Chicken MDA5 Senses Short Double-Stranded RNA with Implications for Antiviral Response against Avian Influenza Viruses in Chicken

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Key Words

Avian influenza virus · Chicken · Melanoma differentiation-associated gene-5 · Retinoic acid-inducible gene-I · Short double-stranded RNA · Type I interferon

Abstract

Mammalian melanoma differentiation-associated gene-5 (MDA5) and retinoic acid-inducible gene-I (RIG-I) selectively sense double-stranded RNA (dsRNA) according to length, as well as various RNA viruses to induce an antiviral response. RIG-I, which plays a predominant role in the induction of antiviral responses against influenza virus infection, has been considered to be lacking in chicken, putting the function of chicken MDA5 (chMDA5) under the spotlight. Here, we show that chMDA5, unlike mammalian MDA5, preferentially senses shorter dsRNA synthetic analogues, poly(I:C), in chicken DF-1 fibroblasts. A requirement for caspase activation and recruitment domains for chMDA5-mediated chicken interferon beta (chIFN β) induction and its interaction with mitochondrial antiviral signaling proteins were demonstrated. We also found that chMDA5 is involved in chIFN β induction against avian influenza virus infection. Our findings imply

that chMDA5 compensates in part the function of RIG-I in chicken, and highlights the importance of chMDA5 in the innate immune response in chicken.

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Introduction

The innate immune system acts as an initial host defense to detect and eliminate invading pathogens. To sense an invader properly, the system utilizes pattern recognition receptors (PRRs) that recognize specific pathogen-associated molecular pattern motifs that are absent in the host [1]. Retinoic acid-inducible gene-I-like receptors (RLRs), including retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5), have been characterized as PRRs for detection of nonself RNAs in the host cytoplasm [2]. Both receptors consist of three major regions: two N-terminal caspase activation and recruitment domains (CARD), a DExD/H-box RNA helicase domain, and a C-terminal regulatory domain (CTD) [3]. The CTD is required for viral RNA binding [4–7], whereas the

CARD are required for interaction with the CARD of mitochondrial antiviral signaling proteins (MAVS; also called IPS-1, VISA and Cardif), which are located in the mitochondrial membrane [8]. CARD-CARD interactions lead to activation of interferon (IFN) regulatory factor 3 and nuclear factor κ -light chain-enhancer of activated B cells (NF κ B), thereby inducing type I IFNs together with IFN-stimulated genes and proinflammatory cytokines to establish a systematic antiviral state, which restricts viral replication and spreads through the host cells [2, 8].

Although both RIG-I and MDA5 exploit a common downstream signaling pathway through MAVS in mammals, they recognize different RNA moieties. RIG-I predominantly recognizes short double-stranded RNA (dsRNA; <1 kbp) and uncapped 5'-triphosphate (5'-ppp) single-stranded RNA [9–11], whereas MDA5 senses long dsRNA (>2 kbp) and higher-order structured RNA [9, 12]. Hence, this difference in ligand specificity has been thought to contribute to differential detection of invading pathogens [9, 13, 14]. In RIG-I/MDA5-deficient mice embryonic fibroblasts, RIG-I was found to detect various kinds of RNA viruses, including orthomyxovirus (influenza A and B virus), paramyxovirus (Sendai virus, Newcastle disease virus and measles virus), flavivirus (hepatitis C virus and Japanese encephalitis virus), and filovirus (Ebola virus), whereas MDA5 could detect picornavirus (encephalomyocarditis virus) [13, 14]. Dengue virus and West Nile virus, which are members of the genus *Flavivirus*, can be detected by both receptors [13, 14].

Considerable attention has recently been given to studies on MDA5 against avian influenza virus (AIV) infection in chicken, which appears to lack RIG-I [15, 16]. Barber et al. [17] suggested that the absence of RIG-I in chicken may lead to insufficient antiviral responses against AIV infection, resulting in increased susceptibility of chickens to infection compared with duck, which possesses RIG-I. It was later shown, however, that knockdown of chicken MDA5 (chMDA5) by RNA interference reduces expression levels of chicken IFN β (chIFN β) mRNA in DF-1 cells and HD-11 macrophage-like cells primed with AIV, suggesting that unlike mouse MDA5, chMDA5 senses AIV [15, 16]. Karpala et al. [15] also reported that silencing of chMDA5 reduces chIFN β expression in DF-1 cells stimulated with poly(I:C) of relatively short (1 kbp) length, as well as medium (3 kbp) and long (6 kbp) lengths. These previous reports suggest that chMDA5 differs in its function from mouse MDA5, in part to compensate for RIG-I function.

In this study, to assess the function of chMDA5 in detail, we determined the preferential length of dsRNA using poly(I:C) of different lengths by measuring induction of chIFN β mRNA in chicken cells transiently overexpressing chMDA5. We also assessed chMDA5-mediated antiviral responses induced by AIV infection, and viral replication levels in DF-1 cells with transient knockdown or overexpression of chMDA5.

Materials and Methods

Viruses and Reagents

A highly pathogenic AIV (HPAIV) of H5N1 subtype, A/chicken/Yamaguchi/7/2004 (HP/Yamaguchi) [18] and a low pathogenic AIV (LPAIV) of H9N2 subtype, A/chicken/Yokohama/aq55/01 (LP/Yokohama) [19] were used in this study. Viruses were propagated in the allantoic cavity of 10- or 11-day-old embryonated eggs at 37°C for 2 days, and harvested viruses, in allantoic fluid, were stocked at –80°C before use. The 50% egg infectious dose titers (EID₅₀/ml) were determined by serial titration of virus stocks in 10- or 11-day-old embryonated eggs, and calculated by the method of Reed and Muench [20]. The experiments using LPAIV and HPAIV were done in biosafety level 2 and 3 facilities, respectively, at the National Institute of Animal Health, Japan. Synthetic dsRNA analogue poly(I:C) of short (0.2–1 kbp) and long (1.5–8 kbp) lengths were purchased (Invitrogen, San Diego, Calif., USA) and stocked according to the manufacturer's instructions.

Cell Cultures and Animals

DF-1, a chicken fibroblast-derived cell line, was obtained from the American Type Culture Collection (CRL-12203; Manassas, Va., USA) [21]. LMH, a liver cancer cell line induced by diethylnitrosamine [22], was purchased from the Japanese Collection of Research Bioresource (JCRB0237; Osaka, Japan). HD11, a continuous chicken myelomonocytic cell line [23], was kindly provided by Dr. John Adams of the Cedars-Sinai Medical Center (Los Angeles, Calif., USA). These cells were maintained in Waymouth's medium (LMH) or Dulbecco's Modified Eagle's Medium (DF-1 and HD11 cells), supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). Madin-Darby canine kidney (MDCK) cells were used to measure 50% tissue culture infectious dose (TCID₅₀) and were cultured in Modified Eagle's Medium, supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. The B cell line, LSCC-RP9, transformed by avian leukosis virus [24], and the T cell line, MDCC-MSB1, transformed by Marek's disease virus [25], both obtained from the repository of the National Institute of Animal Health, Japan, were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, 55 μ M 2-ME and 2.5 μ g/ml Fungizone (Invitrogen). DF-1 cells were maintained at 39°C and 5% CO₂, and the other cell lines were maintained at 37°C and 5% CO₂. Four-week-old male specific pathogen-free White Leghorn chickens (Nisseiken, Yamanashi, Japan) were used for expression analysis. Cherrybelly duck (Hamada, Saitama, Japan) was used for cloning of duck (d)RIG-I. Peripheral blood was collected from the wing vein and leuko-

cytes were enriched by centrifugation using Lymphoprep (Axis-Shield, Dundee, UK). Animal experiments were conducted under protocols approved by the Animal Experiment Committee of the National Institute of Animal Health, Japan.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using an RNeasy Mini Kit or RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. After treatment with RQ1 RNase-free DNase (Promega, Madison, Wisc., USA) or RNase-Free DNase Set (Qiagen), the extracted RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions.

Rapid Amplification of cDNA Ends

The sequence of chMDA5 was determined using the 5'- and 3'-rapid amplification of cDNA ends (RACE) system (Gene Racer Kit, Invitrogen) based on the GenBank accession No. XP_422031 DNA sequence from the National Center for Biotechnology Information chicken genome assembly build 2.1. Total RNA was extracted from HD-11 cells infected with HP/Yamaguchi and used as a template. The determined sequence was deposited into GenBank (accession No. AB371640). chMDA5 comprises a 3,006-bp open reading frame (ORF) encoding 1,001 amino acid residues. For cloning of dRIG-I, total RNA was extracted from white blood cells, intestine and thymus of duck and the RNA mix was then transcribed to cDNA, followed by PCR using degenerate primers. The complete sequence of dRIG-I was determined using the obtained PCR products and the 5'-Full RACE Core Set (Takara Bio, Shiga, Japan) or GeneRacer Kit. dRIG-I comprises a 2,802-bp ORF encoding 933 amino acid residues. Since the base at position 12 (G) of the dRIG-I sequence differs from that of dRIG-I (C at position 12), which was previously identified by Barber et al. [17], the determined sequence was deposited into GenBank (accession No. AB772012). Primer sequences for the determination of chMDA5 and dRIG-I sequences are available upon request.

Construction of Expression Plasmids

The chMDA5 ORF and chMDA5 lacking CARD were amplified by PCR with primer pairs chMDA5-F/chMDA5-R and chMDA5delCARD-F/chMDA5-R, respectively. The dRIG-I ORF and dRIG-I lacking CARD were amplified by PCR with primer pairs dRIG-I-F/dRIG-I-R and dRIG-IdelCARD-F/dRIG-I-R, respectively. All PCR products were cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen). To generate N-terminal Flag fusion proteins, the chMDA5 ORF and chMDA5 lacking CARD fragments were inserted into *Hind*III sites of the p3×FLAG CMV-10 vector (Invitrogen). The dRIG-I ORF and dRIG-I lacking CARD fragments were inserted into *Not*I/*Xba*I sites of the p3×FLAG CMV-10 vector. The HP/Yamaguchi NS1 (YamNS1) ORF (678 bp, accession No. ACZ36647) were amplified by PCR with a primer pair YamNS1-F/YamNS1-R and an amplified product was cloned in *Eco*RI/*Eco*RV sites of the p3×FLAG CMV-10 vector. The chMAVS ORFs were amplified by PCR with primer pairs chMAVS-F/chMAVS-R using total RNA extracted from HD-11 cells infected with HP/Yamaguchi and the obtained PCR products were then cloned using the Zero Blunt TOPO PCR cloning kit. chMAVS comprises a 1,926-bp ORF encoding 641 amino acid residues. Since bases at position 1,247 (A), 1,317 (C)

and 1,318 (A) of the chMAVS sequence differed from that of chMAVS (1,247 G, 1,317 T and 1,318 G), which was previously identified by Liniger et al. [16], the determined sequence was deposited into GenBank (accession No. AB772011). To generate N-terminal GFP fusion proteins, the fragments were inserted into *Eco*RI/*Sall* sites of the pACGFP1-C vector (Clontech, Mountain View, Calif., USA). For the generation of chimeric constructs, the Helicase and CTD domains of dRIG-I fused to CARD of chMDA5 (dRIG-I/chMDA5 CARD) and its complimentary constructs (chMDA5/dRIG-I CARD) were amplified by the overlap PCR method, cloned using the Zero Blunt TOPO PCR cloning kit, and then inserted into the *Xba*I and *Not*I sites, respectively, of the p3×FLAG CMV-10 vector (Invitrogen). dRIG-I/chMDA5CARD-F1, chMDA5/dRIG-ICARD-F1, chMDA5/dRIG-ICARD-R1 and dRIG-I-R were used for construction of dRIG-I/chMDA5 CARD (table 1). dRIG-I-F, dRIG-I/chMDA5CARD-F2, dRIG-I/chMDA5CARD-R1 and chMDA5/dRIG-ICARD-R2 were used for the construction of chMDA5/dRIG-ICARD (table 1). All inserted fragments in the expression vector were verified by sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA).

Transfection of Poly(I:C), siRNA and Expression Plasmids

For stimulation by poly(I:C), DF-1 cells seeded in 12-well plates (5×10^5 cells/well) were transfected with short or long poly(I:C) at a dose of 0.05, 0.5 and 5 μ g/ml for 4 h using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Knockdown of endogenous chMDA5, chMAVS and chicken Toll-like receptor 3 (chTLR3) in DF-1 cells was performed using siRNAs specific for chMDA5 (20 nM, Takara Bio), chMAVS (20 nM, Takara Bio) and chTLR3 (10 nM, Invitrogen) along with the respective nontargeting control siRNA (table 1). The cells in the 12-well plates were transfected with these siRNAs using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions, 24 h before analysis. To check knockdown efficiency, the extracted RNA was subjected to mRNA expression analysis by quantitative real-time PCR (RT-PCR), as described below. The efficiency of silencing was checked by comparing mRNA expression levels of chMDA5, chMAVS or chTLR3 between knockdown cells and control cells. For transient expression of the constructs, DF-1, LMH and HD-11 cells were transfected with Flag-constructs using Lipofectamine 2000 (Invitrogen) 24 h before analysis.

Viral Infection and Titrations

DF-1, LMH and HD-11 cells in 12-well plates (5×10^5 cells/well) were infected with LP/Yokohama or HP/Yamaguchi and incubated at 39°C (DF-1 cells) or 37°C (LMH and HD-11 cells). At 24 and 48 h postinfection, supernatants were collected and titrated in embryonated eggs (DF-1 cells) and MDCK cells (LMH cells). Viral titers were expressed as EID₅₀/ml (DF-1 cells) and TCID₅₀/ml (LMH cells).

Quantitative Real-Time PCR

The synthesized cDNAs were diluted to 1:10 and used as template. Quantitative RT-PCR was performed with equal amounts of cDNA, primers specific for target genes and SYBR Premix Ex Taq (Perfect Real time; Takara Bio) using an Applied Biosystems 7500 apparatus. PCR cycle parameters were as follows: 1 cycle at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60 or 65°C for 34 s. For detection of distribution of chMDA5 mRNA expression,

Table 1. siRNA for silencing as well as primers for plasmid construction and RT-PCR used in this study

Name	Sequence (5' to 3')	Application
chMDA5-F	TAAAGCTTATGTCGGAGGAGTGCCGAG	plasmid construction
chMDA5-R	CAAGCTTAATCTTCATCACTTGAAGGAC	plasmid construction
chMDA5delCARD-F	ACAAGCTTATGAGTGGAAATACAGGAGGAAC	plasmid construction
dRIG-I-F	ATAAGAATGCGGCCGCGATGACGGCGGAGGAGAAGCG	plasmid construction
dRIG-I-R	GCTCTAGACTAAAAATGGTGGGTACAAGTTGG	plasmid construction
dRIG-IdelCARD-F	ATAAGAATGCGGCCGCGATGTGGGATATAAGAGAAGATAATGC	plasmid construction
dRIG-I/chMDA5CARD-F1	GCTCTAGAATGTCGGAGGAGTGCCGAG	plasmid construction
dRIG-I/chMDA5CARD-F2	AAGTGAAGTGAAGTGGAAATACAGGAGGAAC	plasmid construction
dRIG-I/chMDA5CARD-R1	TATTTCCACTCAGTTCACTTGCACGGTAAT	plasmid construction
chMDA5/dRIG-ICARD-F1	AGATGACTTATGGGATATAAGAGAAGATAA	plasmid construction
chMDA5/dRIG-ICARD-R1	TTATATCCCATAAGTCATCTGCAAGGCTTT	plasmid construction
chMDA5/dRIG-ICARD-R2	ATAAGAATGCGGCCGCTTAATCTTCATCACTTGAAGGAC	plasmid construction
chMAVS-F	CTTCGAATTCTGATGGGTTTCGC	plasmid construction
chMAVS-R	CCGTCGACTATTTCTGCAATCGTGTG	plasmid construction
YamNS1-F	ATATCGGAATTCAATGGATCTCAACACTGTGTC	plasmid construction
YamNS1-R	ATATCGGATATCTCAAACCTCTGACTCAATTG	plasmid construction
chMDA5-siRNAcontrol	AAUAGUAACGGUAGAGUAATT	silencing
chMDA5-siRNA	GAACGUGAAGAUGUAAAUAATT	silencing
chMAVS-siRNAcontrol	UACGUAGAGGUUCGGAACGGAGUCA	silencing
chMAVS-siRNA	UACAGGAGGCUUCAAGGAGGUGUCA	silencing
chTLR3-siRNAcontrol	AUUACGGGCCAGUAAUCUATT	silencing
chTLR3-siRNA	GCAGAUUGUAGUCACCUAATT	silencing
chGAPDH-RT-F	CAAACCTCATTGTCATACCAGGAA	RT-PCR
chGAPDH-RT-R	GGTGACAGCCATTCCCTCCAC	RT-PCR
β -actin-RT-F	AAATTGTGCGTGACATCAAGGA	RT-PCR
β -actin-RT-R	AGGCAGCTGTGGCCATCTC	RT-PCR
chMDA5-RT-F1	ATTCCACAGCCGCGAGATTC	RT-PCR
chMDA5-RT-F2	CTACTGTAGCTGAGGAAGGCCTAGA	RT-PCR
chMDA5-RT-R1	CAAGATTGGCACAGATTTTCAGA	RT-PCR
chMDA5-RT-R2	GCATAAGTGCTCTCATCAGCTCGA	RT-PCR
chMAVS-RT-F1	TGTGAGCTCGGATGTTTCCA	RT-PCR
chMAVS-RT-R1	CACAGACCGTGCTTGTCATCA	RT-PCR
chTLR3-RT-F1	CACTACCACTGCTGTGATGCAA	RT-PCR
chTLR3-RT-R1	AAAGCTA TTCTCCACCCTTCAAAA	RT-PCR
chIFN β -RT-F1	CGCATCCTCCAACACCTCTTC	RT-PCR
chIFN β -RT-R1	GTGGCGTGTGCGGTCAATC	RT-PCR

chicken glyceraldehyde-3-phosphate dehydrogenase (chGAPDH) was used as an endogenous control to normalize quantification of chMDA5 expression. The primers used were chGAPDH-RT-F/chGAPDH-RT-R for chGAPDH, and chMDA5-RT-F1/chMDA5-RT-R1 for chMDA5. For detection of mRNA expression levels in the other experiment, β -actin was used as an endogenous control. The primers used were β -actin-RT-F/ β -actin-RT-R for β -actin, chMDA5-RT-F2/chMDA5-RT-R2 for chMDA5, chMAVS-RT-F1/chMAVS-RT-R1 for chMAVS, chTLR3-RT-F1/chTLR3-RT-R1 for chTLR3 and chIFN β -RT-F1/chIFN β -RT-R1 for chIFN β . The relative expression levels of the target genes were analyzed using the $\Delta\Delta$ Ct method [26].

Homology Modeling of chMDA5 and Molecular Docking with Short dsRNA

A three-dimensional homology model of chMDA5 CTD (867–993 aa) was constructed based on the structures of human (hu)MDA5 CTD (893–997 aa; PDB ID 3GA3) [7] using Molecular Operating Environment 2011.10 (Chemical Computing Group Inc., Montreal, Que., Canada). The model was then optimized by energy minimization by Molecular Operating Environment. The stereochemical quality of the obtained model was checked against a Ramachandran plot and Pro-SA web server [27], as described previously [28]. The model of the chMDA5 CTD and structures of huMDA5 CTD (PDB ID 3GA3, chain A) were docked with 12-bp dsRNA (PDB ID: 1YYW, chain EF) using Patchdock software [29].

Fig. 1. Basal expression of chMDA5 in chicken tissues and derived cell lines. chMDA5 mRNA levels in chicken tissues (**a**) and chicken cell lines (**b**) were determined. The expression levels in muscle and DF-1 were set to 1, respectively. WBC = White blood cells.

Confocal Microscopy

DF-1 cells (1×10^6 cells/well) were cultured on coverslips in 6-well culture plates (Becton Dickinson, Franklin Lakes, N.J., USA). The cells were cotransfected with Flag-chMDA5 and GFP-chMAVS at a dose of 500 and 250 ng, respectively. The next day, the cells were transfected with short poly(I:C) at a dose of 0.05 $\mu\text{g}/\text{ml}$ for 4 h and then the coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min and then blocked with 3% bovine serum albumin in PBS for 30 min. To detect Flag-chMDA5, the cells were labeled with mouse anti-FLAG monoclonal antibody, Clone M2 (1:500, F1804, Sigma-Aldrich, St. Louis, Mo., USA) and Alexa Fluor 555-conjugated goat anti-mouse IgG₁ (γ 1; 1:1,000, A21127, Sigma-Aldrich) for 1 h at 37°C. Between incubations, the coverslips were washed three times with 0.05% Tween 20-PBS for 5 min. The coverslips were then mounted on glass slides with SlowFade® Gold antifade reagent (Invitrogen). The samples were observed under a Leica TCS SP5 laser scanning confocal microscope (Leica, Tokyo, Japan). Quantitative analysis at the single-cell level was performed by colocalization software (Leica) according to the manufacturer's instructions.

Results

Expression of chMDA5 in Chicken Tissues and Cell Lines

We firstly examined tissue expression levels of chMDA5 mRNA in chicken by quantitative RT-PCR. chMDA5 mRNA was expressed in all tissues examined, and strong expression was observed in thymus, spleen and duodenum, as consistent with previous reports by Karpala et al. [15] and Lee et al. [30] (fig. 1a). We also

compared expression of chMDA5 among five chicken cell lines. The basal expression in DF-1 cells was relatively low compared to that in other cell lines (fig. 1b). The highest expression was detected in LSCC-RP9, which is derived from retrovirus-induced lymphoblastoid B cells (fig. 1b).

chMDA5 Mediates Poly(I:C)-Induced $\text{chIFN}\beta$ Expression in DF-1 Cells

To ascertain the length of dsRNA that can induce $\text{chIFN}\beta$ expression in DF-1 through the MDA5 signaling pathway in the absence of an RIG-I ortholog in chickens, we firstly assessed mRNA expression of $\text{chIFN}\beta$ in DF-1 cells primed with a viral dsRNA mimic, poly(I:C), of short (0.2–1 kbp) and long (1.5–8 kbp) lengths for 4 h by quantitative RT-PCR. We found that long poly(I:C) induced mRNA expressions of $\text{chIFN}\beta$ and chMDA5 to a greater extent than short poly(I:C) in DF-1 cells at a transfection dose of 0.05 $\mu\text{g}/\text{ml}$ (fig. 2a). Similar tendency was found in the mRNA expression profile of these genes in the cells primed with short and long poly(I:C) at a dose of 0.5 or 5 $\mu\text{g}/\text{ml}$ (data not shown). Incubation of poly(I:C) with DF-1 cells without transfection did not induce $\text{chIFN}\beta$ and chMDA5 expression even at high dose (5 $\mu\text{g}/\text{ml}$), in accordance with a previous report (data not shown) [15].

To determine the major signaling pathway by which poly(I:C) stimulation initiates induction of $\text{chIFN}\beta$ expression in DF-1 cells, we knocked down chMDA5, chMAVS and chTLR3 using gene-specific siRNA and then assessed mRNA expression of $\text{chIFN}\beta$. The mean

Fig. 2. chMDA5 plays a major role in chIFN β induction in DF-1 cells stimulated with poly(I:C) regardless of its length. **a** mRNA expression of chMDA5 and chIFN β in DF-1 cells stimulated with poly(I:C). DF-1 cells were transfected with short (0.2–1 kbp) or long (1.5–8 kbp) poly(I:C) at a dose of 0.05 μ g/ml for 4 h. The extracted RNA was subjected to measurements of chMDA5 and chIFN β expression by RT-PCR, as mentioned in Materials and Methods. The ratio of chIFN β expression levels in poly(I:C)-treated cells to those in mock-transfected cells are shown. The data represent mean $\pm$ SD from at least three independent experiments.

Asterisks represent a significant difference (* $p < 0.05$; Student's t test). **b** DF-1 cells were transfected with siRNA specific for chMDA5, chMAVS or chTLR3 alone or in combination for 24 h and then stimulated with short or long poly(I:C) at a dose of 0.5 μ g/ml for 4 h and chIFN β expression levels were measured. chIFN β expression levels in control siRNA-treated cells after the stimulation was set to 100% and the ratio of expression levels in knockdown cells compared to control siRNA-treated cells are shown. Each datum represents mean $\pm$ SD from three independent experiments (* $p < 0.05$; Tukey-Kramer test).

inhibition of chMDA5, chMAVS and chTLR3 mRNA expression using the corresponding siRNA was 77, 61 and 75%, respectively. Silencing of chMDA5 resulted in 97 and 83% decreases in chIFN β expression in the DF-1 cells primed with short and long poly(I:C), respectively, compared to control siRNA-treated cells (fig. 2b). Silencing of chMAVS in DF-1 cells reduced chIFN β expression by 84 and 70%, respectively (fig. 2b). The lower inhibi-

tory effect of poly(I:C)-mediated chIFN β induction on chMAVS-knockdown cells compared to chMDA5-knockdown cells is probably due to a lower inhibitory efficiency on chMAVS mRNA expression by siRNA against MAVS (61 and 77% reduction for chMAVS and chMDA5 mRNA expression, respectively). Silencing of TLR3 in DF-1 cells was found to reduce chIFN β expression by 57 and 32% after stimulation by short and long

poly(I:C), respectively (fig. 2b). These findings demonstrate that induction of mRNA expression of $\text{chIFN}\beta$ in DF-1 cells by short poly(I:C) stimulation was primarily mediated by chMDA5 . In the case of stimulation by long, but not short poly(I:C), it should be noted that silencing of chMDA5 , chMAVS and chTLR3 in combination significantly reduced $\text{chIFN}\beta$ expression in the cells compared to chMDA5 alone (83% reduction for SiMDA5 ; 98% reduction for SiMDA5 , SiMAVS and SiTLR3 in combination; fig. 2b). chTLR3 and/or an undefined factor(s), which interacts with chMAVS may also be involved in $\text{chIFN}\beta$ induction in DF-1 cells primed with long poly(I:C).

chMDA5 Preferentially Senses Short Poly(I:C) in DF-1 Cells

To define the length dependency of chMDA5 susceptibility to poly(I:C), DF-1 cells were transiently transfected with an expression vector containing Flag-tagged chMDA5 (full length: 1–1001 aa) or dRIG-I (full length: 1–933 aa; fig. 3a) and then mRNA expression of $\text{chIFN}\beta$ in the cells was assessed for 4 h after stimulation. To verify an equal number of binding sites between chMDA5 and huMDA5 [31–33], we used short and long poly(I:C) with equal mass (0.01 $\mu\text{g}/\text{ml}$) for the assay. Expression of chMDA5 in DF-1 cells enhanced $\text{chIFN}\beta$ expression after stimulation with short and long poly(I:C) in a dose-dependent manner, compared to mock-transfected cells primed with a respective ligand [4.3- to 32.9-fold induction after stimulation with short poly(I:C); 1.6- to 3.4-fold induction after stimulation with long poly(I:C); fig. 3b]. We also found that short poly(I:C) induced $\text{chIFN}\beta$ expression in chMDA5 -expressing cells to a greater extent than long poly(I:C) at the same dose of transfected expression vector (2.6-, 4.2-, 8.7- and 9.6-fold at 100, 200, 400 and 800 ng of transfected vector, respectively; fig. 3b). Stimulation with short poly(I:C) also induced $\text{chIFN}\beta$ expression in dRIG-I -expressing cells to a greater extent than long poly(I:C) at transfection doses of 200, 400 and 800 ng (1.5-, 3.1- and 3.5-fold at 200, 400 and 800 ng expression vector, respectively; fig. 3b). These data clearly show that chMDA5 preferentially recognizes short poly(I:C), as does dRIG-I . No $\text{chIFN}\beta$ induction was detected in vector-only transfected cells after poly(I:C) stimulation (fig. 3b). In contrast to the observation that long poly(I:C) induced $\text{chIFN}\beta$ expressions to a greater extent than short poly(I:C) in naïve DF-1 cells in figure 2a, short poly(I:C) stimulates $\text{chIFN}\beta$ more efficiently than long poly(I:C) through ectopically expressed chMDA5 as shown in figure 3b.

This discrepancy appears to indicate that long poly(I:C) stimulates not only the chMDA5 pathway, but also other pathways, such as the TLR pathway and/or others.

Given that mammalian MDA5 mediates $\text{IFN}\beta$ induction via CARD, we constructed an expression vector with chMDA5 devoid of two N-terminal CARD ($\text{chMDA5}\Delta\text{CARD}$, 196–1001 aa; fig. 3a) and assessed mRNA expression of $\text{chIFN}\beta$. As expected, expression of $\text{chMDA5}\Delta\text{CARD}$ in DF-1 cells did not enhance $\text{chIFN}\beta$ expression after stimulation with short and long poly(I:C) (fig. 3b). Also, transfection of $\text{chMDA5}\Delta\text{CARD}$ at a dose of 1,200 ng significantly inhibited chMDA5 -mediated $\text{chIFN}\beta$ expression in DF-1 cells after short poly(I:C) stimulation (71% reduction; fig. 3c). These findings demonstrate that CARD are required for chMDA5 -mediated $\text{chIFN}\beta$ induction in DF-1 cells, as is mammalian MDA5. We also tested the putative dominant-negative effect of dRIG-I devoid of CARD ($\text{dRIG-I}\Delta\text{CARD}$, 185–933 aa) on short poly(I:C)- and chMDA5 -mediated $\text{IFN}\beta$ induction. Expression of $\text{dRIG-I}\Delta\text{CARD}$ in DF-1 cells did not

Fig. 3. chMDA5 preferentially recognizes short poly(I:C). **a** Illustration of chMDA5 , dRIG-I and their deletion and chimera constructs. **b** DF-1 cells were transiently transfected with the indicated plasmid at a dose of 100, 200, 400 and 800 ng, and then stimulated with short or long poly(I:C) at a dose of 0.05 $\mu\text{g}/\text{ml}$ for 4 h. The extracted RNA was subjected to measurement of expression of $\text{chIFN}\beta$. The ratio of $\text{chIFN}\beta$ expression levels in transfected cells stimulated with short or long poly(I:C) compared to untransfected cells stimulated with short or long poly(I:C), respectively, are shown. Each datum represents mean $\pm$ SD from at least three independent experiments (* $p < 0.05$; Mann-Whitney U test). **c** DF-1 cells were transiently transfected with the indicated plasmid and then stimulated with short poly(I:C) at a dose of 0.05 $\mu\text{g}/\text{ml}$ for 4 h. The expression levels in chMDA5 -expressing cells without expression of each mutant were set to 100%. Data represent mean $\pm$ SD from three independent experiments (* $p < 0.05$; Tukey-Kramer test). **d** DF-1 cells were transiently transfected with the indicated plasmid at a dose of 400 ng, and then stimulated with short or long poly(I:C) at a dose of 0.05 $\mu\text{g}/\text{ml}$ for 4 h. Relative $\text{IFN}\beta$ expression levels were assessed as described in **b**. Data represent mean $\pm$ SD from four independent experiments (* $p < 0.05$; Mann-Whitney U test).

Fig. 4. Interactions between chMDA5 and chMAVS . DF-1 cells were cotransfected with Flag- chMDA5 and GFP- chMAVS expression plasmids for 24 h and then stimulated with short poly(I:C) at a dose of 0.05 $\mu\text{g}/\text{ml}$ for 4 h. Colocalization of chMDA5 and chMAVS was visualized under a Leica TCS SP5 laser scanning confocal microscope. **a** Representative confocal microscopic image. Signals of GFP- chMAVS and Flag- chMDA5 are shown in green and red, respectively. **b** Colocalization area and mean intensity of GFP- chMAVS and Flag- chMDA5 within the colocalization area were shown. At least 50 cells were counted in each experiment and the data represent mean $\pm$ SD from three independent experiments (* $p < 0.05$ vs. control group; Mann-Whitney U test).

3

b

	No stimulation	Short poly(I:C) stimulation
Colocalization area, μm	8.7 ± 11.5	15.7 ± 16.6*
Mean intensity of GFP-MAVS	64 ± 16	86 ± 28*
Mean intensity of Flag-MDA5	61 ± 16	83 ± 28*

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inhibit $\text{chIFN}\beta$ expression even at a high transfection dose (1,200 ng), suggesting that the avidity of dRIG-I for short poly(I:C) is less than that of chMDA5 in DF1 cells (fig. 3c).

As shown in figure 3b, short poly(I:C) induced $\text{chIFN}\beta$ expression in dRIG-I-expressing cells to a lesser extent than in chMDA5-expressing cells at the same dose of transfected vector (0.30-, 0.19-, 0.14- and 0.12-fold at 100, 200, 400 and 800 ng, respectively; fig. 3b). The lower level of $\text{chIFN}\beta$ induction may be due to an insufficient interaction between dRIG-I CARD and chMAVS CARD, because of low similarity between their respective CARD (20% similarity). In order to assess our hypothesis, we generated Flag-tagged chimeric proteins containing chMDA5 CARD (1–195 aa) and helicase domain and CTD of dRIG-I (185–933 aa; dRIG-I/chMDA5 CARD, 944 aa) and containing dRIG-I CARD (1–184 aa) and helicase domain and CTD of chMDA5 (196–1001 aa; chMDA5/dRIG-I CARD, 990 aa; fig. 3a). We found that transfection of dRIG-I/chMDA5 CARD in DF-1 cells resulted in a significant increase in $\text{chIFN}\beta$ expression in response to stimulation with short poly(I:C), compared to naive dRIG-I-expressing cells after stimulation (fig. 3d). In contrast, expressions of chMDA5/dRIG-I CARD significantly reduced the $\text{chIFN}\beta$ expression in DF-1 cells primed with short poly(I:C) compared to those of naive MDA5 (fig. 3d). However, expression of dRIG-I/chMDA5 CARD induced chIFN expression after stimulation to a lesser extent than full-length chMDA5 (0.21-fold; fig. 3d).

Homology Modeling of chMDA5 CTD and Molecular Docking with Short dsRNA

huMDA5 CTD (PDB ID 3GA3, chain A) [7] has been shown to be associated with viral RNA binding and amino acid sequences of chMDA5 CTD (867–1001 aa) were 65.9% identical to huMDA5 CTD (893–1025 aa; online suppl. fig. 1a; for all online supplementary material, see www.karger.com/doi/10.1159/000351583). By a homology modeling of chMDA5 CTD (online suppl. fig. 1b), followed by a docking simulation with the 12-bp dsRNA, dsRNA was shown to contact closer to chMDA5 than to huMDA5 (online suppl. fig. 1c). Among the residues within a radius of 4 Å from the 12-bp dsRNA in the docking models, side chains of three residues (T920, V942 and N945) of chMDA5 were longer and directed toward dsRNA, compared to the respective residues (A946, A967 and T971) of huMDA5 (online suppl. fig. 1e). Whether those differences contribute different ligand bindings between chMDA5 and huMDA5 needs to be examined.

chMDA5 Interacts with chMAVS in DF-1 Cells Primed with Short Poly(I:C)

To examine whether chMDA5 and chMAVS interact after ligand stimulation, as reported for mammalian MDA5 and MAVS, DF-1 cells transiently cotransfected with Flag-chMDA5 and GFP-tagged chMAVS were stimulated with short poly(I:C) and localizations of fusion proteins in the cells were observed by fluorescence microscopy for 4 h after stimulation. Yellow fluorescence representing colocalization of Flag-chMDA5 and GFP-chMAVS was observed in DF-1 cells primed with short poly(I:C) (fig. 4a). Quantitative analysis at a single cell level revealed that the stimulation with short poly(I:C) significantly expanded the colocalization area in DF-1 cells [mean colocalization area: 8.7 and 15.7 μm^2 for mock and poly(I:C) transfection groups, respectively; fig. 4b]. The mean intensity of both Flag-chMDA5 and GFP-chMAVS signals within the colocalization area in individual cells was also significantly higher in cells primed with short poly(I:C) than in unprimed cells (fig. 4b). These findings demonstrate that chMDA5 interacts with chMAVS in DF-1 cells following stimulation by short poly(I:C).

Involvement of chMDA5 in $\text{chIFN}\beta$ Induction in DF-1 and LMH Cells Infected with AIV

To examine whether chMDA5 is involved in $\text{chIFN}\beta$ activation by influenza virus infection in DF-1 cells, we firstly investigated the inhibitory effect of chMDA5-, chMAVS- or chTLR3-knockdown on $\text{chIFN}\beta$ induction during AIV infection. Silencing of chMDA5, chMAVS

Fig. 5. chMDA5 is involved in $\text{chIFN}\beta$ induction in DF-1 and LMH cells infected with AIV. **a** DF-1 cells were transfected with siRNA specific for chMDA5, chMAVS or chTLR3 alone or in combinations for 24 h, and then infected with LP/Yokohama at 100 EID₅₀/cell for 24 h. $\text{chIFN}\beta$ expression levels in control siRNA-treated cells after infection were set to 100% and the ratio of expression levels in knockdown cells compared to control siRNA-treated cells are shown. Data represent mean $\pm$ SD from three independent experiments (* $p < 0.05$; Tukey-Kramer test). DF-1 (**c**) and LMH (**d**) cells were transiently transfected with the indicated plasmid at a dose of 100 ng and then infected with LP/Yokohama or HP/Yamaguchi at 0.1 or 10 EID₅₀/cell for 24 h. Data represent mean $\pm$ SD from at least three (DF-1 cells) or four (LMH cells) independent experiments (* $p < 0.05$; Tukey-Kramer test). Growth properties of LP/Yokohama in knockdown DF-1 cells (**b**) and LP/Yokohama and HP/Yamaguchi in chMDA5-expressing DF-1 (**e**) and LMH (**f**) cells. At 24 and 48 h postinfection, supernatants were titrated in embryonated eggs (**b**, **e**) or MDCK cells (**f**). Viral titers were expressed as EID₅₀/ml (**b**, **e**) or TCID₅₀/ml (**f**). Data represent mean $\pm$ SD from at least three independent experiments.

and chTLR3 in DF-1 cells significantly reduced chIFN β expression by 84, 79 and 85%, respectively, at 24 h after LP/Yokohama infection compared to control siRNA-treated cells (fig. 5a). Silencing of chMDA5, chMAVS and chTLR3 in combination was the most effective, leading to a 95% decrease in chIFN β expression (fig. 5a). These results suggest that both chMDA5 and chTLR3 are involved in chIFN β activation in DF-1 cells following LP/Yokohama infection.

Next, DF-1 cells were transiently transfected with Flag-chMDA5 and then mRNA expression of chIFN β in the cells was assessed 24 h after AIV infection. We found that LP/Yokohama induced chIFN β expression in chMDA5-expressing cells to a greater extent than in the mock-transfected cells at infection doses of 0.1 and 10 EID₅₀/cell (13.5- and 15.0-fold, respectively; fig. 5c). Expression of vector alone or chMDA5 Δ CARD in the cells did not elicit chIFN β expression during viral infection (fig. 5c). In contrast, HP/Yamaguchi alone significantly ($p < 0.05$) induced chIFN β expression in the mock-transfected cells compared to LP/Yokohama (34.8- and 17.0-fold at doses of 0.1 and 10 EID₅₀/cell, respectively; fig. 5c). HP/Yamaguchi infection also enhanced chIFN β expression in the cells transfected with chMDA5 Δ CARD. As a consequence, no enhancement of chIFN β expression was observed in chMDA-expressing cells by HP/Yamaguchi infection (fig. 5c). We also measured chIFN β expression levels in LMH or HD-11 cells transfected with chMDA5 during AIV infection. Both viruses elicited chIFN β inductions in chMDA5-expressing LMH cells to a greater extent than in mock-transfected cells at infection doses of 0.1 and 10 EID₅₀/cell (fig. 5d), whereas transfection of chMDA5 in HD-11 cells did not induce the expressions during the infection of both viruses (data not shown).

Although chMDA5 was shown to be involved in chIFN β activation during AIV infection, neither the chMDA5 single knockdown nor the chMDA5/chMAVS/chTLR3 triple knockdown in DF-1 cells affected viral replication at 24 or 48 h postinfection, compared to non-knockdown cells infected with LP/Yokohama (fig. 5b). We also found that expression of chMDA5 in DF-1 and LMH cells did not inhibit viral replication of LPAIV or HPAIV (fig. 5e, f).

Discussion

Here, we provide the first evidence that chMDA5, unlike mammalian MDA5, preferentially senses short poly(I:C) (0.2–1 kbp) than the longer one (1.5–8 kbp) in

chicken DF-1 cells. This observation extends the previous notion by Karpala et al. [15] that chMDA5 plays a role in chIFN β induction in the cells stimulated with different lengths (1, 3 and 6 kbp) of poly(I:C). In mammals, viral dsRNA is reported to be selectively recognized by RIG-I and MDA5 according to its length [9]. Among birds, RIG-I is present in duck, geese, zebra finch and pigeon, but has not been identified in chicken [34, 35]. It is also absent from several fish species [34]. Deddouche et al. [36] reported that invertebrates possess the Dicer-like helicase family related closely to mammalian RLRs including RIG-I and MDA5. They also suggested that drosophila Dicer-2 senses viral RNA in the infected cells and thereby induces antiviral response, as do mammalian RLRs [36]. Taking above observations into account, loss of RIG-I in chicken appears to have occurred during the evolutionary process after separation of bird lineage from tetrapod lineage. Our studies clearly show that chMDA5 is a pivotal receptor that mediates antiviral responses driven by recognition of short dsRNA and AIV in the absence of RIG-I in chicken. Chicken TLR21 is reported to sense CpG-DNA, which is recognized by TLR9 in the case of mammals. It has been considered that chicken TLR21 compensates for the function of TLR9, which is lost during evolution [37]. Our study implies that chMDA5 compensates, in part, the function of RIG-I to induce protection against various RNA virus infections in chicken in the absence of RIG-I lost during evolution.

Comparison between docking models of chMDA5 and huMDA5 CTDs with short dsRNA suggests that chMDA5 CTD may have a strong interaction with short dsRNA compared to huMDA5 CTD, and thereby sense short poly(I:C) preferentially (fig. 3b; online suppl. fig. 1d, e). huMDA5 CTD is reported to bind 25 bp dsRNA much more weakly than huRIG-I, and this low binding affinity may lead to dominant detection of short dsRNA by RIG-I in mammals [6]. Given that closer proximity between protein and RNA leads to stronger interaction, 3 amino acids (T920, V942 and N945), located in the vicinity of dsRNA only in the chMDA5 docking model, seem to be potential candidates for strengthening the binding between chMDA5 CTD and short dsRNA (online suppl. fig. 1e). Such molecular alterations could be required to compensate for the lack of RIG-I. Further studies comparing the binding affinity to dsRNA between chMDA5 and huMDA5 CTDs, as well as mutation analysis of the above-mentioned amino acids would be required to prove this hypothesis. Recent structural analysis of huMDA5 revealed that it cooperatively assembles into filamentous oligomers along with dsRNA and the heli-

case domain is required for preferential recognition of long dsRNA by huMDA5 [31–33, 38]. The overall structure of chMDA5 would be needed to elucidate the molecular mechanism by which this protein preferentially senses short dsRNA.

Although chMDA5 was found to be capable of sensing AIV leading to induction of chIFN β expression in DF-1 cells, the induced chIFN β did not affect viral replication in our experimental setting (fig. 5c, e). It is known that type I IFNs secreted from virus-infected cells induce IFN-stimulated genes including myxovirus-resistance protein and dsRNA-dependent protein kinase (PKR) in self and in neighboring uninfected cells through autocrine and paracrine mechanisms, respectively, and thereby establish the antiviral state that impedes viral replication [39]. It was reported that pretreatment of MC57 with 100 units of IFN α effectively suppresses HPAIV and A (H1N1) pdm09 virus replication in vitro [40]. This implies that, even in cells responsive to type I IFN, autocrine and paracrine effects of IFNs from virus-infected cells may not be sufficient to suppress viral replication in vitro. Since AIVs have a short duplication time, they readily infect neighboring uninfected cells before there is time to establish the antiviral state by IFNs secreted paracinely by cMDA5-mediated induction in AIV-infected cells. Previous reports also demonstrated that transient expression of RIG-I inhibited viral titers by only 2- to 6-fold in comparison to untransfected cells [17, 41].

Transient expression of chMDA5 elicited significant IFN β induction in DF-1 cells, effective against LPAIV infection but not HPAIV infection, compared to the respective untransfected cells (fig. 5c). There are three possible explanations for this observation. Firstly, HPAIV induced IFN β to the limit of the infected cells through endogenous PRRs, including chMDA5, and additive induction could thus not be observed by expression of exogenous chMDA5. In support of this possibility, Deng et al. [42] reported that overexpression of TLR4 in sheep fetal fibroblasts did not enhance IL6, IL8 or TNF α mRNA expression levels after stimulation with lipopolysaccharide at time points when high expression levels of cytokine were observed in untransfected cells after stimulation. Secondly, there is the implication of negative-feedback reactions against type I IFN responses induced by HPAIV at the early stage of infection. Rothenfusser et al. [43] reported that overexpression of laboratory of genetics and physiology 2 inhibited IFN signaling induced by Sendai virus in mammalian cells, due to sequestration of dsRNA from RIG-I, thereby acting as a negative regulator of RIG-I signaling to prevent excessive production of type I IFNs.

The previous report that overexpression of chicken laboratory of genetics and physiology 2 inhibits chIFN β promoter activity induced by HPAIV infection in DF-1 cells would appear to support this idea [16]. The third possibility is that HPAIV, but not LPAIV, used in this study could inhibit the type I IFN response in the cells. HPAIV NS1 and PB2 are reported to inhibit chIFN β promoter activity mediated by chMDA5 in DF-1 cells [16, 44], suggesting that these segments may inhibit IFN β expression to some extent in our study. Indeed, we found that transfection of YamNS1 significantly inhibited chIFN β inductions in chMDA5-expressing DF-1 and LMH cells stimulated with short poly(I:C) (online suppl. fig. 2). NS1 protein of influenza A virus is reported to bind to CPSF30 and thereby block cellular mRNA processing in mammalian cells infected with influenza A virus [45]; therefore, YamNS1 possibly inhibits maturation of chIFN β mRNA in the infected cells. However, mRNA binding activity of CPSF30 of two strains used in this study is yet to be determined. To understand mechanisms by which LPAIV, but not HPAIV, elicits IFN β induction in DF-1 cells, comparison of mRNA binding activity of CPSF30 between both strains would be needed.

There are considerable differences between chicken and mammalian innate immune systems. Our study provides another example of such differences. Overall understanding of the chicken innate immune system, including TLRs and RLRs, and comparison with the mammalian system will contribute to the elucidation of a comprehensive mechanism against invading microbes, including HPAIV, in chickens.

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Disclosure Statement

We report no conflicts of interest.

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