allantoic fluid from eggs at 3 days post-injection of inactivated G7, as described above. Primary embryo fibroblast cells were prepared from 12-day-old embryonated duck eggs, as previously described (Vaheri et al., 1973), and cultured in Dulbecco's modified Eagle's medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Two-week-old Changbai ducks were purchased from a duck farm in Lanzhou and grouped in ventilated cages. All animal experiments were carried out in ABSL-3 facilities and approved by the guidelines for Animal Experimentation of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (SYXK2010-003).

2.2. RT-PCR amplification of complete dViperin gene

A pair of oligonucleotide primers from 5′-terminal untranslated regions (5′UTR) and 3′-terminal coding sequence (CDS) were designed based on the reported incomplete viperin sequence (GenBank Accession Nos. NW_004676922.1 and XM_005018580.1) as shown in Table 1. Total RNA was extracted from duck peripheral blood mononuclear cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and then served as the templates for reverse transcription of cDNA. The complete viperin gene was subsequently amplified by PCR with an initial denaturation step at 98 °C for 2 min; followed with 30 cycles of denaturation, annealing and extension at 98 °C for 10 s, 56 °C for 15 s and 72 °C for 70 s; and a final elongation step at 72 °C for 5 min. The PCR products were purified and inserted into pMD18-T vector (Takara, Dalian, China) and then sequenced (GENEWIZ, Beijing, China). The complete coding sequence of viperin gene was obtained by assembling all sequenced fragments using Lasergene software (DNA-STAR, WI, USA).

Table 1
PCR primers used in this study.

Primer	Sequence of oligonucleotide (5′–3′)	Purpose
Dkvip-ncd-f	GACAGGATGGGGCTTTTAAGAGAGG	Gene cloning
Dkvip-ncd-r	TTACCAGTCCAGTTTCATGTCTGCT	
DkViperin-f	ATGCATCTGGGCGCGCTGCT	Gene cloning
DkViperin-r	TTACCAGTCCAGTTTCATGTCTGCT	
qDkViperin-f	GCCGAGAGTATGCTGTTGCTT	qRT-PCR
qDkViperin-r	AATGAGCAGGCACTGGAACAC	
qGapdh-f	ATGAGAAGTATGACAAGTCC	qRT-PCR
qGapdh-r	ACTGTCTTCGTGTGTGGCT	qRT-PCR
qNDV-F-f	AGCGCCATTAGAGGCATATAACA	
qNDV-F-r	CTTGGATCTTGCGGATGGA	

2.3. Bioinformatics analysis

The domain structure of dViperin was predicted and generated through a Conserved Domains (CDD) online search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Amino acid sequences of Viperin from different species were aligned using MEGA software and edited with ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The phylogenetic trees were generated based on the representative viperin sequences of *aves*, *mammalia*, and *pisces* classes from GenBank using the MEGA software (version 5.0). Phylogenetic and molecular evolutionary analyses were then calculated by neighbor-joining method with 1000 bootstrap replicates. Secondary and partial 3D structures of dViperin protein were deduced using PHYRE 2 online server and PyMOL software.

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

To investigate whether dViperin expression could be induced by G7 infection, duck embryo fibroblast (DEF) cells plated and cultivated in 12-well plates were infected with live or inactivated G7 at a dose range of 0–10 multiple of infection (MOI) for 24 h. Meanwhile, DEF cells treated with different concentrations of poly(I:C) in the range 1.25–10 μg/ml were used as positive control. The total RNA of DEF cells was extracted and subjected to qRT-PCR analysis. Primers (Table 1) of dViperin and NDV fusion (F) gene were designed using Primer5 software and their specificity was determined by dissociation curves. qRT-PCR was subsequently performed using an ABI7500 Fast Real-Time PCR system with a One Step SYBR Green® PrimeScript™ PLUS RT-PCR Kit (Takara). In brief, the reaction volume was 20 μl, containing 200 ng of total RNA for each test, 10 μl 2× One Step RT-PCR Buffer and 0.8 μM of forward and reverse primers. The RT-PCR procedure was carried out at 42 °C for 10 min, 95 °C for 10 s, and followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 10 s. The relative mRNAs of dViperin were presented and calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH (GU564233.1) as an internal control (Schmittgen and Livak, 2008), whereas the relative mRNA level of NDV F gene was normalized by using the level of the NDV F gene at 12 hpi.

2.5. Western blot analysis

Primary duck embryo fibroblast cells were infected with different doses of G7 strain and harvested at 24 hpi. The total protein of DEF cells and duck tissues from the animal experiment was extracted and lysed using RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% NP-40, and 1 mM PMSF. The samples were denatured at 100 °C for 10 min prior to loading onto 10% gels by SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were then transferred onto nitrocellulose membranes and blocked with 5% non-fat milk in PBS with 0.1% Tween-20 for 2 h at room temperature. Membranes were then incubated for 2 h with rabbit anti-dViperin polyclonal antibody, which was prepared and stored in our laboratory by immunizing the rabbits with the purified Viperin protein. After three washes, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H + L) for 1 h at room temperature. Target proteins were detected through exposure.

2.6. Animal experiment

To examine the physical distribution of viperin in different tissues and their kinetic changes upon NDV infection, 60 Changbai ducks (2 weeks old) were purchased from a duck farm in Lanzhou and randomly divided into three groups of 20. The ducks were housed in isolators. The ducks in each group were infected with G7 at 10^6 EID_{50} and inactivated G7 via intranasal or injected with PBS as a negative control. At 0, 12, 24, 48 and 72 hpi, three ducks in each group were sacrificed,