Developmental and Comparative Immunology 84 (2018) 71–81

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Broad-spectrum antiviral functions of duck interferon-induced protein with tetratricopeptide repeats (AvIFIT)

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ARTICLE INFO

Article history:
Received 18 September 2017
Received in revised form
7 February 2018
Accepted 7 February 2018
Available online 8 February 2018

Keywords:
Duck
AvIFIT
Influenza A virus
Growth arrest
Nucleoprotein

ABSTRACT

Mammalian interferon-induced proteins with tetratricopeptide repeats (IFITs) play important roles in many cellular processes and host innate immune response to viruses. However, the functions of IFIT proteins in birds are largely unknown. Here, we first describe that the only one avian IFIT protein is orthologous to ancestor of mammalian IFITs. We find that the predicted structure of duck AvIFIT protein is similar to that of human IFIT5. We also find that duck AvIFIT protein shows antiviral activity to a broad range of specific RNA and DNA viruses like mammalian IFIT proteins. Further analysis indicates that overexpression of duck AvIFIT protein in DF1 cells leads to a remarkable accumulation of cells at G1/S transition associated with growth arrest and may promote apoptosis. Moreover, duck AvIFIT binds to nucleoprotein (NP) of H5N1 influenza virus and upregulates the expression of genes involving the IFN pathway in DF1 cells. In summary, our findings support that duck AvIFIT protein plays critical role in host immune response to viruses, at least H5N1 virus, through affecting function of viral NP protein, magnifying the IFN signaling and arresting cell growth.

1. Introduction

Virus infections can cause devastating consequences for the host and must therefore be resisted quickly and effectively. Host pattern-recognition receptors (PRRs) sense 'non-self' molecular signatures associated with microbe (Akira et al., 2006; Goubau et al., 2013). Pathogen-associated molecular patterns (PAMPs) delivered and generated during the viral life cycle activate those receptors to initiate the innate antiviral defense (Pichlmair and Reis, 2007). Interferons (IFNs) are a family of proteins secreted by host cells in response to various pathogens, thereby activating and stimulating a cascade of pathways for antiviral factors, including hundreds of IFN-stimulated genes (ISGs) (Schoggins and Rice, 2011). Among them, IFN-induced tetratricopeptide repeat (TPR) proteins (IFITs) is a family characterized by multiple repeats of TPR helix-turn-helix motifs that mediate a variety of protein-protein interactions involved in translation inhibition, sequestering viral proteins or RNA, cell migration, and proliferation (Diamond and Farzan, 2013; Fensterl and Sen, 2015; Vladimer et al., 2014).

Human IFIT1 was the first IFIT protein to be discovered and named as "P56", a 56 kDa protein (Chebath et al., 1983; Kusari and Sen, 1987). So far, IFIT genes have been reported in mammals, birds, fish and amphibians (Fensterl and Sen, 2011). Four canonical members, known as IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60 or IFIT4), IFIT5 (ISG58), and an uncharacterized IFIT1B as well as a pseudogene IFIT1P1, have been characterized in humans. However, mouse lacks IFIT5 but presents one additional IFIT3 (Ifit3b) and two IFIT1 members (Ifit1b and Ifit1c) (Fensterl and Sen, 2011, 2015). Normally, IFIT genes are silent or expressed at very low constitutive levels. IFIT gene expression can be induced by many stimuli including viral and bacterial infections, as well as independently of type I IFNs (Barnes et al., 2004; Lou et al., 2009). In addition, IFIT

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https://doi.org/10.1016/j.dci.2018.02.007
0145-305X/

proteins localize within the cytoplasm and ostensibly lack any enzymatic activity (Fensterl and Sen, 2011, 2015).

Mammalian IFIT proteins were initiated to characterize their functions in gene transcription and cellular process. For example, human and mouse IFIT1/Ifit1 and IFIT2/Ifit2 were reported to block binding of the eukaryotic translation initiation factor 3 (eIF3) to the eIF2-GTP-Met-tRNA ternary complex by interacting with eIF3e or eIF3c (Hui et al., 2003; Terenzi et al., 2005, 2006). Human IFIT1 and IFIT3 inhibit cell proliferation (Hsu et al., 2011; Xiao et al., 2006), whereas IFIT2 promote cellular apoptosis (Stawowczyk et al., 2011). Recently, mammalian IFIT proteins were well re-characterized as important immune genes. Human IFIT1 blocks HCV (hepatitis C virus) replication through targeting eIF3-dependent steps in the viral RNA translation initiation and mediates binding of a larger protein complex together with IFIT2 and IFIT3 to inhibit HPV (human papillomavirus) infection (Pichlmair et al., 2011; Terenzi et al., 2008; Wang et al., 2003; McDermott et al., 2012). Human IFIT1 and IFIT5 can also function as sensors for viral RNA by recognizing and sequestering an uncapped 5′-PPP-RNA or cap0 RNA (Abbas et al., 2013; Katibah et al., 2014; Kumar et al., 2014; Pichlmair et al., 2011), whereas IFIT2 appears to prefer binding AU-rich dsRNA (Yang et al., 2012). Moreover, rabbit IFIT1B shows very high affinity to cap-proximal regions of cap0-mRNAs (Kumar et al., 2014). However, such sophisticated and functional divergent IFIT gene family in mammals still keeps a door for viruses. For example, 2′-O methylation of the viral RNA subverts innate host antiviral response through escape of IFIT1-, IFIT2- and IFIT5-mediated suppression (Daffis et al., 2010; Habjan et al., 2013; Kumar et al., 2014; Szretter et al., 2012). In contrast, birds have a highly contractive IFIT family including only one member (Huang et al., 2013; Liu et al., 2013; Wang et al., 2015), and its function is largely unknown.

Here, we first describe that ancient tetrapod IFIT has been lineage-specifically expanded in mammals, but not in birds. Structural analysis indicate that duck AvIFIT protein holds a structure being more similar to that of human IFIT5 (representing an ancient homolog) than other two new gained IFIT proteins (IFIT1 and IFIT2). We also demonstrate that duck AvIFIT exhibits a broad-spectrum antiviral activity, which inhibits the replication of a strain of positive single-stranded RNA virus, two strains of double-stranded RNA viruses, three strains of negative single-stranded RNA viruses and a strain of double-stranded DNA virus. Further *in vitro* experiments indicated that duck AvIFIT protein could inhibit these virus replications through one or more pathways. When infected with CK/0513 H5N1 influenza A virus, duck AvIFIT protein might directly or indirectly affect viral replication in DF1 cells. These effects include binding and/or sequestering viral NP protein, arresting cell growth, inducing cell apoptosis and magnifying the IFN signaling.

2. Materials and methods

2.1. Molecular phylogenetic and structural analysis of IFIT proteins

IFIT coding and protein sequences were collected from GenBank (www.ncbi.nlm.nih.gov/) and Ensembl (www.ensembl.org/) as detailed in Additional file 1. Multiple sequence alignments were performed using the PRANK (Phylogeny-aware alignment with PRANK, v140603). Maximum-likelihood (ML) trees were constructed using the iq-tree (W-IQ-TREE, v1.3.11) with a bootstrap value of 1000 and displayed using the FigTree (v1.4.2). The tertiary structure of duck AvIFIT protein was predicted using the I-TASSER (http://zhanglab.ccmb.med.umich.edu/) and aligned using the PyMOL (v1.8.2.3) software.

2.2. Cell culture and viral infections

DF1 (chicken embryonic fibroblasts cells), Vero (Aferical green monkey kidney cells), MDCK (Madin-Darby canine kidney cells), PK15 (porcine kidney epithelial cells), IBRS2 (porcine kidney cells) and BHK21 (baby hamster kidney fibroblast cells) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) in an atmosphere of 5% CO_2 at 37 °C.

Four strains of negative single-strand RNA viruses (DK/49 H5N1, GS/65 H5N1, CK/0513 H5N1 and NDV/La Sota), one strain of positive single-strand RNA viruses (FMDV/O/Mya), two strains of double-strand RNA viruses (IBDV/B87 and REOV/Z97/C10) and two strains of double-stranded DNA viruses (FPV/CVCC/AV1003 and PRV/Henan/2014) were selected to test the antiviral activity of duck AvIFIT protein to RNA viruses. Among them, the DK/49 (a high pathogenic) and GS/65 (a weak pathogenic) H5N1 viruses were isolated from a duck and a goose, respectively, during the avian influenza outbreak of 2005 in China (Chen, 2009; Song et al., 2011). Studies of these two H5N1 viruses were conducted in a biosecurity level 3 + laboratory approved by the Chinese Ministry of Agriculture. The CK/0513 (a weak pathogenic H5N1) virus was maintained in a biosecurity level 2 + laboratory approved by the China Agricultural University. The NDV/La Sota, IBDV/B87, REOV/Z97/C10, FPV/CVCC/AV1003, PRV/Henan/2014 and FMDV/O/Mya viruses were maintained in a biosecurity level 2 + laboratory approved by China Institute of Veterinary Drug Control or Lanzhou Veterinary Research Institute.

These viruses were propagated in 10-*d*-old chicken embryos. Three cell samples inoculated with a multiplicity of infection (MOI) of 0.001 with the DK/49, GS/65 or CK/0513 virus after 12, 24, 36, 48, 60 and 72 h were collected to monitor virus replication. Similarly, three samples of cells infected with an MOI of 0.01 with NDV, IBDV, REOV and PRV after 24 or 48 h were collected to probe virus replication. In addition, three samples of cells infected with an MOI of 0.1 with the FPV and FMDV viruses after 6, 12, 18 or 24 h were collected to measure virus replication. Titers were calculated by the EID50 individuals using the Reed and Muench method (DK/49, GS/65 and NDV), monitored TCID50 of the cytopathic effect of end-point dilutions (CK/0513, PR8, IBDV, REOV, PRV and FMDV), or quantified through quantitative PCR with the primer listed in Table S2 (FPV).

2.3. Establishment of stable recombinant DF1 cell lines

The coding regions of duck AvIFIT (KF956064.1) and RIG-I (NM_001310380.1) were amplified from duck lung tissues infected with the DK/49 virus using the primers in Table S3 (Huang et al., 2013). The coding region of mouse Mx1 was obtained from mouse C2C12 cells cDNA based on the mMx1 sequence (NM_010846.1). Full-length AvIFIT, dRIG-I and mMx1 were cloned into the piggyBac (containing a Flag-tag; (Wu et al., 2007), respectively (Table S3). Recombinant plasmids were verified by DNA sequencing.

Cells were transfected with the recombinant plasmids using Lipofectamine 3000 (Thermo Fisher, Carlsbad, CA) according to the manufacturer's protocol. They were sorted by flow cytometry and then selected with neomycin (800 μg/mL) as described previously (Wu et al., 2007). During the early passages in culture, clusters of spontaneously contracting cells were seen on several distinct areas of the culture dishes. These were isolated with a glass cloning ring, dissociated with 0.25% Typsin-EDTA (Gibco, Carlsbad, CA), and removed to other culture dishes to establish stable monoclonal cell lines. All cells in the present study were all monoclonal cell lines.