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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 TLR7.3,5 Furthermore, a novel TLR gene, TLR15, identified in chicken, is upregulated in response to heat-killed Salmonella enterica but shows no definitive 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...
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. Interleukin-6 (IL-6) activity was determined using the IL-6-dependent mouse B cell line 7TD1 as described.

**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 Nhel restriction sites of plasmid pCA. 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'-CCAGATTATTGCAACCTACCC-3' were used in a first PCR. A second PCR was done using the same forward primer and reverse primer 5'-CCTTCGAATTCATGACTGTG-3'. For amplification of the 3' fragment of ChTLR3 cDNA, forward primer 5'-TGTACACGAGTACATACATCAGTCC-3' and reverse primer 5'-GAGAGCTAGCTGAGCCATTTACATTAG-3' were used for the first PCR. A second PCR was done using the forward primer 5'-GGTCATGAATTCAGCCG-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 rikenl-19d10r1 using forward primer 5'-GAGAGCGGCCGCTATGGTACATCCTCGACAGG-3' and reverse primer 5'-GAGAGCTAGCTGAGCCG-3', and the resulting product was cloned between the NotI and Nhel restriction sites of the pCA vector. To generate expression plasmid ChTLR7-short, PCR was performed using the same reverse primer and forward primer 5'-GGTCATGAATTCAGCCG-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 DQ783042. Expression plasmids for HuTLR7 and HuTLR9 (InvivoGen) and NF-kB-luciferase reporter construct p55A2Luc 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
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 Escherichia 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. 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. 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 (designated 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 but instead possess at least one TLR gene (TLR15) with no obvious counterpart in mammals. 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.

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REFERENCES

3. Fukai A, Inoue N, Matsumoto M, Nomura M, Yamada K, 
   Mat-suda Y, Toyoshima K, Seya T. Molecular cloning and functional 
   characterization of chicken toll-like receptors. A single chicken toll 
   covers multiple molecular patterns. J. Biol. Chem. 2001;276: 
   47143–47149.
   and sequence analysis of chicken toll-like receptors. Immunogenet- 
5. Iqbal M, Philbin VJ, Withanage GS, Wigley P, Beal RK, Good- 
   child MJ, Barrow P, McConnell I, Maskell DJ, Young J, Bumstead 
   N, Boyd Y, Smith AL. Identification and functional characterization 
   of chicken toll-like receptor 5 reveals a fundamental role in 
   the biology of infection with Salmonella enterica serovar ty- 
   N, Young J, Smith AL. Identification and characterization of a 
   functional, alternatively spliced toll-like receptor 7 (TLR7) and ge-
   nomic disruption of TLR8 in chickens. Immunology 2005:114: 
   507–521.
7. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, 
   Hood LE, Aderem A. The evolution of vertebrate toll-like recep-
8. Higgs R, Cormican P, Cahalane S, Allan B, Lloyd AT, Meade K, 
   James T, Lynn DJ, Babiuk LA, O’Farrelly C. Induction of a novel 
   chicken toll-like receptor following Salmonella enterica serovar ty-
9. He H, Crippen TL, Farnell MB, Kogut MH. Identification of CpG 
   oligodeoxynucleotide motifs that stimulate nitric oxide and cyto-
   kine production in avian macrophage and peripheral blood 
10. Leveque G, Forgets V, Morroll S, Smith AL, Bumstead N, Bar-
    row P, Loredo-Osti JC, Morgan K, Malo D. Allelic variation in 
    TLR4 is linked to susceptibility to Salmonella enterica serovar ty-
11. Schwarz H, Harlin O, Ohnemus A, Kaspers B, Staeheli P. Syn-
    thesis of IFN-β by virus-infected chicken embryo cells demonstr-
    ated with specific antiserum and a new bioassay. J. Interferon Cy-
12. Schneider K, Klaus R, Kaspers B, Staeheli P. Chicken interleukin-
    2001;286:4200–4206.
13. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-ex-
    pression transfectants with a novel eukaryotic vector. Gene 1991; 
    Akira S, Lipford G, Wagner H, Bauer S. Species-specific recogni-
    tion of single-stranded RNA via toll-like receptor 7 and 8. Science 
    2004;303:1526–1529.
15. Yoneyama M, Suhara W, Fukuhara Y, Fukuda M, Nishida E, Fu-
    jita T. Direct triggering of the type I interferon system by virus in-
    fection: activation of a transcription factor complex containing IRF-
16. Sick C, Schultz U, Munster U, Meier J, Kaspers B, Staeheli P. Pro-
    moter structures and differential responses to viral and nonviral in-
    ducers of chicken type I interferon genes. J. Biol. Chem. 1998;273: 
    9749–9754.

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