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Molecular cloning and functional characterization of a novel isoform of chicken myeloid differentiation factor 88 (MyD88)

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Summary
Myeloid differentiation factor 88 (MyD88) is an adaptor protein involved in the interleukin-1 receptor- and Toll-like receptor-induced activation of nuclear factor-κB (NF-κB). A novel isoform of chicken MyD88, designated chicken MyD88-2, has been cloned and functionally characterized. Its open reading frame is of length 900 bp, and it encodes a predicted 299 residue protein, similar in length to its mammalian orthologues, but, respectively, 77 and 69 amino acids shorter than the previously described chicken MyD88-1 and -3. The amino acid sequence of chicken MyD88-2 displays 96.9%, 96.9%, 70.4% and 70.2% identity with, respectively, chicken MyD88-1, -3, human and mouse MyD88. Chicken MyD88-2 expression was detected in a range of tissues tested, but no expression of either chicken MyD88-1 or -3 was observed. The over-expression of chicken MyD88-2 significantly induced the activation of NF-κB *in vitro*, suggesting that chicken MyD88-2 plays an important role in the innate immune responses of chicken.

Introduction

Microorganisms which invade a vertebrate host are initially recognized by the innate immune system, via germline-encoded pattern-recognition receptors (PRRs). One class of PRRs is represented by the mammalian toll-like receptors (TLRs) family, which is engaged by microbial components and triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense [1]. The myeloid differentiation factor 88 (MyD88) is a TLR adaptor protein that is involved in the interleukin-1 receptor (IL-1R)- and TLR-induced activation of nuclear factor-κB (NF-κB) signal pathway and plays an essential role in TLR-mediated antimicrobial host defense [2].

MyD88 was first named in 1990 as a protein that was induced during the terminal differentiation of M1D$^+$ myeloid precursors in response to IL-6. The 'MyD' part of the name stands for myeloid differentiation and '88' refers to the gene number in the list of induced genes [3]. The length of

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the open reading frame (ORF) of human and mouse MyD88 is 891 bp, and the sequence encodes a 296-residue protein composed of a C-terminal Toll/IL-1R homology (TIR) domain, an N-terminal death domain and a small intermediate domain (ID) [4–6].

MyD88 is a key adaptor for almost all TLRs [7]. Upon stimulation, MyD88 recruits IL-1R-associated kinase 4 (IRAK-4) to TLRs via an interaction between the death domains of both molecules, and facilitates the IRAK-4-mediated phosphorylation of IRAK-1. The activated IRAK-1 then associates with tumour-necrosis-factor-receptor-associated factor 6, leading to the activation of the complex of transforming-growth-factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 2, which enhances activity of the NF-κB kinase (IKK) complex. Once activated, the IKK complex induces phosphorylation and subsequent degradation of IκB, which leads to activation of NF-κB [2]. In addition to the activation of NF-κB, MyD88 is also required for the activation of interferon regulatory factors-1, -5 and -7, which play important roles in the induction of pro-inflammatory cytokines and type I interferons during the defense response of the host [2].

The over-expression of MyD88 has been shown to induce downstream cellular signaling in the absence of TLRs or TLR ligands [8], and so has been thought to have potential as a genetic adjuvant for DNA vaccine to enhance DNA-raised immune responses [9]. To explore the possibility of chicken MyD88 as a genetic adjuvant for chicken DNA vaccines, we set out to clone chicken MyD88 cDNA, exploiting its sequence which has been reported elsewhere [10]. The analysis of this sequence, however, revealed that what had been cloned was a novel isoform of chicken MyD88. In the present report, we give a molecular characterization of this novel isoform of chicken MyD88.

Materials and methods

Experimental tissues, cells and antibodies

Experimental tissues were collected from 14-day-old specific-pathogen-free (SPF) White Leghorn chickens, following procedures set down by the Guidelines for Animal Experimentation of the Shanghai Veterinary Research Institute. Samples were taken from five chickens immediately after slaughter, snap-frozen in liquid nitrogen and stored at $-70\,^{\circ}C$. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old SPF chicken embryos and cultured using standard techniques. 293T cells were cultured in Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 mg/ml of each) and grown under 5% CO_2 at $37\,^{\circ}C$. The commercial antibodies employed were an anti-FLAG monoclonal antibody (M2, Sigma, St Louis, MO, USA) and an anti-β-actin monoclonal antibody (AC-15, Sigma).

Cloning of chicken MyD88 cDNA

Total RNA was extracted from the bursa of Fabricius using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using reverse transcriptase [either AMV Reverse Transcriptase (TaKaRa, Otsu, Japan), SuperScript™ II Reverse Transcriptase (Invitrogen) or Sensiscript Reverse Transcriptase (QIAGEN, Venlo, The Netherlands)] and oligo (dT) primer. The full-length MyD88 ORF was amplified by primer pair MyD88f1/MyD88r1 (Table 1), which correspond, respectively, to positions 1–20 and 1109–1131 of GenBank accession NM_001030962 (Figure 2). Either *Tfi* DNA Polymerase (Invitrogen), *Ex Taq*™ DNA Polymerase (TaKaRa) or Easy-A® High-Fidelity PCR Cloning Enzyme (Stratagene, Cedar Creek, TX, USA) were used as a DNA polymerase. The PCR conditions consisted of a denaturation step of $95\,^{\circ}C/120$ s, followed by 30 cycles of $94\,^{\circ}C/30$ s, $57\,^{\circ}C/60$ s and $72\,^{\circ}C/120$ s, and completed with a 10 min extension at $72\,^{\circ}C$. The PCR products were cloned into the pMD18-T vector (TaKaRa) for sequence analysis.

Reverse transcription-PCR/restriction fragment length polymorphism (RT-PCR/RFLP)

A reverse transcription reaction based on 500 ng of total RNA extracted from either spleen, liver, kidney, bursa of Fabricius, muscle or lung was PCR amplified as described above. Subsequently, 1 μl of the first PCR was used as the template for a second PCR, the products of which were separated by agarose electrophoresis, purified from the gel and subjected to digestion by *Bst*X I. The digested PCR products were separated by 1% agarose gel electrophoresis, and the signal visualized by ethidium bromide staining. Positive controls for chicken MyD88-1, -2 and -3 were amplified from, respectively, FLAG-MyD88-1, -2 and -3 template.

PCR analysis of genomic DNA

Total genomic DNA was isolated from the bursa of Fabricius using the QIAGEN DNaeasy Tissue kit, according to the manufacturer's instructions. A partial nucleotide sequence of exon 5 of chicken MyD88 gene was amplified using the

Table 1 Primer sequences.

Primer	Sequence
MyD88f1	5′-ATATAATGGCTACGGTACCCGTGGG-3′
MyD88r1	5′-GCTCACCAAGTGCTGGATGCTATTG-3′
MyD88f2	5′-CCCTGACTGCTACTTGAGTGACATG-3′
MyD88r2	5′-GCTCACCAAGTGCTGGATGCTATTG-3′
MyD88f3	5′-CCAATCCTTGCACCAAAAAAATGGTTCTGG-ACAAGACTGGC-3′
MyD88r3	5′-GCCAGTCTTGTCCAGAACCATTTTTTTGG-TGCAAGGATTGG-3′
MyD88f4	5′-CACTGCACAACTTGGATCCTTGCAGTCA-GCATTTGGAGCCC-3′
MyD88r4	5′-GGGCTCCAAATGCTGACTGCAAGGA-TCCAAGTTGTGCAGTG-3′
MyD88f5	5′-GGTGTTCCTACTTAATTCAGTGAGCAATA-GCATCCAGCACT-3′
MyD88r5	5′-AGTGCTGGATGCTATTGCTCACTGAATTAA-GTAGGAACACC-3′