Molecular Immunology 49 (2011) 84–96

Contents lists available at ScienceDirect

Molecular Immunology

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

Three novel *Anas platyrhynchos* avian β-defensins, upregulated by duck hepatitis virus, with antibacterial and antiviral activities

Deying Ma[a,**,1], Lijuan Lin[a,b,1], Kexin Zhang[a,b], Zongxi Han[b], Yuhao Shao[b], Xiaoli Liu[b], Shengwang Liu[b,*]

[a] *Department of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, PR China*
[b] *Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, PR China*

ARTICLE INFO

Article history:
Received 16 May 2011
Received in revised form 3 July 2011
Accepted 27 July 2011

Keywords:
Avian β-defensins
Duck
Antibacterial activity
Antiviral activity
Induction

ABSTRACT

Three novel *Anas platyrhynchos* avian β-defensins (Apl_AvBDs), Apl_AvBD4, 7 and 12, were identified successfully and characterized in tissues from Peking ducks in the present study. The cDNA fragment of Apl_AvBD4 contained 171 bp, and encoded 56 amino acids. The complete nucleotide sequences of Apl_AvBD7 and 12 contained 204 bp and 198 bp open reading frames, which encoded 67 and 65 amino acids, respectively. Both recombinant and synthetic forms of the three Apl_AvBDs showed antibacterial activity against most of the bacteria investigated, including Gram-negative and Gram-positive bacteria, except for *Salmonella choleraesuis*. In addition, the antibacterial activity of all the three Apl_AvBDs decreased significantly in 150 mM NaCl. Significant antiviral activity of the three Apl_AvBDs was shown against duck hepatitis virus (DHV). However, none of the Apl_AvBDs showed significant hemolytic activity. Additionally, the expressions of the three Apl_AvBDs in response to DHV infection was highly variable, and significant upregulation of Apl_AvBD7 in liver was found in response to infection at different time points. Expression of Apl_AvBD4 in thymus, and of Apl_AvBD7 in bone marrow was induced in a time-dependent fashion by DHV infection. In contrast, expression of Apl_AvBD12 was found to be significantly decreased, and was hard to detect in cecal tonsil, spleen, bursa of Fabricius, and thymus of ducks at some time points after DHV infection. The present results demonstrate that Apl_AvBDs play vital roles in the immune response of ducks against bacterial and viral pathogens.

1. Introduction

Duck hepatitis virus (DHV) is an acute and fatal disease of young ducklings that is characterized by its rapid transmission (Woolcock, 2003). The major pathologic change in infected ducklings is hepatitis, which is the most common health problem of domestic ducks. Vaccination frequently does not provide a sufficiently rapid immune response to prevent infection of ducks (Lu et al., 1993; Sung and Kim, 2000). As a consequence, other methods to suppress infection with the virus are needed. One of the alternatives may be to stimulate the innate immune system of the ducks by dietary modulation.

In recent years, defensins have been recognized as key mediators of the innate immune response in many vertebrate species, and they provide the first line of defense against potential pathogens (Zasloff, 2002; Sugiarto and Yu, 2004). Defensins are a family of cationic peptides, which are produced by neutrophils, macrophages or epithelial cells and are divided into α-, β- and θ-defensins according to their structural properties (Ganz, 2003). Human β-defensins show direct inhibitory activities against viruses such as human immunodeficiency virus (HIV) and influenza virus (Quiñones-Mateu et al., 2003; Leikina et al., 2005), as well as against Gram-positive and Gram-negative bacteria (Krishnakumari et al., 2006). In addition, β-defensins have immunostimulatory functions, including chemotaxis and activation of antigen presenting cells (Ganz, 2003). Thus, besides being effector molecules of innate immunity, defensins also provide a link between innate and adaptive immunity.

In poultry, only the β-defensins have been reported to exist (Lynn et al., 2007). Avian β-defensins (AvBDs) are studied actively in birds, and so far over 30 AvBDs have been identified in several avian species. All of these AvBDs have been shown to display a wide range of bactericidal activities against Gram-positive and Gram-negative bacteria, and against fungi (Evans et al., 1994, 1995; Harwig et al., 1994; Thouzeau et al., 2003; Lynn et al., 2004; Higgs et al., 2005; van Dijk et al., 2007, 2008; Ma et al., 2008; Soman

* Corresponding author. Tel.: +86 451 85935065; fax: +86 451 82734181.
** Corresponding author. Tel.: +86 451 55190862; fax: +86 451 85935065.
E-mail addresses: mdy296@yahoo.cn (D. Ma), swliu@hvri.ac.cn (S. Liu).
[1] Deying Ma and Lijuan Lin contributed equally to the study.

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doi:10.1016/j.molimm.2011.07.019

Table 1
PCR primer sequences and predicted product lengths.

Target mRNA	Sense primer	Antisense primer	Product size (bp)	Accession no.
Apl_AvBD4 (RT-PCR)	5′-ATCGTGCTCCTCTTTGTGGCAGTTCA-3′	5′-CTACAACCATCTACAGCAAGAATACT-3′	171	–
Apl_AvBD7 (RT-PCR)	5′-GGAGACAGAAGGCAGCGGTGAT-3′	5′-TGCCAGAGAAGCCATTTGGTAGA-3′	328	JF831960
Apl_AvBD12 (RT-PCR)	5′-ATGAGGAACCTTTGTTTCGTGT-3′	5′-TCAGGTCTTGGTGGGAGTTG-3′	198	–
Apl_AvBD4 (protein expression)	5′-GAATTCTGATCGTGCTCCTCT-3′	5′-GTCGACCTACAACCATCTACAG-3′	186	–
Apl_AvBD7 (protein expression)	5′-GAATTCATGCTGCTGCTGTCTG-3′	5′-GTCGACTCAGCTCCTCCATCC-3′	204	JF831960
Apl_AvBD12 (protein expression)	5′-GAATTCATGTTCGTGTTCATCTT-3′	5′-GTCGACTCAGGTCTTGGTGG-3′	198	–
Apl_AvBD4 (real-time PCR)	5′-ATCGTGCTCCTCTTTGTGGCAGTTCA-3′	5′-CTACAACCATCTACAGCAAGAATACT-3′	153	–
Apl_AvBD7 (real-time PCR)	5′-ACCTGCTGCTGTCTGTCCTC-3′	5′-TGCACAGCAAGAGCCTATTC-3′	173	JF831960
Apl_AvBD12 (real-time PCR)	5′-GGAACCTTTGTTTCGTGTTCA-3′	5′-GAGAATGACGGGTTCAAAGC-3′	155	–
Duck β-actin (real-time PCR)	5′-CCGTAAGGACCTGTACGCCAACAC-3′	5′-GCTGATCCACATCTGCTGGAAGG-3′	208	AY251275

et al., 2009; Ma et al., 2009a, 2009b; Wang et al., 2010). Additionally, most of the AvBDs can be either constitutively expressed or induced in response to microbial infection, and regulation is often dependent on the site of synthesis (van Dijk et al., 2008). It has been shown that AvBD3 is constitutively expressed in the chicken skin and tongue but is inducible in the trachea (Zhao et al., 2001); Gal4, 7, and 9 were constitutively expressed in the chicken small intestine and Gal7 was induced in chicken liver following infection with *Salmonella enteriditis* (SE) (Milona et al., 2007). Furthermore, treatment with lipopolysaccharide (LPS) or SE infection induced the expression of certain AvBDs in the reproductive tract of the chicken (Ohashi et al., 2005; Ebers et al., 2009). These findings suggest that an AvBD-mediated immune system exists in the chicken. It probably plays a role in the recognition of LPS and of pathogens such as *Salmonella* species, and in the initiation of the immune response to protect the specified tissues to allow successful development.

Three *Anas platyrhynchos* avian β-defensins (Apl_AvBDs), named Apl_AvBD2, 9, and 10, have been isolated from ducks in previous studies (Soman et al., 2009; Ma et al., 2009a, 2009b). All of these three Apl_AvBDs exhibited a broad spectrum of potent antibacterial activity against Gram-positive and Gram-negative bacteria. Both Apl_AvBD2 and Apl_AvBD9 have been detected in a range of tissues, including the liver of ducks. In contrast, Apl_AvBD10 was only highly expressed in the liver and kidney of ducks at different ages (Soman et al., 2009, 2010; Ma et al., 2009a, 2009b). In addition to the three Apl_AvBDs identified, it is possible that other AvBDs exist in ducks, because 14 AvBDs have been found in the chicken. Furthermore, while the antibacterial activities and tissue distribution of the three Apl_AvBDs have been studied in detail, their expression in response to bacteria or viruses and their antiviral activity remain unknown.

Given the importance of AvBDs in the innate immunity of birds, Apl_AvBDs may offer novel routes for the prevention and treatment of diseases, including DHV, by acting as antimicrobials, either after topical application or by upregulation of defensin production in the host cell, and by acting as mediators to stimulate the innate immune response. This study aimed, therefore, to identify the presence of other novel Apl_AvBDs in ducks (*A. platyrhynchos*). In addition, the antibacterial activity, and the antiviral activity against DHV, of these novel Apl_AvBDs were studied in vitro. Finally, the effect of DHV infection on expression of the mRNAs encoding the AvBDs in duck tissues has been studied.

2. Materials and methods

2.1. Animals

Forty-five 11-day-old specific pathogen free (SPF) Peking ducklings were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, China. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

2.2. RNA extraction, reverse transcriptase polymerase chain reaction amplification, and sequencing

Approximately 1 g each of bone marrow, Harderian glands, and lung tissue obtained from five healthy 11-day-old ducklings was used to process tissue fluid, and the total cellular RNAs were extracted from 100 μL aliquots of the respective tissue fluid using TRIzol reagent (Invitrogen, Beijing, PR China) according to the manufacturer's instructions. Reverse transcriptions (RT) were performed using oligo-dT primers in a 40 μL reaction mixture containing 20 μL RNA. The specific cDNAs obtained were amplified by PCR using Ex-Taq polymerase (TAKARA Bio Inc., Otsu, Shiga, Japan) with three sets of primers designed internally on the basis of the coding sequences of chicken AvBD4, 7, 12 (previously known as gallinacin-7, -5, -10) (Lynn et al., 2004) (Table 1), respectively. The PCR protocol was as follows: an initial denaturation for 5 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and polymerization at 72 °C for 1 min. The final polymerization step was performed at 72 °C for 10 min. The PCR products were cloned into the pMD18-T vector (TAKARA) to confirm amplification, followed by sequencing of the recombinant plasmids.

2.3. Sequence analysis of Apl_AvBD cDNAs

Basic searches were conducted with a local alignment search tool (BLAST) using the three entire AvBD cDNAs from the ducks. Sequences of the other known AvBDs, and some mammalian β-defensins, including β-defensins from humans (*Homo sapiens*), cattle (*Bos taurus*), pigs (*Sus scrofa*), Norway rats (*Rattus norvegicus*), and house mice (*Mus musculus*), were selected for sequence comparison with the three novel Apl_AvBDs. Multiple alignment and phylogenetic analyses were performed using the Clustal V routine of the MegAlign program provided in the DNAStar package (Windows 4.05, DNAStar, Madison, WI, USA) (Higgins and Sharp, 1988). The signal peptides of the three novel Apl_AvBDs were analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/Services/signalP).

2.4. Protein expression and purification

The cDNA fragments that encode the Apl_AvBDs were amplified by PCR from the plasmids described above using the primers for protein expression shown in Table 1. The PCR products, which contained either coding sequence of Apl_AvBD4, Apl_AvBD7, or Apl_AvBD12 flanked by *EcoR* I and *Sal* I, were inserted into the pGEX-6p-1 vector (Amersham) at the respective sites. The resultant plasmids were designated as recombinant Apl_AvBD4-pGEX,