Toll-like Receptors of the Ascidian Ciona intestinalis

PROTOTYPES WITH HYBRID FUNCTIONALITIES OF VERTEBRATE TOLL-LIKE RECEPTORS

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Key transmembrane proteins in the innate immune system, Toll-like receptors (TLRs), have been suggested to occur in the genome of non-mammalian organisms including invertebrates. However, authentic invertebrate TLRs have been neither structurally nor functionally investigated. In this paper, we originally present the structures, localization, ligand recognition, activities, and inflammatory cytokine production of all TLRs of the ascidian Ciona intestinalis, designated as Ci-TLR1 and Ci-TLR2. The amino acid sequence of Ci-TLR1 and Ci-TLR2 were found to possess unique structural organization with moderate sequence similarity to functionally characterized vertebrate TLRs. ci-tlr1 and ci-tlr2 genes were expressed predominantly in the stomach and intestine as well as in hemocytes. Ci-TLR1 and Ci-TLR2 expressed in HEK293 cells, unlike vertebrate TLRs, were localized to both the plasma membrane and endosomes. Intriguingly, both Ci-TLR1 and Ci-TLR2 stimulate NF-κB induction in response to multiple pathogenic ligands such as double-stranded RNA, and bacterial cell wall components that are differentially recognized by respective vertebrate TLRs, revealing that Ci-TLRs recognize broader pathogen-associated molecular patterns than vertebrate TLRs. The Ci-TLR-stimulating pathogenic ligands also induced the expression of Ci-TNFα in the intestine and stomach where Ci-TLRs are expressed. These results provide evidence that the TLR-triggered innate immune systems are essentially conserved in ascidians, and that Ci-TLRs possess “hybrid” biological and immunological functions, compared with vertebrate TLRs. Moreover, it is presumed that chordate TLR ancestors also acquired the Ci-TLR-like multiple cellular localization and pathogen-associated molecular pattern recognition.

Toll-like receptors (TLRs)2 play pivotal roles in mammalian host defenses via the innate immune system. All TLRs are type I transmembrane proteins, containing an intracellular Toll/interleukin-1 receptor (TIR) domain and leucine-rich repeat (LRR) motifs in the extracellular domain (1–3). The diversity in the numbers and organization of LRR domains enables the specific and sensitive recognition of pathogenic molecular species by respective TLRs (1–3). TLRs are expressed mainly in immune cells such as lymphocytes, macrophages, and dendritic cells. TLR1, TLR2, TLR4, TLR5, and TLR6 are responsible for recognition of extracellular microbial pathogenic components on plasma membranes, whereas TLR3, TLR7, TLR8, and TLR9, present on endosomes, participate in defensive responses to viral DNA or RNA incorporated into the cytoplasm (1–5). Stimulation of TLRs initiates signal transduction pathways via adaptor proteins (MyD88, TIRAP, TRIF, and TRAM) followed by activation of a wide range of inducible transcriptional factors such as NF-κB, AP-1 and IRF, leading to production of inflammatory cytokines, chemokines, and type I interferon (1–5).

Recent genomic surveys detected a great variety of TLR-like genes in fish and deuterostome invertebrates: ascidian (2 TLR-related genes), amphioxus (72 TLR-like genes), sea urchin (222 TLR-like genes), and Fugu (23 TLR-like genes) (6–9). Furthermore, primary structures, localization, authentic ligands, and/or the resultant signaling of several fish and cyclostome TLRs have been documented (9, 10). These findings support the view that the TLR family is also involved in the innate immune system of non-mammalian animal species, and diverges in the number, structural organization, and biological roles among animal species. In insects and hydra, pathogenic components for Drosophila Toll and hydra Toll-related receptors were identified (11–13). However, the molecular mechanisms underlying their ligand recognition and signal transduction are distinct from those of vertebrate TLRs, leading to the presumption that they are not regarded as models of the vertebrate-type innate immune system. Moreover, the active protein forms, pathogen-associated molecular patterns (PAMPs), cellular localization of respective deuterostome invertebrate TLRs, or TLR-like receptors and an evolutionary origin of the vertebrate TLR family have never been structurally or functionally elucidated.

The ascidian Ciona intestinalis is regarded as the invertebrate chordate most closely related to vertebrates (14, 15). Moreover, C. intestinalis, unlike traditional protostome model animals including Drosophila melanogaster and Caenorhabditis elegans, has been found to possess the developmental, neuropeptidergic, and endocrine systems prototypic to those of vertebrates (14–17), indicating the possibility that prototypes of vertebrate innate immune systems are also conserved in the protochordate, and that identification of Ciona TLRs provides critical clues to the investigation of the evolutionary origin of TLR pathways.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs S1–S3 and Table S1.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB495261 and AB495262.

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2. The abbreviations used are: TLR, Toll-like receptor; Ci-TLR, Ciona intestinalis TLR; hTLR, human TLR; LRR, leucine-rich repeat; TIR, Toll/interleukin-1 receptor; HEK, human embryonic kidney; PAMP, pathogen-associated molecular pattern; TNFα, tumor necrosis factor α; RT, reverse transcriptase; HKLP, heat-killed L. pneumophila.
In the present study, we identified the structures, tissue distribution, cellular localizations, and ligand-specific activities of all Ciona TLRs, Ci-TLR1 and -2. Our data verified both similar and unique propensities of Ci-TLRs, compared with vertebrate TLRs, suggesting the original features of chordate TLRs. To the best of our knowledge, this is the first report on molecular and functional characterization of TLRs that are localized to the plasma membrane and endosomes and recognize multiple PAMPs.

**EXPERIMENTAL PROCEDURES**

*Animals*—Adults of *C. intestinalis* were cultivated and collected at the Maizuru Fisheries Research Station of Kyoto University, and kept in seawater at 18 °C.

**Cloning of Ci-TLRs**—Total RNA was extracted from *Ciona* hemocytes. The complete sequences of the *ci-tlr* genes were obtained by 5' - and 3' -rapid amplification of the cDNA ends using a 3' - and 5' -rapid amplification of the cDNA ends system for the rapid amplification of cDNA ends kit (Invitrogen). The products were sequenced with primers used for RT-PCR and universal primers (SP6 and T7 primers).

**Molecular Phylogenetic Tree of the TLR Family**—The amino acid sequences were aligned using the CLUSTAL program. The alignment was checked by hand. After removing gaps, the verified alignments were used to construct phylogenetic trees. The trees were calculated using the MEGA program based on the neighbor-joining method. The sequences used were as follows: chicken TLR1, BAD67422; chicken TLR2, NP_989609; chicken TLR3, XP_420675; chicken TLR4, XP_415518; chicken TLR5, ABW07794; chicken TLR7, XP_416836; chicken TLR15, XP_419294; chicken TLR21, XP_413989; human TLR1, NP_003254; human TLR2, NP_003255; human TLR3, NP_003256; human TLR4, NP_612564; human TLR5, NP_003259; human TLR6, NP_006059; human TLR7, XP_057646; human TLR8, NP_619542; human TLR9, NP_059138; human TLR10, NP_112218; Amphioxus TLR1, ABD58972; Fugu TLR1, AAW69370; Fugu TLR2, AAW69373; Fugu TLR5, AAW69374; Fugu TLR7, AAW69375; Fugu TLR8, AAW69376; Fugu TLR9, AAW69377; Fugu TLR14, AAW69369; Fugu TLR21, BAC66138; Fugu TLR22, AAW69372; Fugu TLR23, AAW70378; zebrafish TLR1, NP_001124066; zebrafish TLR2, NP_997977; zebrafish TLR3, NP_001013287; zebrafish TLR7, XP_701101; zebrafish TLR9, NP_001124066; zebrafish TLR18, NP_001082819; zebrafish TLR19, XP_690254; zebrafish similar TLR21, AA163075; and zebrafish similar TLR22, NP_001122147. The amino acid sequence of *Xenopus* homologs was obtained as previously described (18).

**Whole Mount in Situ Hybridization of Ci-TLR mRNAs**—The Ci-TLR1 cDNA fragment (nucleotides 1614–2356) and the Ci-TLR2 cDNA fragment (nucleotides 1115–1654) were inserted into the pCRII TOPO vector according to the manufacturer's instructions. Whole mount *in situ* hybridization was performed as previously reported (19, 20).

**RT-PCR**—Total RNA (1 μg) was reverse transcribed using oligo(dT)20 primer, and PCR for Ci-TLR1, Ci-TLR2, and Ci-β-actin (CLSTR00046) was performed using EX Taq polymerase (TaKaRa, Ohtsu, Japan). The PCR program was 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min. All primers for PCR are summarized under supplemental Table S1. PCR products were electrophoresed with 1.5% agarose gel.

**Cell Culture and Transfection**—HEK293 MSR cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% carbon dioxide. The cells were transfected using F1-reagent (Targeting System, El Cajon, CA) with the pcDNA6/myc-His vector A containing no insert, ci-tlr1, or ci-tlr2 (Invitrogen). The stable transfectants were selected in medium containing 20 μg/ml blasticidin. Each hTLR-stable HEK293 transfectant was purchased from InvivoGen (San Diego, CA).

**Ligands**—All TLR ligands (1, 21–35), summarized in Table 1, were purchased from InvivoGen or Sigma.

**NF-κB Reporter Assay**—Stable transfectants with empty vector, ci-tlr1 or ci-tlr2 were transiently transfected using F1-reagent with the NF-κB-responsiveness of each TLR ligand according to the manufacturer’s instructions. Alexa 488- and Alexa 594-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) were used for visualization. All immunoreactivities were detected using a confocal microscopy FLUOVIEW FV1000 (Olympus, Tokyo, Japan).

**Quantification of Ci-TNFα in Ciona Tissues**—Adult tissue was dissociated into cells, treated with each ligand, and incubated for 24 h at 25 °C. Real-time PCR for Ci-TNFα using the SYBR Green master mixture (Applied Biosystem, Foster City, CA) was performed three times. As an internal control, Ci-β-actin was employed. All primers for real time PCR are shown under supplemental Table S1. To calculate relative quantification of gene expression, we used the comparative C_T method.

**Statistical Analysis**—Data are expressed as mean ± S.D. Differences in the mean values assessed for significance by one-way analysis of variance, Scheffe’s F-test (see Fig. 6), and Fisher’s PLSD (see Fig. 7). p values (p < 0.05) were considered significant.

**RESULTS**

**Identification of ci-tlr Genes**—We initially performed BLAST searches on the genome data base of *C. intestinalis* with each typical domain of various vertebrate TLRs as a query, and detected only two independent clones, gene numbers ci0100137574 and cibd077g18. This is compatible with a previous *Ciona* genome survey (6), and suggested that only two TLR-like genes are present in *C. intestinalis*. The two full-length cDNA sequences were cloned from *Ciona* hemocytes, and the open reading frames of ci0100137574 and cibd077g18 were found to encode 883 and 948 amino acids, respectively. SMART protein domain analyses revealed that both of the deduced proteins harbor an intracellular TIR domain, a transmembrane domain, and multiple extracellular LRRs, which are remnis-
cent of vertebrate TLRs (Fig. 1). We thus designated the two genes as ci-tlr1 and ci-tlr2, respectively. Ci-TLR1 and Ci-TLR2 bear 7 and 13 putative LRRs, respectively (Fig. 1). Moreover, Ci-TLR1 contains one LRR C-terminal domain, which is flanked by a cysteine-rich domain, although three LRR C-terminal domains are present in Ci-TLR2 (Fig. 1). Sequence homology of Ci-TLRs to vertebrate counterparts is confounding, compared with vertebrate TLRs (Fig. 1). The overall amino acid sequences of Ci-TLR1 and -2 were most homologous to human TLR (hTLR)7 (26%) and hTLR8 (26%), respectively. The TIR domains of Ci-TLR1 and Ci-TLR2 were most similar to hTLR4 and hTLR6, respectively. The LRRs at 1–330 in Ci-TLR1 are most similar to hTLR5, whereas the LRR at amino acids 331–681 displays the highest similarity to hTLR9 in the corresponding region. In contrast, each portion of the LRR in Ci-TLR2 was most similar to hTLR8. The phylogenetic tree of TLRs and their putative orthologs (Fig. 2) demonstrate that Ci-TLRs undoubtedly belong to the vertebrate TLR family, and suggest the pivotal positions of Ci-TLRs as prototypes of vertebrate TLRs.

Expression of Ci-TLRs—Whole mount in situ hybridization demonstrated that ci-tlr1 and ci-tlr2 genes were expressed intensively in the stomach, intestine (Fig. 3, A and C), and numerous, but not all, hemocytes (Fig. 3, B and D) in Ciona juveniles. RT-PCR also showed that both Ci-TLRs were detected in various adult tissues with prominent expression in the stomach, and/or anterior and middle intestines (Fig. 4), whereas Ci-TLR2 expression was slightly less intensive than that of Ci-TLR1 (Fig. 4).

Cellular Localization of Ci-TLRs—To verify the intracellular localization of Ci-TLRs, we performed double immunofluorescent confocal microscopy observations of localization of the myc-tagged ci-tlr1 or ci-tlr2 stably expressed in HEK293 MSR cells (Fig. 5, A and D). Moreover, syntaxin-7, which was shown
FIGURE 2. A phylogenetic tree of the TLR family constructed by the neighbor-joining method. The number beside each branch indicates the percentage of times that a node was supported in 1000 bootstrap pseudoreplications. These bootstrap values that support >85% are represented. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per protein.
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FIGURE 3. Whole mount in situ hybridization of the ci-tlr mRNAs. Prominent expression of the ci-tlr1 (A and B) and ci-tlr2 (C and D) in the alimentary tracts and hemocytes in the Ciona juveniles is shown. Scale bars, 200 μm.

FIGURE 4. RT-PCR detection of the ci-tlr mRNA. Expression of the ci-tlr1 and -2 were examined in the anterior intestine (lane 1), middle intestine (lane 2), posterior intestine (lane 3), ovary (lane 4), endostyle (lane 5), neural complex (lane 6), and stomach (lane 7) by RT-PCR. ci-β-actin was amplified as an internal control.

FIGURE 5. Localization of Ci-TLRs in 293 MSR cells. Localization of Ci-TLR1 (A) and Ci-TLR2 (D) were observed by immunostaining using anti-myc antibody followed by Alexa 488-conjugated secondary antibody (green). Late endosomes (red) were visualized with anti-syntxin 7 antibody followed by Alexa 594-conjugated secondary antibody (B and E). The merged images clarify that Ci-TLR1 (C) and 2 (F) are localized to endosomes as well as plasma membrane. Scale bar, 10 μm.

TABLE 1
Applied ligands for Ci-TLRs

| Ligand                                | hTLR | Ref. |
|---------------------------------------|------|------|
| Pam3CSK4 (a synthetic triacylated lipopeptide) | TLR1/2 | 1 |
| Heat-killed Listeria monocytogenes (a Gram-positive bacterium) | TLR2 | 21 |
| Zymosan (S. cerevisiae cell wall)*    | TLR2 | 22 |
| 1,3-β-Glucan (laminarin)             | TLR2 | 23 |
| Heat-killed L. pneumophila (Gram-negative bacterium)* | TLR2 | 24 |
| Heat-killed Staphylococcus aureus (HKSA, Gram-positive bacterium) | TLR2 | 25 |
| Poly(I-C) (double-stranded RNA)*      | TLR2 | 26 |
| LPS (lipopolysaccharide from Escherichia coli K12, the major component of outer wall) | TLR4 | 27 |
| Lipid A (a synthetic diphosphoryl lipid) | TLR4 | 28 |
| Flagellin S. typhimurium (the major component of the bacterial flagellar filament)* | TLR5 | 29 |
| FSL1 (a synthetic lipoprotein derived from Mycoplasma salivarium) | TLR6 | 30 |
| Imiquimod (an imidazoquinolone amino acid analogue to guanosine) | TLR7 | 31 |
| Single-strand RNA40/LyoVec (GC-rich single strand RNA) | TLR8 | 32 |
| ODN2006 (a synthetic oligonucleotide containing unmethylated CpG dinucleotide) ssDNA (endotoxin-free preparation of bacteria single strand DNA) | TLR9 | 33 |
| E. coli DNA (bacterial DNA containing unmethylated CpG motif) | TLR9 | 35 |

* Ligands used in Fig. 6.

to be co-localized with human TLR3 (36), was stained as a marker for late endosomes (Fig. 5, B and E). Intriguingly, Ci-TLR1 and Ci-TLR2, unlike any mammalian TLRs, were found to be present on both the plasma membrane and a number of late endosomes (Fig. 5, C and F), whereas more intense expression of Ci-TLR2 was detected in endosomes than in the plasma membrane (Fig. 5, C and F).

Elucidation of Pathogenic Ligands of Ci-TLRs—All mammalian TLRs eventually transactivate a transcriptional factor, NF-κB, in response to their specific ligands (1–3). Moreover, the homologous ascidian genes encoding the innate immunity relevant adapter proteins, except MD-2 and transcriptional factors including NF-κB, were detected in the genome (6). Therefore, we employed a NF-κB-responsive secreted alkaline phosphatase reporter assay to elucidate PAMPs of Ci-TLRs. HEK293 MSR cells were stably transfected with an empty, ci-tlr1, or ci-tlr2-inserted pcDNA6.1 vector, and treated with 16 known TLR ligands (Table 1) at concentrations that were found to exhibit significant activation of the respective hTLRs (21–35). Unexpectedly, Ci-TLR1 and Ci-TLR2 activated NF-κB in response to multiple TLR ligands (Fig. 6), which are recognized by different mammalian TLRs. Zymosan (Saccharomyces cerevisiae cell wall) for hTLR2, heat-killed Legionella pneumophila (HKLP, a Gram-negative bacterium) for hTLR2, double-stranded RNA, poly(I-C) for hTLR3, S. typhimurium flagellin (the major component of the bacterial flagellar filament) for hTLR5 elicited a dose-dependent induction of NF-κB in the ci-tlr1- or ci-tlr2-expressing cells (Fig. 6). Additionally, the potencies of these four ligands at Ci-TLRs were comparable with those at hTLR2, -3, and -5 (results not shown). In contrast, no significant responses to these ligands were detected in empty vector-transfected cells (supplemental Fig. S1), and other ligands failed to induce NF-κB transactivation in ci-tlr1- or ci-tlr2-transfected cells (supplemental Figs. S2 and S3). Notably, both Ci-TLRs showed equipotent NF-κB activation in response to the same ligands (Fig. 6). These data clarified PAMPs of Ci-TLRs, and indicated that Ci-TLRs have more extensive PAMPs than vertebrate TLRs. In addition, these functional analyses proved that
Ci-TLRs, like vertebrate TLRs (1–3), directly recognize various pathogenic compounds and trigger signal transduction, which is in contrast with functionally characterized Drosophila Toll and hydra Toll-related receptors that require the specific associated proteins for ligand recognition and/or initiation of signal transduction cascades (11–13). These findings confirmed that Ci-TLRs share the essential ligand recognition and signaling mechanisms with the vertebrate TLR family.

**Induction of TNFα by Ci-TLR Ligands in Ciona Tissues**—In the mammalian TLR signaling pathway, activation of NF-κB leads to an increase in gene expression of an inflammatory cytokine, TNFα. We examined whether gene expression of Ci-TNFα (37) was up-regulated by the Ci-TLR ligands (Fig. 6) in the intestine or stomach, where ci-tlr1 and ci-tlr2 are abundantly expressed (Figs. 3 and 4). Real-time PCR revealed that poly(I-C) induced 4- and 10-fold Ci-TNFα expression in the anterior (Fig. 7A) and middle (Fig. 7B) intestine, respectively, compared with controls. Flagellin caused 6-fold and more than 10-fold elevation of Ci-TNFα in the stomach (Fig. 7D) and middle intestine (Fig. 7E), respectively. In contrast, neither poly(I-C) nor flagellin induced any significant Ci-TNFα expression in the Ciona posterior intestine (Fig. 7, C and F) where expression of ci-tlrs was not detected (Figs. 3 and 4). These results suggest that Ci-TLRs respond to the pathogenic ligands in Ciona tissues.

**DISCUSSION**

The innate immune system is expected to play pivotal roles in defensive responses to pathogens in invertebrates, where adaptive immune systems are absent. In vertebrates, the innate immunity is functionally linked to the adaptive immunity (1, 2). However, high molecular divergence in the numbers and organizations of the LRRs among fish and invertebrate TLR (or TLR-like) genes hinders sequence homology-based elucidation or prediction of their crucial functions: PAMPs, cellular localization, and signaling pathways (6–9). To address these issues, we explored all TLRs of the ascidian C. intestinalis the closest group of vertebrates. The striking feature is that Ci-TLRs show unprecedented cellular localization and PAMPs. Ci-TLRs are localized to both the plasma membranes and late endosomes (Fig. 5), whereas vertebrate TLRs have so far been shown to be present exclusively either on the plasma membrane (TLR1, -2, -4, -5, and -6) or on endosomes (TLR3, -7, -8, and -9). Trafficking and localization of hTLR3 and -7 to endosomes are likely to be regulated by several regions between the transmembrane and TIR domains (4, 5), although the underlying molecular mechanism has yet to be elucidated. Because Ci-TLRs share no significant sequence homology with hTLRs in the corresponding region,
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the molecular mechanism underlying the multiple localization of Ci-TLR awaits further study. Also of particular biochemical and immunological significance is the multiple PAMP recognition by Ci-TLRs. Ci-TLR1 and -2 are responsive to identical multiple PAMPs (Fig. 6), which are differentially recognized by respective vertebrate TLRs: poly(I-C) for hTLR3, flagellin for hTLR5, zymosan for hTLR2, and HKLP for hTLR2 (1–3, 9, 21, 22, 24, 26, 29). Such PAMPs of Ci-TLRs were not predicted solely on the basis of sequence homology and molecular phylogenetic analyses (6–8) (Fig. 2) due to no consistency of PAMP specificity with their sequence homology to hTLRs (Figs. 1 and 22, 24, 26, 29). Moreover, up-regulation of TNFα was demonstrated in the Ciona stomach and anterior and middle intestine in response to the ligands of Ci-TLRs (Fig. 7). Unfortunately, neither gene silencing nor gene knock-out techniques for adult ascidians including C. intestinalis have ever been established, and we cannot absolutely exclude the possibility that other receptors than Ci-TLRs are also involved in the induction of Ci-TNFα in these tissues. Instead, we also showed that no up-regulation of TNFα was detected in the posterior intestine where ci-tlrs were not expressed (Figs. 3, 4, and 7).

In conclusion, we have identified novel TLRs, Ci-TLRs, in the ascidian C. intestinalis. The unique hybrid cellular localization and PAMP recognition of Ci-TLRs pave the way to investigate an evolutionary origin of vertebrate TLRs and species-specific molecular divergences of TLRs that are correlated with lifespans and environmental pathogens specific to each organism. Our study also sheds new light on TLRs of lower animals. For instance, unique and diverse PAMP recognition modes of TLRs in each species are expected to provide novel targets for highly specific agonists and antagonists of TLRs in a certain animal species, which will lead to the unprecedented protection and/or expansion of endangered species or elimination of invading organisms with high efficiency and species specificity. Such studies are underway.

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