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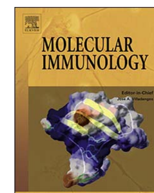

Maintenance of cyclic GMP–AMP homeostasis by ENPP1 is involved in pseudorabies virus infection

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A B S T R A C T

In a previous study, we demonstrated that porcine cyclic GMP–AMP (cGAMP) synthase (cGAS) catalyzes cGAMP production and is an important DNA sensor for the pseudorabies virus (PRV)-induced activation of interferon β (IFN-β). Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) has recently been identified as the hydrolase of cGAMP in rodents, but its role in porcine cells is not clear. Our recent study demonstrated that porcine ENPP1 is responsible for the homeostasis of cGAMP and is critical for PRV infection. Porcine ENPP1 mRNA is predominantly expressed in muscle. PRV infection was enhanced by ENPP1 overexpression and attenuated by silencing of ENPP1. During PRV infection, the activation of IFN-β and NF-κB was reduced in ENPP1 overexpressed cells and promoted in ENPP1 knockdown cells. Investigation of the molecular mechanisms of ENPP1 during PRV infection showed that ENPP1 hydrolyzed cGAMP in PRV-infected or cGAMP-transfected cells and inhibited IRF3 phosphorylation, reducing IFN-β secretion. These results, combined with those for porcine cGAS, demonstrate that ENPP1 acts coordinately with cGAS to maintain the reservoir of cGAMP and participates in PRV infection.

1. Introduction

Mammals are constantly challenged by pathogens, including viruses and bacteria. The innate immune system reacts to microbial molecules recognized as non-self, called ‘pathogen-associated molecular patterns’, using a limited number of germline-encoded receptors called ‘pattern recognition receptors’ to mount an appropriate immune response to the pathogen. One pivotal type of PAMP includes pathogenic nucleic acids, such as DNA, and cytosolic pattern recognition receptors that sense microbial nucleic acids in the cytoplasm have recently been discovered (Kato et al., 2011; Keating et al., 2011).

Pseudorabies virus (PRV), an enveloped double-stranded DNA (dsDNA) virus, is a member of the subfamily *Alphaherpesvirinae* in the family *Herpesviridae* (Pomeranz et al., 2005). It has been reported that DNA-dependent activator of interferon (IFN)-regulatory factors (DAI) is a DNA sensor of the PRV genome and triggers the production of type I IFN. DAI knockdown significantly reduced the PRV-induced activation of IFN-β (Xie et al., 2010). However, DAI deficiency did not affect the production of type I IFNs in response to poly(dA:dT), a synthetic analogue of B-DNA, suggesting that redundant cytosolic DNA sensors exist (Ishii et al., 2008). DEAD (Asp–Glu–Ala–Asp) box polypeptide 41 (DDX41), a member of the DEXDc helicase family, has since been identified as another intracellular DNA sensor of the genomic DNA of PRV and is involved in the dsDNA-virus-mediated type I IFN signaling pathway in porcine kidney cells (Zhu et al., 2014).

Cyclic GMP-AMP (cGAMP) synthase (cGAS), also called Mab-21 domain containing 1 (MB21D1), belongs to the nucleotidyltransferase family and is a newly identified cytoplasmic DNA sensor (Sun et al., 2013). cGAS recognizes cytosolic DNA and directly interacts with DNA through its amino-terminal domain, which is required for the activation of cGAS to synthesize cGAMP from GTP and ATP (Gao et al., 2013b; Li et al., 2013). cGAMP is a second messenger that activates the STING/TBK1/IRF3 axis and then induces IFN-β expression (Ablasser et al., 2013a; Diner et al., 2013; Sun et al., 2013). In a previous study, we demonstrated that cGAS is involved in the PRV-mediated induction of IFN-β. Using cGAS overexpression and RNA interference (RNAi), we also demonstrated that cGAS senses cytosolic PRV DNA and promotes IFN-β expression through STING and IRF3 (Wang et al., 2015).

Ectonucleotide pyrophosphotase/phosphodiesterase (ENPP1), also known as plasma cell membrane glycoprotein 1 (PC-1), is a type II transmembrane glycoprotein with pyrophosphatase and phosphodiesterase activities, which is expressed in a wide range of tissues and cell

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types, including the heart, kidney, vascular smooth muscle cells, and chondrocytes (Johnson and Terkeltaub, 2005; Nitschke et al., 2011). ENPP1 is thought to be involved in bone formation, insulin signaling, and glucose homeostasis (Anderson, 2003; Frittitta et al., 1996; Maddux et al., 1995; Meyre et al., 2005). Extracellular ATP is hydrolyzed to AMP and inorganic pyrophosphate (PP_i), which inhibits bone mineralization and calcification (Hessle et al., 2002). The overexpression of ENPP1 inhibits insulin receptor function, resulting in extreme human insulin resistance (Maddux and Goldfine, 2000; Maddux et al., 1995). ENPP1 has also been identified as the dominant 2′3′-cGAMP hydrolase in cultured cells, tissue extracts, and blood (Li et al., 2014), and recombinant ENPP1 efficiently hydrolyzes 2′3′-cGAMP. Tissue extracts and plasma derived from *Enpp1*$^{-/-}$ mice lack 2′3′-cGAMP degradation.

Whether ENPP1 is involved in viral infection is unclear. Therefore, in this study, the role of ENPP1 in PRV infection was investigated. Our results demonstrate that ENPP1 is involved in PRV infection, regulating cGAMP homeostasis. ENPP1 overexpression negatively regulated the STING/TBK1/IRF3 axis in PRV-infected or cGAMP-transfected cells, evident as reduced IRF3 phosphorylation and reduced IFN-β production. These results provide new insight into the role of ENPP1 in promoting PRV infection.

2. Materials and methods

2.1. Cells, virus, and tissues

PK13, PK15, 3D4/21 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco) at 37 °C 5% CO_2. The virulent PRV isolate QXX and porcine tissues were used as previously mentioned (Wang et al., 2017). The recombinant PRV strain of PRV-GFP, derived from PRV Hubei strain with TK gene replaced by GFP expression cassette from pEGFP-N1 plasmid, was kindly donated by Han-Zhong Wang from Wuhan Institute of Virology, Chinese Academy of Sciences.

2.2. Plasmids and transfection

The coding sequence of porcine ENPP1 gene was amplified from the cDNA of the porcine muscle with the primers shown in Table 2. The PCR product was cloned into p3 × Flag–CMV-14 (Sigma, Saint Louis, MI) to generate ENPP1–Flag. IFN-β–Luc was used as previously mentioned (Wang et al., 2015). NF-κB–Luc, containing four copies of the NF-κB-binding positive regulatory domain of IFN-β promoter, was kindly donated by Yong-Tao Li from the College of Animal Sciences and Veterinary Medicine, Henan Agricultural University. Transfection of plasmids was performed with Lipofectamine® 3000 (Invitrogen, Grand Island, NY), according to the manufacturer's instructions. Each assay was performed in triplicate.

2.3. Functional domains and phylogenetic analyses

The transmembrane domain of ENPP1 was predicted in TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The functional domains of ENPP1 were analyzed at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The protein sequences of ENPP1 from different species were taken from GenBank database and aligned by Clustal Omega. A phylogenetic tree was constructed with the neighbor-joining method in the MEGA 6 software. A bootstrap analysis was performed with 1000 replicates to assess the robustness of the tree branches.

Table 1
The sequences of siRNA used for gene knockdown.

Name	Sequence(5′–3′)	Position
siControl	UUCUCCGAACGUGUCACGU	/
siENPP1-1	GCGAGGCACUUUACCCUAU	1959–1977
siENPP1-2	GCACCGUCUGUCUGCUUUA	2006–2024
siENPP1-3	CCAUCCGUAAUCAAGAAAU	2456–2474

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

Total RNAs were extracted from cells or tissues with Trizol Reagent (TaKaRa, Otsu, Shiga) and reversely transcribed into cDNA with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa), according to the manufacturer's instructions. qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa) with the primers (Table 2) in an Eppendorf Mastercycler® ep realplex system. The comparative cycle threshold method was used to analyze the relative expression of mRNAs. Porcine 18S rRNA (for tissues) or actin (for cells) was used as internal controls against which to normalize the gene expression levels. Each assay was performed in triplicate.

2.5. Luciferase reporter assays

Cells cultured in 12-well plate were cotransfected with 0.02 μg/well pCMV–Renilla (normalization plasmid) and 0.1 μg/well IFN-β–Luc or NF-κB–Luc (luciferase reporter plasmid) using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. After 24 h, luciferase reporter assays was performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), according to the manufacturer's instructions. The luminescent signal was detected with a Fluoroskan Ascent™ FL Microplate Fluorometer (Thermo Scientific, Waltham, MA). Each assay was performed in triplicate.

2.6. IFN-β quantification

Cell supernatants were collected 24 h p.i. and secreted IFN-β was quantified by ELISA (Shanghai Tongwei Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Assay sensitivity was 2 pg/ml. Each assay was performed in triplicate.

2.7. Fluorescent microscopy

Cells grown on glass coverslips (Thermo Fisher Scientific, Waltham, MA, USA) were first infected with PRV-GFP for 24 h and then fixed with 4% paraformaldehyde for 20 min. After washing with phosphate-buffered saline (PBS), the glass coverslips were mounted and subjected to fluorescent microscopy (IX73, Olympus Corporation, Tokyo, Japan). Each assay was performed in triplicate.

2.8. Immunoblot analysis

Whole-cell lysates were extracted with RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM $MgCl_2$) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). The protein concentrations in the lysates were quantified with a BCA Protein Assay Kit (DingGuo, Beijing), detected with a microplate reader (Awareness Technology Inc., Palm City, FL). Protein samples (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes (Millipore, Billerica, MA), and incubated in 5% nonfat milk (Sangon) at room temperature for 1 h. The membranes were incubated with the primary antibody at °C overnight and then incubated with a horseradish-peroxidase-conjugated donkey anti-mouse IgG antibody (diluted 1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA) or donkey anti-rabbit IgG antibody (diluted 1:5000; Jackson ImmunoResearch Laboratories) at room temperature for 1 h. The target proteins were detected with Luminata Crescendo Western HRP Substrate (Millipore)

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