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(continued)

Cover art: The nuclear body organizing protein PML (also known as TRIM19) co-localizes in the cytoplasm with rabies virus P protein. Chinese hamster ovary cells stably expressing PML protein (detected with anti-PML and Texas red, *middle panel*) were transiently transfected with a Protein GFP fusion protein (*left panel*). The merged image is shown in the *right panel.*

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Chicken Toll-like Receptor 3 Recognizes Its Cognate Ligand When Ectopically Expressed in Human Cells

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ABSTRACT

Recognition of pathogens by toll-like receptors (TLRs) causes activation of signaling cascades that trigger cytokine secretion and, ultimately, innate immunity. Genes encoding proteins with substantial homology to mammalian TLR1, TLR2, TLR3, TLR4, TLR5, and TLR7 are present in the chicken genome, whereas orthologs of TLR8, TLR9, and TLR10 seem to be defective or missing. Except for chicken TLR2 (ChTLR2), which was previously shown to recognize lipopeptides and lipopolysaccharides (LPS), the ligand specificity of ChTLRs had not been determined. We found that polyI:C, LPS, R848, S-28463, and ODN2006, which are specifically recognized by TLR3, TLR4, TLR7/8, and TLR9 in mammals, induced substantial amounts of type I interferon (IFN) and interleukin-6 (IL-6) in freshly prepared chicken splenocytes. To determine the ligand specificity of ChTLR3 and ChTLR7, we used a standard reporter assay frequently employed for analysis of mammalian TLRs. Neither S-28463 nor any other TLR ligand induced reporter activity in human 293 cells expressing ChTLR7. However, human 293 cells expressing ChTLR3 strongly and specifically responded to polyI:C, demonstrating that this chicken receptor represents a true ortholog of mammalian TLR3.

INTRODUCTION

THE TOLL-LIKE RECEPTOR (TLR) multigene family codes for important recognition receptors of the innate immune system. In humans and other mammals, 10 or more TLRs with mostly distinct ligand specificity have been recognized.[1,2] The chicken genome contains genes with homology to TLR1, TLR2, TLR3, TLR4, TLR5, and TLR7.[3–7] Furthermore, a novel TLR gene, TLR15, identified in chickens, is upregulated in response to heat-killed *Salmonella enterica* but shows no decisive similarity to the extracellular domain of any known mammalian TLR.[8] Interestingly, chickens appear to lack an ortholog of TLR9,[4] although chicken macrophages and peripheral blood mononuclear cells (PBMCs) can respond to CpG oligonucleotides.[9] In light of these differences and peculiarities of the chicken TLR system, it is important to know if the various chicken TLR orthologs have the same ligand specificities as their mammalian counterparts.

Chicken TLR2 (ChTLR2) was shown to act as a receptor for bacterial lipoprotein and, similar to studies of human TLR2 (HuTLR2), was demonstrated to recognize lipopolysaccharide (LPS) in the presence of MD2.[3] Genetic evidence suggests that ChTRL4 plays a role in resistance to *Salmonella* infections,[10] suggesting that it has a function similar to that of TLR4 of mammals. For other ChTLRs, the ligand specificity has not been determined experimentally. We show here that cultured chicken spleen cells readily respond to TLR ligands that activate TLR3, TLR4, TLR7/8, and TLR9 in mammals. By analyzing the response to various TLR ligands of transfected human 293 cells expressing full-length cDNAs for ChTLR3 and ChTLR7, we demonstrate that double-stranded RNA (dsRNA) is specifically recognized by ChTLR3. Imidazoquinolines, such as R848 or S-28463, and CpG oligonucleotides failed to activate transfected 293 cells expressing either ChTLR3, ChTLR7, or both, leaving the identity of the chicken receptors recognizing these TLR ligands undefined.

MATERIALS AND METHODS

Spleen cell cultures

Spleens from adult White Leghorn chickens were dissociated mechanically using stainless steal sieves, washed with

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phosphate-buffered saline (PBS), suspended in RPMI medium containing 10% fetal bovine serum (FBS), and seeded into 48-well culture plates. TLR ligands were added to the culture medium at the indicated concentrations for 24 h.

TLR ligands

Ultrapure LPS, polyI:C, ODN2006, R848, and purified DNA from *Escherichia coli* were purchased from InvivoGen (San Diego, CA). S-28463 was a gift from Dr. M. Tomai (3M Pharmaceutical, St. Paul, MN).

Cytokine assays

Quail CEC32 cells stably transfected with a chicken Mx promoter-luciferase reporter construct were used for titrating type I interferon (IFN) as described.[11] Interleukin-6 (IL-6) activity was determined using the IL-6-dependent mouse B cell line 7TD1 as described.[12]

Expression plasmids

To construct the ChTLR3 expression plasmid, two PCR fragments representing the 5′ and 3′ halves of ChTLR3 cDNA, respectively, were cloned between the NotI and NheI restriction sites of plasmid pCA.[13] cDNAs were amplified by doing semi-nested PCR on cDNA derived from bronchial-associated lung tissue. For amplification of the 5′ fragment, forward primer 5′-AAATGCGGCCGCTATGGGATGCTCTATTCCTTGC-3′ and reverse primer 5′-CCAGATTATTGCAACCTACC-3′ were used in a first PCR. A second PCR was done using the same forward primer and reverse primer 5′-CCTTCGAATTCATGAC-CTGTG-3′. For amplification of the 3′ fragment of ChTLR3 cDNA, forward primer 5′-TGTACAGCAACCAAATCAC-3′ and reverse primer 5′-GAGAGCTAGCTCAGCGCACTTTAC-TATTAG-3′ were used for the first PCR. A second PCR was done using the forward primer 5′-GGTCATGAATTCGAAG-GTCTC-3′ and the same reverse primer. The sequence of our ChTLR3 clone was deposited at GenBank under accession number DQ780341.

To construct expression plasmid ChTLR7-long, PCR was performed on EST clone riken1-19d10r1 using forward primer 5′-GAGAGCGGCCGCTATGACAAATCTTTCAGAGG-3′ and reverse primer 5′-GAGAGCTAGCCTAAACAGTTTC-CTGGAGAAG-3′, and the resulting product was cloned between the NotI and NheI restriction sites of the pCA vector. To generate expression plasmid ChTLR7-short, PCR was performed using the same reverse primer and forward primer 5′-GAGAGCGGCCGCTATGGTACATCATGCCAAGG-3′, and the 5′-truncated product was cloned into the same restriction sites of the pCA vector. The sequences of our ChTLR7-long and ChTLR7-short were deposited at GenBank under accession number DQ780342. Expression plasmids for HuTLR7 [14] and HuTLR9 (InvivoGen) and NF-κB-luciferase reporter construct p55A2Luc[15] were described previously.

TLR activity assay

HEK293 cells in 6-well plates were transfected with 250 ng of reporter plasmid p55A2Luc and 1 μg of the various TLR expression plasmids using Metafectene (Biontex, Munich, Germany). About 15 h posttransfection, the cells were removed from the culture plates using trypsin and seeded into 24-well dishes. Some 9 h later, the cells were treated with TLR ligands for 15 h. The cells were then lysed in 100 μL of lysis buffer (Promega, Madison, WI), and the luciferase activity in 10-μL samples was determined in a Berthold LB9010 luminometer using 50 μL of luciferase substrate (Promega).

RESULTS

Chicken spleen cells readily respond to various TLR ligands

To determine the extent of the TLR response in chickens, we stimulated cultures of freshly explanted spleen cells with either polyI:C, LPS, R848, or ODN2006. Culture supernatants were harvested at 24 h poststimulation and assayed for the presence of type I IFN and IL-6 using appropriate bioassays. Supernatants of unstimulated spleen cells did not contain detectable levels of cytokine activity, whereas supernatants of

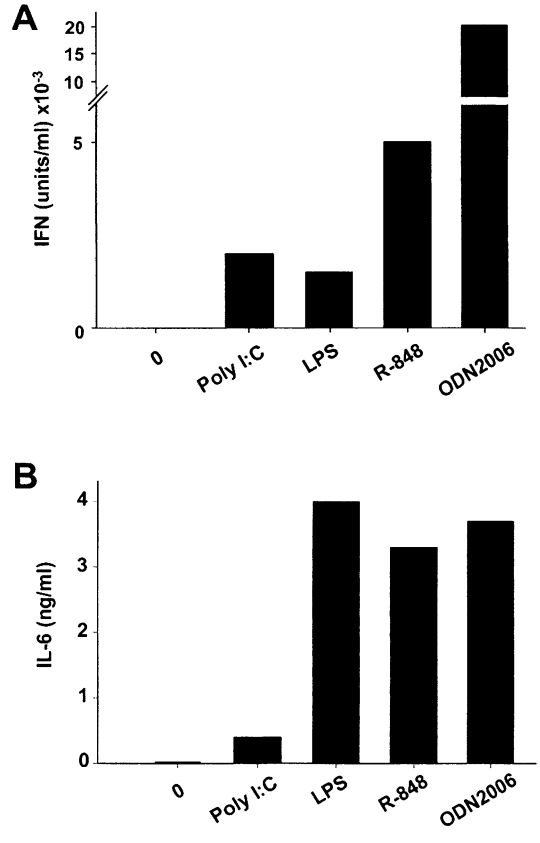

FIG. 1. Chicken spleen cells readily respond to TLR ligands. Triplicate cultures of freshly explanted chicken spleen cells were stimulated for 24 h with either polyI:C (25 ng/mL), LPS (10 μg/mL), R848 (1 μg/mL), or ODN2006 (1 μM), as indicated. Splenocyte supernatants were then analyzed for the presence of (**A**) type I IFN using the CEC32-511 indicator cell line, which carries an Mx promoter-controlled luciferase reporter gene, or (**B**) IL-6 by measuring IL-6-dependent proliferation of the murine hybridoma cell line 7TD1. Average values are shown. Variation between the triplicates was minimal.

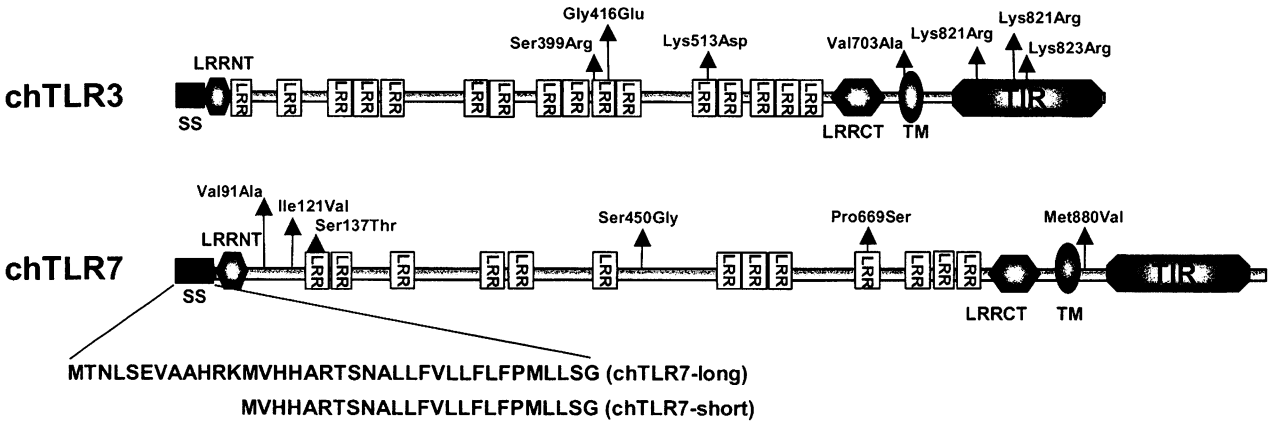

FIG. 2. Domain structures of ChTLR3 and ChTLR7. Domains of the mature protein were identified using the SMART software package. The signal sequence of ChTLR3 was determined using the SignalP3.0 program. SS, signal sequence; LRR, leucine-rich repeat; TM, transmembrane domain; TIR, TIR domain; LRRNT, cysteine-rich region flanking LRR domain at the N-terminus; LRRCT, cysteine-rich region flanking the LRR domain at the C-terminus. Arrows mark differences from published amino acid sequences of ChTLR3 and ChTLR7 (accession numbers AY633575 and AY633577). Two different signal sequences found in ChTLR7 resulting from putative translation initiation of the mRNA at two different AUG codons are shown in single amino acid code.

cells treated with TLR ligands contained substantial amounts of both IFN and IL-6 (Fig. 1). None of the TLR ligands induced luciferase activity when added directly to the indicator cells (data not shown), excluding possible artifacts of the assay system. In several independent experiments, ODN2006 was a potent inducer of IL-6 and IFN activity. LPS and R848 induced high amounts of IL-6 but lower levels of IFN. Cytokine induction by polyI:C was comparably weak. IFN and IL-6 activity was also detected in supernatants of spleen cells treated with S-28463 or purified DNA from *E. coli* (data not shown). Collectively, these results demonstrated that chicken immune cells are equipped with receptors that recognize ligands that specifically activate TLR3, TLR4, TLR7/8, and TLR9 in mammals.

Full-length cDNAs encoding ChTLR3 and ChTLR7

ChTLRs with high homology to mammalian TLR3 and TLR7 were identified previously.[4,6] Full-length cDNAs for these two molecules used in our laboratory showed several nucleotide differences compared with database entries AY633575 and AY633577, resulting in the amino acid changes indicated in Figure 2. It should be noted that our cDNA clone for ChTLR7 contains additional nucleotides at the 5′ end that extend the open reading frame (ORF) at the N-terminus compared with another EST clone.[6] Consequently, the putative full-length translation product of our cDNA clone (designated ChTLR7-long) contains a signal sequence that is 12 amino acids longer than the signal sequence in the previously described molecule[6] (designed as ChTLR7-short).

Ligand specificity of ChTLR3 and ChTLR7

To determine the ligand specificity of ChTLR3 and ChTLR7, we employed a reporter assay system frequently used for analysis of mammalian TLRs. HEK293 cells were transfected with an NF-κB-luciferase reporter construct and expression plasmids for TLRs. At 24 h posttransfection, the cells were stimulated for 15 h with TLR ligands before measurement of luciferase activity in the cell lysates. Under these experimental conditions, ChTLR3 conferred a high degree of responsiveness to polyI:C but not LPS, S-28463, and ODN2006 (Fig. 3A), demonstrating identical TLR specificities of chicken and human TLR3. When expressed in HEK293 cells, neither the long nor the short form of ChTLR7 mediated a response to S-28463 (Fig. 3B). HuTLR7, which served as positive control in these transfections, was active, indicated by the fact that transfected HEK293 cells readily responded to S-28463. None of the other TLR ligands used for these experiments, including polyI:C, LPS, R848, and ODN2006, was recognized by HEK293 cells expressing ChTLR7 (Fig. 3B; data not shown).

To evaluate the remote possibility that ChTLR7 might recognize its ligands only if complexed with TLR3 and to determine if heterodimers of ChTLR3 and ChTLR7 might recognize unmethylated dsDNA, the two cDNAs were coexpressed in HEK293 cells. Expression of ChTLR7 had no negative effect on TLR3 activity, but it did not induce the formation of heterodimeric complexes with specificity for S-28463 or ODN2006 (Fig. 3C). HuTLR9, which served as positive control for this experiment, conferred responsiveness to ODN2006 and, to a lesser extent, to polyI:C (Fig. 3C).

DISCUSSION

Available genetic information indicates that chickens lack functional orthologs of mammalian TLR8, TLR9, and TLR10[4] but instead possess at least one TLR gene (TLR15) with no obvious counterpart in mammals.[8] This constellation raises the question whether chicken cells are capable of responding to TLR agonists known to be active in mammals and whether certain ChTLRs might have unexpected ligand specificities. Our studies with freshly explanted spleen cells revealed that the chicken has no obvious deficits in recognition of pathogen-as-

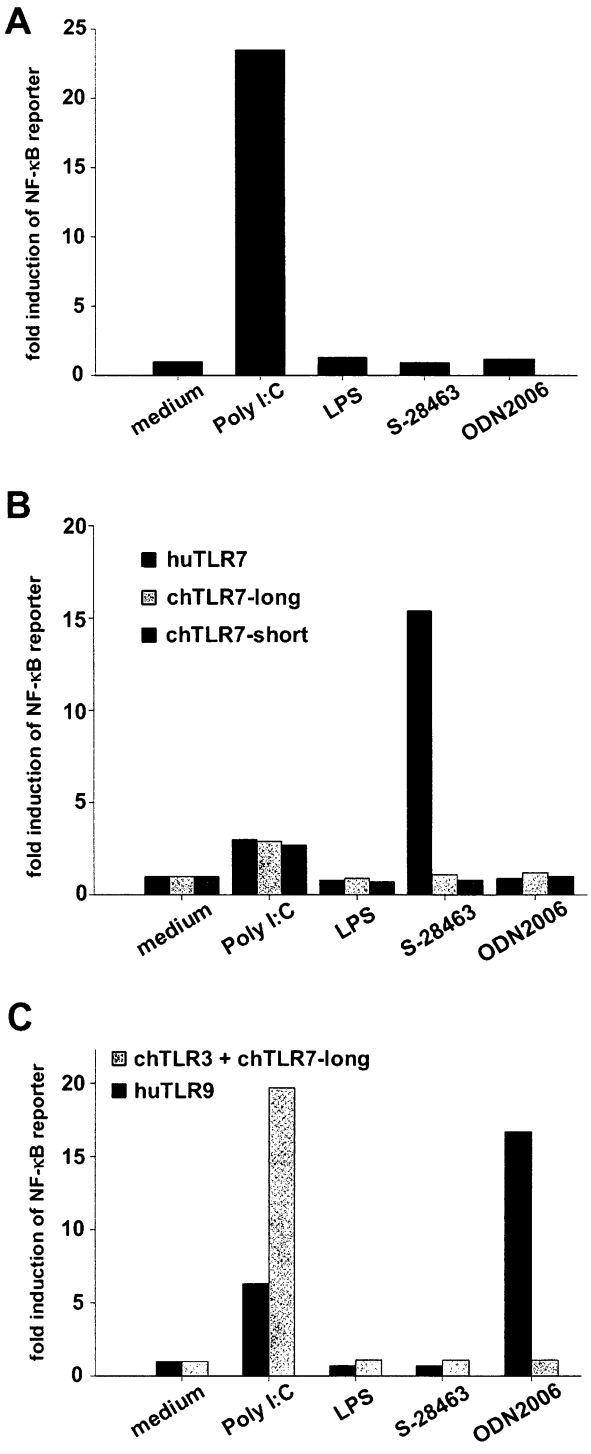

FIG. 3. ChTLR3 confers responsiveness of human cells to polyI:C. HEK293 cells were transfected with an NF-κB-luciferase reporter construct and expression plasmids for either (**A**) ChTLR3, (**B**) short or long version of ChTLR7, and (**C**) ChTLR3 and ChTLR7. Expression plasmids encoding HuTLR7 and HuTLR9 served as positive controls. At 24 h posttransfection, the cells were stimulated for 15 h with either polyI:C (25 ng/mL), LPS (10 μg/mL), S-28463 (1 μg/mL), or ODN2006 (1 μM). TLR-mediated activation of NF-κB was assessed by measuring luciferase activity in the cell lysates.

sociated molecular patterns. We found that ligands expected to be recognized by TLR3, TLR4, TLR7/8, and TLR9 readily induced the synthesis of type I IFN and IL-6. These results confirm and extend earlier studies that showed that IFN-α genes are activated in the spleen of chickens after oral treatment with S-28463[16] and that chicken heterophils are responding to CpG oligonucleotides.[9]

Responsiveness to a broad panel of TLR agonists in the absence of certain TLR orthologs inevitably leads to the hypothesis that certain TLRs of the chicken might exhibit broader ligand specificity than their mammalian counterparts. A simple way to analyze ligand specificity of ChTLRs is to employ well-established technology that was successfully used to decipher the specificity of mammalian TLRs. A potential limitation of this approach is that ChTLRs might not reveal their activity because of the lack of species-specific accessory factors that are not present in human cells. However, prior to our work, it was demonstrated that ChTLR2 is functional when expressed in the human HEK293 cell line.[3] Our own experiments unambiguously showed that ChTLR3 is active in HEK293 cells and that it exclusively recognizes dsRNA, like its mammalian counterpart.

The experimental approach with HEK293 cells failed to show the ligand specificity of ChTLR7. We evaluated the possibility that two putative ChTLR7 variants with long and short leader peptides might exhibit different activities, but both forms of ChTLR7 yielded negative results in this assay. We further evaluated the possibility that ChTLR7 might recognize variant imidazoquinolines with different efficacy. However, neither S-28643 nor R848 was recognized by HEK293 cells expressing ChTLR7. We also examined if polyI:C or unmethylated DNA, such as ODN2006, would activate HEK293 cells expressing ChTLR7. All these attempts failed to yield evidence for ChTLR7 activation by known TLR ligands. Coexpression of ChTLR3 and ChTLR7 similarly failed to induce heterodimeric forms that would have recognized imidazoquinolines or unmethylated DNA. Thus, at least two possibilities remain that might explain our findings. First, it is possible that our cDNA clone, which differs from the published ChTLR7 sequence by six amino acid changes, represents an inactive molecule. Second, ChTLR7 may require an unknown accessory factor that is not present in human HEK293 cells.

From our work, it is clear that the chicken ortholog of mammalian TLR3 specifically recognizes polyI:C, like its mammalian counterpart. However, the ligand specificity of the chicken ortholog of TLR7 remains undefined. Further studies are needed to determine if ChTLR7 can mediate the responses of chicken cells to such ligands as R848 and S-28463. It remains unclear if ChTLR7 is involved in recognition of unmethylated DNA, like ODN2006, or if the recently discovered unique TLR of chickens might play a role in the recognition of unmethylated DNA in chickens.[8]

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