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Molecular identification and comparative transcriptional analysis of myxovirus resistance GTPase (Mx) gene in goose (*Anser cygnoide*) after H9N2 AIV infection

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ABSTRACT

Interferon (IFN)-induced myxovirus resistance (Mx) GTPases belong to the family of dynamin-like GTPases and control a diverse range of viruses. In this study, the identified goose Mx (goMx) mRNA is 2009 bp long, shares partially conserved exons with other homologues, and shares highly conserved domains in its primary structure. The amino acid position 629 (629 aa) of the goMx protein was identified as serine (Ser), in contrast to the Ser located at 631 aa in chicken Mx, which is considered to be responsible for the lack of chicken Mx antiviral activity. In addition, the goMx 142 aa residue in the dynamin family signature differs from that of other functional Mx proteins. Transcriptional analysis revealed that goMx was mainly expressed in the digestive, respiratory and immune systems in an age-specific manner. GoMx transcript levels in goose peripheral blood mononuclear cells (PBMCs) were found to be significantly up-regulated by various agonists and avian viruses. Furthermore, a time course study of the effects of H9N2 avian influenza virus (AIV) on goMx expression in infected goslings suggested that H9N2 AIV affected goMx expression. However, significant changes in goMx expression were observed in the trachea, lung and small intestine of infected birds. Altogether, these results indicate that goMx protein may have acquired its broad antiviral activity by changing only a few amino acids at select sites, even as it shares a conserved architectures with species.

1. Introduction

The evolutionary difference in immune genes between the palmipeds and other vertebrates justifies deeper investigation into key antiviral mediators of the bird immune system [1,2]. As intracellular antiviral proteins with direct and classical effects, the interferon-induced myxovirus resistance GTPases (Mx) are critical host restriction factors. Mx proteins can inhibit multiple viruses, such as influenza and human immunodeficiency viruses, and have been identified in many mammals and other vertebrates, including amphibian and avian species [3]. Mx proteins have molecular weights of approximately 70 kDa–80 kDa, and contain three domains in their primary structure: a GTPase domain (G-Domain), which is found in the conserved N-terminus and contains the tripartite GTP-binding motif; a middle linker domain element (MD) abundant in α-helices; and a GTPase effector domain (GED) characterized by conserved leucine zipper (LZ) and divergent L4 motifs [3,4]. The MD is important for oligomerization and virus target recognition, whereas the folded LZ motif, combined with the G-Domain, forms the enzymatically active centre of Mx proteins [5–7].

When cultured cells or organisms are treated with Interferon (IFN) or other inducers, Mx expression increases significantly above its low constitutive expression [8–10]. When the stimulus is herpes simplex virus-1, human MxA mRNA was demonstrated to undergo alternative splicing, resulting in the expression of a novel isoform that supports viral infection, instead of restricting viral infection

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[11]. This contrasts with the well-known status of human MxA protein as the key intracellular restriction molecule against influenza virus, especially avian influenza virus (AIV). AIV often adapts to a non-fatal form in waterfowl, which plays a critical role in viral transmission and dissemination [2,12–14]. However, the function and contribution of Mx as an antiviral effector protein in chickens is complicated because of polymorphisms at amino acid position 631 (631 aa) [15,16]. Compared with terrestrial birds, aquatic birds have advantageous defences against some viruses, which has led to theories involving the effect of water ecology temperatures on the virus targeted receptor [17,18]. However, the role of interferon-stimulated genes (ISGS) in the IFN system of the goose is unclear, and the antiviral function of Mx in birds is still controversial.

Commercially selected goose lines under artificial selection pressure are subjected to attack by a range of viruses, including gosling plague virus (GPV), gosling new type viral enteritis virus (NGVEV), Tembusu virus (TMUV) and AIV [12,19–21]. Because this species is usually a resistant, asymptomatic carrier of AIV, H9N2 AIVs were classified as a low pathogenic subtype in geese. However, H9N2 AIVs currently threaten wild birds and domestic poultry, and have been documented as a pathogen transmissible to mammals [22]. In addition, influenza A virus (IAV) was reported to use an immune escape strategy where non-structural protein 1 (NS1) inhibits host IFN expression [23,24]. Because human MxA possesses viral nucleoprotein-dependent sensitivity towards avian IAV strains [25,26], avian Mx may similarly resist human influenza strains.

In this report, the cDNA of Chinese goose Mx (goMx) is identified and characterized for the first time. Furthermore, quantitative real-time PCR (qPCR) was used to analyse tissue expression profiles and goMx expression in goose peripheral blood mononuclear cells (PBMCs) treated with agonists and goose-adapted viruses. The expression of goMx was also examined in the tissues of goslings infected with H9N2 subtype influenza virus. Our data will help illuminate the antiviral immunological characteristics of goMx protein while underlining the importance of further research into the molecular regulation of goMx.

2. Materials and methods

2.1. Viruses, agonists and animals

DTMUV (6.3×10^6 $TCID_{50}$/mL) and GPV ($10^{6.6}$ EID_{50}/0.2 ml) were provided by the Avian Diseases Research Center of Sichuan Agricultural University. The AIV strain ($7.14 \times 10^{-12.6}$ copies/mL, A/chicken/JS/C1/2008 (AIV H9N2 strain)) was kindly provided by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The agonists Poly (I:C) (Sigma, USA), R848 (InvivoGen, USA), ODN2006 (Sangon, Shanghai), and LPS (InvivoGen, USA) were used to mimic treatment with double-stranded RNA, single-stranded RNA, synthetic oligonucleotides and lipopolysaccharides, respectively.

Healthy geese and goslings were purchased from a commercial farm in Chengdu, China. Two-week-old goslings and adult geese were maintained and observed for a week in laboratory animal rooms prior to experiments. Animal welfare was ensured during the sampling process. Five healthy goslings and five adult geese were prepared for the tissue expression profile study, whereas a total of 15 goslings were used for the study of H9N2 stimulated individuals.

Biological samples consisting of different tissues and organs were collected from 2-week-old and adult geese. The tissue samples included brain (B), blood (BL), liver (Li), heart (H), lung (LU), thymus (T), spleen (SP), calaec tonsil (CT), Harderian gland (HG), pancreas (P), kidney (K), bursa of Fabricius (BF), caecum (CE), skeletal muscle (M), small intestine (SI), gizzard (GI), proventriculus (PR), skin (SK), and trachea (TR). The samples were snap-frozen in liquid nitrogen, minced, and then stored in 1 ml of TRIzol reagent (Invitrogen, USA) at −80 °C until use.

2.2. Generation of cDNA

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNA concentrations were determined by measuring the ratio of absorbance at 260 and 280 nm using a Thermo Scientific NanoDrop 2000. The cDNA was immediately synthesized using the 5× All-in-One RT MasterMix (ABM, USA) for qPCR or HiScript 1st Strand cDNA Synthesis Kit (Vazyme, USA) according to the manufacturer's instructions and then stored at −80 °C.

2.3. Full cDNA cloning of goMx

Total RNA from goose caecum was used as the template for cDNA synthesis. Based on Mx regions conserved across species, three partial sequences of goMx were amplified with the degenerate primers F1, R1, F2, R2, F3, and R3 (listed in Table 1). Subsequently, the full-length cDNA of the Mx gene was obtained by 5′ rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). Nested PCR was utilized to accurately amplify the target gene. The 5′ RACE primers GSP1 and AAP were used for the first round of nested PCR, and the 5′ RACE primers GSP2 and AUAP, for the second round. RACE amplicons were cloned into the pMD19-T vector (Takara Co., Ltd., Japan), with the bacteria clones screened by PCR and sequenced with the ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA). The 5′ RACE PCR protocol was as follows: an initial 5 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, annealing at 53 °C + 0.4 °C/cycle, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The resulting product was diluted 100-fold and amplified again with the same protocol, except that the annealing temperature was set to 55 °C + 0.3 °C/cycle.

2.4. Bioinformatics analysis

Potential open reading frames (ORFs) were analysed with the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and translated into the corresponding amino acid sequences with DNAMAN software. The amino acid sequence, protein molecular weight (MW), isoelectric point (pI), and hydropathy profile of goMx were predicted using ExPASy (http://us.expasy.org/tools/protparam.html). The secondary and three-dimensional protein structure were predicted with the I-TASSER software package (http://zhanglab.ccmb.med.umich.edu/). To investigate the mRNA instability motif, six mRNA splice variants with untranslated regions (UTR) and exons were predicted with Splign software (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). Multiple alignments of Mx sequences were performed with ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2).

To identify a reference template for modelling the goMx structure, the UniProt database (http://www.uniprot.org/blast/) was searched using BLAST, which identified a single recently resolved crystal structure of human MxA protein. Pairwise sequence alignment was then used for homology modelling of goMx. Nuclear localization signal (NLS) was predicted for the goMx amino acid sequence by NLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). The constructed phylogenetic trees on the method of Neighbor-Joining algorithm and a Jukes–Cantor distance model based on the desk of MEGA 6, with the Aligned sequences bootstrapped 1000 for times, only the bootstrap values higher than 50 were taken into consideration for the consensus tree.