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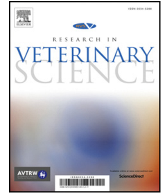

Molecular characterization and biological activity of bovine interferon-omega3

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ABSTRACT

Bovine interferon-omega3 (BoIFN-ω3) gene was amplified from bovine liver genomic DNA, which encodes a 195-amino acid protein containing a 23-amino acid signal peptide. Analysis of the molecular characteristics revealed that BoIFN-ω3 evolving from IFN-ω, contained four cysteine residues and five alpha helices, showing that BoIFN-ω3 presented the typical molecular characteristics of type I interferon. BoIFN-ω3 exhibited antiviral and antiproliferative activities, which exerted a protective effect against VSV in several mammalian cell lines, as well as against BEV, IBRV, and BVDV in MDBK cell. Moreover, BoIFN-ω3 was shown to be highly sensitive to trypsin, but remaining stable despite changes in pH and temperature. Additionally, BoIFN-ω3 induced the transcription of Mx1, ISG15, and ISG56 genes, as well as the expression of Mx1 protein in a time-dependent manner. These findings will be useful to further study BoIFN-ω in host's defence against infectious diseases, particularly viral infections. Furthermore, results will facilitate further research on the bovine interferon family.
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1. Introduction

Interferons (IFNs), which are produced in response to viral infections, contribute to host defence by establishing an antiviral state in target cells wherein viral replication is blocked or impaired as a result of the synthesis of a number of enzymes interfering with cellular and viral processes (Staeheli, 1990; Sen, 2001). Type I IFNs are a family of cytokines with pleiotropic activities including inhibition of viral replication and cell proliferation, as well as activation of the immune system (Stark et al., 1998). Their administration has been proposed as an immunomodulatory therapy (Domenech et al., 2011) to treat several viral and immunomediated diseases. Type I IFNs, including IFN-α, IFN-β, IFN-ω, IFN-δ, IFN-τ, IFN-ε, IFN-ν, and IFN-κ, are produced by virus-infected cells and exert nonspecific antiviral activities on adjacent non-infected cells (Krause and Pestka, 2005). They display a general mechanism of action based on their interaction with specific cell-surface receptors and the subsequent induction of IFN-stimulated genes (ISGs) expression, thereby encoding direct antiviral effectors or molecules with the potential to positively and negatively regulate IFN signaling and other host responses, including enzymes, signaling proteins, chemokines, antigen-presenting proteins, transcription factors, and apoptotic proteins (Der et al., 1998). Overall, a number of ISGs that act to enhance pathogen detection and innate immune signaling (Schneider et al., 2014), such as ISG15, ISG56, and GTPase Mx1, have been shown to function as antiviral effectors. The Mx1 gene product, which is one of the first described inhibitors of virus entry, is broadly inhibitory and acts prior to genome replication at an early postentry step of the virus life cycle (Schneider et al., 2014). ISG15 demonstrates numerous antiviral functions, including inhibition of virus release, lysis of both viral and host proteins, and immunomodulatory cytokine-like properties in its unconjugated form (Schoggins, 2014). ISG56, which is induced in response to type I IFNs, dsRNAs, and viruses (Terenzi et al., 2006), has been implicated in antiviral actions of IFNs against West Nile virus and lymphocytic choriomeningitis virus (Wacher et al., 2007).

IFN-ω, which was first discovered by Hauptmann and Swetly (Hauptmann and Swetly, 1985), is a type I IFN secreted by virus-infected leukocytes, that are encoded by multiple IFN- or IFN-like genes present across mammalian groups, including cats, porcine and rabbit (Hauptmann and Swetly, 1985; Roberts et al., 1998; Charlier et al., 1993a; Mege et al., 1991). Like other type I IFNs, IFN-ω presents species-restricted biological activity in vitro and can bind to the same type I IFN receptor complex as other type I IFNs, thus exerting similar antiviral, antiproliferative, and immunomodulatory effects (Adolf, 1995). However, its antigenic structure is distantly related to IFN-α, -β, and -γ, as it does not cross react with antibodies against them (Adolf, 1995). Interferon-ω has been used for antiviral treatment in humans and many other mammalian species (Tiefenthaler et al.,

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1997; Hagelstein et al., 1998; Gil et al., 2014; Leal et al., 2014; Martin et al., 2002). Although human IFN-ω (HuIFN-ω) can exert in vivo antitumor effects in several models of human cancer (Horton et al., 1999), Feline IFN-ω (FeIFN-ω) shows a proven antiviral effect, both in vitro (Mochizuki et al., 1994; Ohe et al., 2008; Litzlbauer et al., 2014) and in vivo (Gil et al., 2014; Leal et al., 2014; Martin et al., 2002; Horton et al., 1999; Mochizuki et al., 1994; Ohe et al., 2008; Litzlbauer et al., 2014), against canine and feline parvovirus, herpesvirus, calicivirus, coronavirus, and rotavirus, and has been licensed for use in veterinary medicine (Virbagen®, Virbac) in Europe, Japan, Australia, New Zealand and Mexico (Domenech et al., 2011). Research on BoIFN-ω subtypes was not much reported, in 1998, BoIFN-ω1 was reported to have biological activation, and can sufficiently prevent luteolysis, without being deleterious to embryonic survival (Rodriguez et al., 1998a); in 2015, BoIFN-ω24 has been characterized and can be used as a candidate antiviral therapeutic reagent (Luo et al., 2015). The newfound BoIFN-ω3, which presents a potential as a novel antiviral therapeutic agent, exerts antiviral activity against VSV in several mammalian cell lines and a protective effect against bovine virus that including BEV, IBRV and BVDV. In this study, we present a novel type I IFN named BoIFN-ω3 on the basis of the location in the bovine genome. BoIFN-ω3 demonstrates typical characteristics of type I IFN, exerts antiviral activity in several mammalian cell lines, and displays low cytotoxicity in vitro. Additionally, BoIFN-ω3 can induce the transcription of Mx1, ISG15, and ISG56 genes, as well as the expression of Mx1 protein in a time-dependent manner. The current findings will be useful to further study BoIFN-ω in host's defence against infectious diseases, particularly viral infections, and should facilitate research on the bovine interferon family.

2. Materials and methods

2.1. Cells, viruses and antibody

Liver was collected from Holstein cows in a dairy farm in Harbin, Heilongjiang in Northeast China. Madin–Darby bovine kidney (MDBK) cells, primary bovine testicular (BT) cells, primary embryo bovine lung (BL) cells, feline kidney (F81) cells, Madin-Darby Canine Kidney (MDCK) cells, baby hamster Syrian kidney (BHK-21) cells, porcine kidney (PK-15) cells and rabbit kidney (RK-13) cells were preserved in our laboratory. Vesicular stomatitis virus (VSV) was purchased from the China Institute of Veterinary Drug Control. Bovine intestinal virus (BEV), bovine infectious bovine rhinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV) were preserved in our laboratory. Rabbit polyclonal antibody (PAb) against BoIFN-ω24 were prepared and preserved in our laboratory (Luo et al., 2015). Rabbit PAb against Mx1 (GTX110256) and GAPDH (GTX100118) were purchased from GeneTex (CA, USA). HRP-conjugated goat anti-rabbit IgG was purchased from ZSGB (Beijing, China).

Table 1
Sequences of the primers.

Primers	Sequences(5′–3′)
BoIFNWS	GATCCCTGGGCTGTGACYTGTCT
BoIFNWA	CTTCTCTTKCAGGTAGAYATGGAT
BoIFN-ω3S	TCGGGATCCTGTGACCTGTCTCAGAACCA
BoIFN-ω3A	AGGCTCGAGTCAAGGTGAGTTCAGATCTC
BoGAPDHS	TTCAACGGCACAGTCAAGG
BoGAPDHA	ACATACTCAGCACCAGCATCAC
BoMx-1S	TCAACCTCCACCGAACTG
BoMx-1A	TCTTCTTCTGCCTCCTTCTC
BoISG15S	GCAGCCAACCAGTGTCTG
BoISG15A	CCTAGCATCTTCACCGTCAG
BoISG56S	TGGACTGTGAGGAAGGATGG
BoISG56A	AGGCGATAGACAACGATTGC

The restriction enzyme sites that were introduced in primers are underlined.

2.2. Clone of BoIFN-ω3

Genomic DNA extracted from bovine liver (Sambrook and Green, 2012) was used as the PCR template. Degenerate primers BoIFNWS and BoIFNWA (Table 1) designed according to the alignment of BoIFN-ω subtypes, were used as the PCR primers with the following thermal profile: initial denaturation at 94 °C for 5 min, 30 amplification cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. The PCR products obtained were cloned into the pMD18-T cloning vector (TaKaRa, Japana) and sequenced.

2.3. Sequence analysis of BoIFN-ω3

Sequences analysis was performed on the DNAStar program (DNASTAR Inc., USA). The obtained sequences were identified by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the putative amino acid sequence was compared with its counterparts of other animals by using the program ClustalX. Signal peptide was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). The glycosylation sites were analyzed by NetGlycate 1.0 Server (http://www.cbs.dtu.dk/services/NetGlycate/) online. Multiple alignments and phylogenetic tree were constructed with ClustalX and MEGA5.0 using UPGMA method, and the availability of the branch stem was verified with 500 bootstrap replicates. Secondary structure elements were predicted using the algorithms available from NPS (http://www.npsa-pbil.ibcp.fr).

2.4. Expression and purification of recombinant BoIFN-ω3

pET32a (Invitrogen, CA, USA) was used as the expression vector. Express primers BoIFN-ω3S and BoIFN-ω3A (Table 1) containing the *Bam*H I and *Xho* I sites were designed. Mature BoIFN-ω3 gene was cloned into the pET-32a vector to generate pET32a-BoIFN-ω3, which contains an N-terminal His tag for facilitating protein purification. Recombinant plasmid was transformed into *Escherichia coli* Rosetta (DE3) Lys cells and then recombinant protein His-BoIFN-ω3 was induced with IPTG (Sigma-Aldrich, St. Louis, MO), the recombinant His-BoIFN-ω3 was analyzed through 12% reduced SDS-PAGE. The whole cells were collected and treated with ultrasonic, supernatants and sedimentations were isolated by centrifuge and analyzed by 12% reduced SDS-PAGE, which were used to identify the solubility of His-BoIFN-ω3. Then the recombinant protein His-BoIFN-ω3 was purified using a nickel-chelated column (GenScript, Nanjing, China) according to the manufacturer's instructions. The purified protein was renatured through dialysis with TGE (50 mM Tris–HCl, 0.5 mM EDTA, 50 mM NaCl, 5% glycerinum, pH 8.0) under a urea gradient to reduce the concentration from 6 M to 0 M. After denaturation and renaturation, soluble homogeneous protein was obtained and concentration was quantified with the Bradford Protein Assay kit (Beyotime, Shanghai China) according to the instructions.

2.5. Antiviral activity and antibody blocking assay in vitro

First, titers of viruses described above were determined by an endpoint dilution assay and the titers were expressed as the tissue culture infectious dose 50 ($TCID_{50}$) per milliliter using the Reed-Muench method (Guo et al., 2015). Virus titers were calculated by determining the dilution giving 50% of wells containing cells that displayed cytopathic effect. Antiviral activity was determined with a standard cytopathic effect assay (Rubinstein et al., 1981) with some modifications (Boue et al., 2000; Bracklein et al., 2006; Rodriguez et al., 1998b; Shao et al., 2015a). Briefly, the monolayers cells were seeded in 96 well plates, then inoculated with four-fold serial diluted BoIFNs. After overnight cultivation, 100 $TCID_{50}$ viruses was added to each well and the plate was then re-incubated under the conditions of 37 °C in a humidified 5% CO_2 atmosphere for 18–24 h. Eight wells without the viruses were

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