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Lamprey TLRs with Properties Distinct from Those of the Variable Lymphocyte Receptors

Akihiro Ishii,* Aya Matsuo,* Hirofumi Sawa,† Tadayuki Tsujita,‡ Kyoko Shida,* Misako Matsumoto,‡* and Tsukasa Seya*‡

Fish express mammalian-type (M-type) TLRs consisting of leucine-rich repeats (LRRs) and Toll-IL-1R (TIR) homology domain for immunity, whereas invertebrates in deuterostomes appear to have no orthologs of M-type TLRs. _Lampetra japonica_ (lamprey) belongs to the lowest class of vertebrates with little information about its TLRs. We have identified two cDNA sequences of putative TLRs in the lamprey (laTLRs) that contain LRRs and TIR domains. The two laTLRs were 56% homologous to each other, and their TIRs were similar to those of members of the human TLR2 subfamily, most likely orthologs of fish TLR14. We named them laTLR14a and laTLR14b. We raised a rabbit polyclonal Ab against laTLR14b and identified a 85-kDa protein in a human HEK293 transfectant by immunoblotting using the Ab. FACS, histochemical, and confocal analyses showed that laTLR14b is expressed intracellularly in lamprey gill cells and that the overexpressed protein resides in the endoplasmic reticulum of human and fish (medaka) cell lines. Because natural agonists of TLR14 remained unidentified, we made a chimera construct of extracellular CD4 and the cytoplasmic domain of laTLR14. The chimera molecule of laTLR14b, when expressed in HEK293 cells, elicited activation of NF-κB and, consequently, weak activation of the IFN-β promoter. LaTLR14b mRNA was observed in various organs and leukocytes. This lamprey species expressed a variable lymphocyte receptor structurally independent of laTLR14 in leukocytes. Thus, the jawless vertebrate lamprey possesses two LRR-based recognition systems, the variable lymphocyte receptor and TLR, and the M-type TLRs are conserved across humans, fish, and lampreys. The Journal of Immunology, 2007, 178: 397–406.

The first line of host defense against pathogen invasion is assigned by the innate immune system. This system involves a number of microbe pattern recognition receptors such as complement receptors, lectins, and TLRs. TLRs have recently been identified as the main receptors for the recognition of microbe-specific pattern molecules (1). TLRs structurally consist of extracytoplasmic leucine-rich repeats (LRRs) and an intracytoplasmic Toll-IL-1R (TIR) homology domain (2–4), similar to _Drosophila_ Toll (5). The LRRs sense microbes and the TIR transmits a signal for infectious response. The results of vertebrate and invertebrate genome projects have suggested that the TLR pattern recognition system is conserved across mammalian groups and probably most vertebrate species (6). In contrast, some invertebrates, based on their genomes, have only a few TLR-like proteins, whereas others have >100 members of the TLR family that are structurally unlinked to those of mammals (7). Toll homologues in insects and worms are often related to developmental functions rather than immunity (7, 8). In deuterostomes, based on their genome information invertebrates harbor TLR-like proteins with no homology to human TLRs (huTLRs), whereas gnathostomes, including fishes, amphibians, reptiles, birds, and mammals, have homologs of huTLRs (6), which we designated the mammalian-type (M-type) TLR system (9). It is becoming clear that M-type TLR family members are mostly related to microbial pattern recognition for immunity (10). Thus, species-specific development of TLR expansion appears to have independently occurred in each division of insects, deuterostome invertebrates, and jawed vertebrates, most of which happen to have a TLR repertoire consisting of multiple members of Toll or TLRs with differential functions (7, 8). It remains unclear whether jawless vertebrates possess the TLR system comparable to that in humans.

Jawed vertebrates generate a lymphocyte receptor repertoire of sufficient diversity to discriminate the antigenic component of any pathogen (11–13). In jawed vertebrates including mammals, lymphocytes recognize peptides mounted on APCs to induce clonal lymphocyte proliferation and activate effector cells, leading to elimination of the pathogen. The initiation of these adaptive immune responses is triggered by the preceding activation of TLR signaling in the APCs. The Ig domains are used for the cellular interactions in the acquired immune system in jawed vertebrates. In contrast, the surviving jawless fish (agnathans), lamprey and hagfish, have diverse lymphocyte Ag receptor genes encoding LRRs (12–14). These cell surface receptors are designated variable lymphocyte receptors (VLRs) (12). VLRs are GPI-anchored proteins retained on the cell surface.

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4 Abbreviations used in this paper: LRR, leucine-rich repeat; ER, endoplasmic reticulum; fgTLR, fugu TLR; huTLR, human TLR; laTLR, lamprey TLR; M-type, mammalian-type; poly(I:C), polyinosinic-polycytidylic acid; TIR, Toll-IL-1R (homology domain); TICAM, TIR domain-containing adaptor molecule; VLR, variable lymphocyte receptor.

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membranes or released in the fluid phase like Abs (7). Although the Ag-presenting mechanism, including the machinery for Ag presentation, has not been elucidated in agnaths, recombinatorial mechanisms for the generation of anticipatory receptors thus evolved in both jawless and jawed vertebrates (7). However, the role of innate immunity in the triggering of the VLR response has not been elucidated.

In humans and mice, the TLR system with a multigene family has been characterized as a functional entity specifically recognizing microbial pattern molecules independently of the acquired system (15, 16). To investigate whether jawless fish possesses the TLR family with a functional profile similar to that in humans, we used Lampetra japonica (lamprey) to analyze the TLR system. We searched lamprey tissues and cDNA libraries for fish TLR orthologs and found that the lamprey harbors two isoforms of the TLR2 subfamily. One of them resides inside lamprey cells and serves to deliver signals to activate NF-κB and, to a lesser extent, type I IFN in human cells. These results together with their subcellular localization profiles in human and fish cells suggest that the lamprey expresses immune-related TLR orthologs independently of the VLR system.

Materials and Methods

Lampreys

Live lampreys were purchased from Ebetsu Gyokyo. Sectioned tissues of the lamprey were stored at −80°C until use. Whole blood of the lamprey was drained from the dorsal aorta using a syringe, immediately added to an equal volume of Percoll (Amersham Biosciences), and then centrifuged at 2000 rpm for 10 min at 4°C, and the buffy coat was harvested to prepare leukocytes as previously described (17).

Because the chromosomes in jawless fish cells are unstable (18), no cell line is yet available. In addition, jawless fish genome information is difficult to obtain. For these reasons, we used human and fish (medaka, Oryzias latipes) cells instead of lamprey cells for the analysis of lamprey proteins. Although the data have been only partially published, the signal pathways of fish TLRs can be principally reconciled in human cells (19).

Cloning of lamprey TLRs (laTLRs) and construction of their constitutive active forms

laTLRs and their construction of their constitutive active forms

laTLRs were amplified by PCR with degenerated primers from a cdna library of lamprey embryo mRNA as previously described (20). The primers were designed on the basis of the conserved TIR domain of 200-bp regions of fugu TLR (fgTLR) 1, huTLR1, huTLR6, and huTLR10. Three and 5'-RACE followed by consecutive PCR allowed us to identify two distinct sequences of laTLR-like proteins. The expected sizes of the two cdna fragments were obtained by the PCR (data not shown). Full-length cdnas were extended by consecutive PCR and then the two distinct cdnas of TLRs were cloned into pEFBOS vector (named laTLR1a and laTLR1b; GenBank accession nos. AB109402 and AB109403, respectively). A FLAG tag was attached to the C terminus of the protein in this vector. N-terminal FLAG-tagged laTLR14b was constructed in a pCMV-FLAG vector (Sigma-Aldrich) as described previously (21).

To analyze the signaling pathway of the TLRs, TIR domains of laTLR14 (TIR1a and TIR1b) were fused with the human CD4 extracellular domain and cloned into the pEFBOS vector as constitutive active forms (1, 22). Each DNA fragment was amplified by PCR, and the obtained products were digested by XhoI and BgII (CD4), BgII and NotI (TIR1a), and BamHI and NotI (TIR1b). The digested DNA fragments of the TIR and the CD4 fragment were cloned into the pEFBOS vector. Sequences of the primers used are shown in Table I.

Abs, cells, and proteins

Anti-human CD4 Ab was purchased from BD Biosciences. Anti-FLAG mAb was purchased from Sigma-Aldrich.

Human HEK293 HeLa cells were obtained from the American Type Culture Collection. RK13 cells (derived from rabbit kidney) were obtained from the RIKEN Cell Bank. HEK293 and RK13 cells were cultured in DMEM containing 10% heat-inactivated FCS. The HeLa cells were cultured in MEM with 2 mM l-glutamine and 10% FCS. These cells were transfected with expression vectors. The medaka fish cell line OL-17 (23) was a gift from Dr. H. Mitani (Tokyo University, Tokyo, Japan). Cells were cultured in L15 medium containing 10 mM HEPES buffer (pH 7.5) with 20% FCS at 33°C.

Rabbit anti-laTLR14b polyclonal Ab was produced by the method established in our laboratory (24). Briefly, RK13 cells (1 × 10⁵) were transiently transfected with a pFLAG CMV-His × 6 with full-length laTLR14b construct using LipofectAMINE PLUS reagent (Invitrogen Life Technologies). After 48 h, transfected RK13 cells were collected in 10 mM EDTA-PBS, washed three times with PBS, and suspended in 0.5 ml of PBS. The RK13 cell suspensions were then mixed and emulsified with 0.6 ml of Freund’s complete adjuvant (Difco) and used for the immunization of rabbits. Immunization was performed four times at 7-day intervals, and the rabbits were boosted 3 days before drawing blood. Titers of the anti-laTLR14b Ab were roughly estimated by immunoblotting with lines of cell lysate containing laTLR14b (data not shown). IgG was purified by precipitation with 33% ammonium sulfate, dialyzed against PBS, and stored at −80°C until use.

Reporter assay

HEK293 cells (1 × 10⁶ cells/well) were transiently transfected in 6-well plates by using LipofectAMINE 2000 reagent (Invitrogen Life Technologies) with reporter plasmid p-125 luc (IFN-β promoter) or luciferase-linked E-selectin (ELAM) promoter (NF-κB, 1 μg) and an internal control vector (pβRL-TK, 0.2 ng; Promega). The total amount of DNA used for transfection was kept constant at 4 μg by adding an empty vector. After 24 h, cells were harvested and added to 96-well plates (5 × 10⁴ cells/well) and stimulated with PBS, 10 μg/ml Staphylococcus aureus peptidoglycan (Furuka-Chemie), 100 nM macrophage-activating lipopeptide-2 (Biologica), 100 ng/ml Pam3 (BioLinks), 100 ng/ml LPS (Difco), 10 μg/ml polyinosinic-polycytidylic acid (polyI:C); Amersham Biosciences), 1 μg/ml Vibrio anguillarum flagellin (19), and 1/100 volume of sonicated Closstridium sporogenes and Salmonella typhimurium cell lysates (25). After 6 h, cells were lysed in lysis buffer (Promega) and assayed for firefly and Renilla luciferase activities. Firefly luciferase activity was normalized with that of Renilla and expressed as fold stimulation relative to the activity of vector-transfected cells or PBS-stimulated cells. In some experiments, HEK293 cells (2 × 10⁵ cells/well) in 24-well plates were transfected with pEFBOS/human CD4-laTLR14b plasmid (0.2 μg), the reporter plasmid (0.2 μg) internal control vector (0.1 ng), and the indicated expression vectors with dominant negative adapters (0.4 μg). After 12 h, cells were lysed and assayed for reporter activity. The SD was calculated from three separate experiments, each performed in triplicate.

RNA preparation and RT-PCR analysis

Individual female lampreys were dissected and the skin, gills, heart, liver, gut (stomach and intestine), kidneys, muscle, eyes, and eggs were harvested. A section above the vertebral column (called marrow) was aspirated into a syringe. One hundred milligrams of each tissue was homogenized in 1 ml of TRizol reagent (Invitrogen Life Technologies) using a Dounce-type glass homogenizer and then total RNA was extracted by the TRizol RNA preparation method. Five hundred nanograms of total RNA was treated with RQ1 RNase-free DNase and reverse transcribed with Moloney murine leukemia virus reverse transcriptase and random primers. For amplification of laTLR14 fragments, PCR was performed by denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and extension at 72°C for 30 s. L. japonica VLR DNA fragments were also amplified similarly for 30 cycles. Ex-Taq polymerase (Takara) and the indicated primers were used for both reactions (Table I).

Gene analysis

Assembling and editing of the determined DNA sequences were performed with ATGC and GENETYX-MAC version 12.1 software (Software Development). The sequences of the predicted open reading frames and TIR domains were compared with other sequences in a homology search by the BLAST program (www.ncbi.nlm.nih.gov/BLAST). TLR14 members of Xenopus tropicalis (GenBank accession nos. AC156431 and AAW96369), Xanipo ThereforeRNA and Xenopus tropicalis (Department of Energy Joint Genome Institute identifiers 190020, 30694, 421728, and 421736) were identified in the BLAST database. Alignment of amino acid sequences and unrooted phylogenetic analysis of TLRs were performed using the ClustaW program (www.ddbj.nig.ac.jp/ search/blastw/blastw.html). Functional domains of the proteins were predicted by the SMART program (http://smart.embl-heidelberg.de/).

Flow cytometry and immunoblotting

Transfected HEK293 cells were analyzed for protein expression by flow cytometry and immunoblotting. For flow cytometric analysis, cells were

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stained with anti-TLR14b Ab (250-fold diluted in PBS with 0.2% BSA) or anti-FLAG Ab (5 μg/ml), washed in PBS containing 0.2% BSA and 0.1% sodium azide, and then incubated with FITC-conjugated goat anti-mouse IgG (American Qualex). Cells were washed and fluorescence intensity was measured (FACS Calibur; BD Biosciences). For immunoblotting, various lamprey tissues were solubilized in lysis buffer (1% (v/v) Nonidet P-40, 0.14 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl (pH 7.4), 1 mg/ml iodoacetamide, and 1 mM PMSF) using a Dounce-type homogenizer. After incubation at 4°C for 30 min, each lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatants were collected and protein concentration was measured using a protein estimation kit (Bio-Rad). Equal amounts of total cellular protein from each lysate were resolved by SDS-PAGE (7.5% gel) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with either anti-laTLR14b Ab (1,000-fold diluted in PBS containing 0.02% Nonidet P-40) or anti-FLAG Ab (5 μg/ml), washed in PBS containing 0.02% Nonidet P-40, and tagged by HRP-linked goat anti-rabbit second Ab (BioSource International). Proteins reacting to the Ab were visualized by ECL detection system (Amersham Bioscience).

**Confocal microscope analysis**

HeLa cells or OL-17 cells of the Hd-rR strain of medaka (23) (1.5 × 10^5 cells/well) were plated onto coverslips in a 24-well plate. Cells were then transiently transfected with vectors expressing FLAG-tagged proteins. At timed intervals, the adherent cells were fixed for 30 min with 3% formaldehyde in PBS and permeabilized with 0.5% saponin in 1% BSA and PBS for 30 min and then washed four times with PBS. After the cells had been soaked in 1% BSA and PBS, they were treated for 1 h at room temperature with 5 μg/ml mouse anti-FLAG Ab (Sigma-Aldrich). The cells were then washed with 1% BSA and PBS and treated for 30 min at room temperature with Alexa 488-conjugated goat anti-mouse IgG (MOLECULAR PROBES) (1/400) in PBS. To see subcellular localization of the FLAG-tagged laTLRs, cells were treated with anti-FLAG Ab and Alexa 488 as described above and simultaneously stained with organella marker Abs; that is, rabbit anti-calnexin Ab (StressGen Biotechnologies) as an endoplasmic reticulum (ER) marker (1/200) or rabbit anti-EEA1 Ab (Affinity BioReagents) as an early endosome marker (4 μg/ml) and then Alexa 568-labeled goat anti-rabbit IgG for staining. The Golgi apparatus was stained with Texas Red-conjugated wheat Germ agglutinin (1/100; Molecular Probes) and F-actin was stained with Phalloidin (1/500; Sigma-Aldrich). For acidic organelle staining, cells were pretreated with LysoTracker (final concentration of 1 mM) for 1 h before fixation. The stained cells were visualized at ×60 magnification under a FLUOVIEW microscope (Olympus). Images were captured using the attached computer software FLUOVIEW (26).

**Immunohistochemical analysis**

Formalin-fixed and paraffin-embedded sections were deparaffinized with xylene and rehydrated through a graded ethanol series. For Ag retrieval, sections were immersed in citrate buffer (pH 6.0) and heated using a pressure cooker. Thereafter, sections were treated with normal serum to eliminate nonspecific binding of Abs and incubated with 0.3% H2O2 methanol to quench endogenous peroxidase activity. After treatment, sections were incubated with rabbit anti-laTLR14b Ab (1/1000) at 4°C overnight. After incubation with EnVision goat anti-rabbit HRP-labeled second Ab (Dako-Cytomation), immunoreaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride (27, 28).

**Table I. Primers used in this study**

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AFW, Forward; RVS, reverse.

Many trials for the molecular cloning of lamprey TLRs were made with cDNAs prepared from an embryo of *L. japonica*. PCR amplification was successful using the primer set TLR16101 and TLR101R1 (Table I), which yielded a 210-bp cDNA fragment. A set of degenerate PCR primers was designed from conserved sequences (Fig. 1A; open rectangle) based on a homology search for TLR2 of various species (9, 29–31).

The cDNA fragment we cloned contained two distinct sequences encoding putative LRRs similar to those in huTLR2. By using the RACE method, the two sequences were extended over the pmE185-containing lamprey cDNA libraries (20). Finally, we identified two nucleotide sequences with putative open reading frames of LRRs and the TIR. We have prepared mRNAs from individual lampreys coming up the Ishikari river every year. The presence of this message was confirmed with adult lamprey gill mRNAs by sequencing 12 independent RT-PCR amplicons.

Deduced amino acid sequences from the cDNAs were analyzed by SMART programs, and the amino acid sequences are shown in Fig. 1A. The presence of the two LRR motifs and one TIR domain were predicted to exist. A hydrophobicity plot suggested that laTLR14a and 14b proteins are type I membrane proteins with signal peptides. These structures are shown in the Fig. 1B. These findings suggest that *L. japonica* has proteins of a TLR-like structure.

The two cloned laTLRs were highly homologous to each other (56% amino acid identities) (Fig. 1C). Recent BLASTP homology search analysis revealed that the laTLRs are most similar to the *T. rubripes* (pufferfish) TLR14 (fgTLR14) in the BLASTP database (Fig. 1C and Table II). We named these novel LRR-containing lamprey proteins laTLR14a and laTLR14b. The laTLR14a cDNA consisted of 2,486 bp, including an incomplete polyadenylation signal and poly(A) tail. This encoded a predicted protein of 815 amino acids whereas the laTLR14b cDNA consisted of 2,510 bp,
FIGURE 1. Amino acid alignment of cloned lamprey TLRs. A, Predicted amino acid sequences deduced from lalTLR14a and lalTLR14b cDNAs are aligned. Identical residues are indicated by asterisks, and each functional domain identified by the SMART program is shown by arrows (LRR-NT and LRR), gray box (TM), dotted arrow (C-terminal flanking region), dotted line (signal peptides), and solid line (TIR).

B, Models of the predicted domain structures of lalTLR14a and lalTLR14b. The LRR-CT domain of lalTLR14a was not identified by SMART analysis.

C, Percentage homology between fugu TLR14 (fgTLR14) and lalTLR14a/b. The LRR and TIR regions are separately compared in the two bottom tables.
encoding a predicted protein of 822 amino acids (Fig. 1A). laTLR14a consisted of one unique N-terminal LRR-like motif (named LRR-NT), seven typical LRR motifs, one unique C-terminal LRR motif (named LRR-CT), followed by a transmembrane domain and a TIR domain (Fig. 1C). TLR14b structurally resembled TLR14a except for the lack of LRR-NT sequence. The TIRs of laTLR14a/b are most similar to that of fgTLR14 (>50% of amino acid identities). However, laTLR14a and laTLR14b exhibited only 29–34% identity to fgTLR14 in overall similarity (data not shown). The LRR domains of laTLR14 were <30% homologous to those of the huTLR2 subfamily, huTLR1, 2, 6, and 10, although their TIR domains were >50% homologous (Table II). Bootstrap probability analysis suggested that TLR14 evolved from M-type TLR2 before the lamprey species diverged from fish (Fig. 2). Hence, we suggest that the agnathan lamprey has structural orthologs of the gnathostome TLR system.

Protein expression of laTLRs

Protein expression and subcellular localization of laTLRs were analyzed in human HEK293 cells. We used pEFBOS-FLAG vector, which has the elongation factor promoter. Proteins of laTLR14a and laTLR14b with FLAG (either C-terminal or N-terminal) were detected by immunoblotting, flow cytometry, and immunofluorescence staining. laTLR14b (C-terminal FLAG) gave a thick protein band of 85 kDa and a thin band of 105 kDa by immunoblotting using an anti-FLAG Ab (Fig. 3A). This two-band profile was reproducible on the blot by using an Ab against laTLR14b (Fig. 3A). Only the 85-kDa band was detected in the lane with laTLR14a (C-terminal) in the blot using anti-FLAG Ab (Fig. 3A). The laTLR14b protein could be detected with the anti-laTLR14b Ab as well as anti-FLAG Ab (Fig. 3A). The molecular mass of 85 kDa is consistent with those expected from the two cDNA sequences of laTLRs. The 105-kDa protein form may be generated through posttranslational modifications.

By using flow cytometry with an anti-FLAG Ab, HEK293 cells expressing N-terminal laTLR14a or 14b were analyzed. laTLR14a (data not shown) and laTLR14b (Fig. 3B) were not detected on the surface of the transfected HEK cells. The laTLR14b with C-terminal FLAG showed a similar result (data not shown). When the cells were permeabilized and probed with anti-FLAG Ab (Fig. 3C), laTLR14b (C-terminal FLAG) was detected in the M1-positive gate by flow cytometry. Ultimately, ~10% of the transfected HEK cells expressed laTLR14b inside the cells.

We next probed laTLR14b in lamprey cells with the Ab against laTLR14b. The lamprey gill specimen was immunohistochemically stained using the Ab against laTLR14b. Some of cells in the epithelium of the lamprey gill were laTLR14b-positive (Fig. 3D), although the signal of laTLR14b was not very strong. laTLR14b appeared to reside inside the cells, consistent with the results of ectopic expression studies on laTLR14.

To see the localization of laTLRs in organelles, cells overexpressing laTLR14a and 14b were analyzed by a confocal microscope (Fig. 4). Two cell lines of human and fish were used for laTLR14 localization analysis. In human HeLa cells, FLAG-tagged laTLR14a and 14b were merged with the ER marker calnexin (Fig. 4A, upper rows), but not with markers for the Golgi apparatus (Fig. 4A, lower rows), endosomes, lysosomes, and F-actin (data not shown). In medaka OL-17 cells, the majority of the laTLR14 populations were localized to the ER, although some of them were distributed in other organelles (Fig. 4B, upper rows) but not merged with the Golgi apparatus (Fig. 4B, lower rows). Thus, like TLR9 as reported previously (32), laTLR14a and 14b are largely localized to the ER and only partly moved to other organelles.

**Table II. Amino acid identities between fugu and lamprey TLRsa**

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*The values of identities are indicated as a percentage, and the highest values are shown by bold numbers. Low, Percentage of similarity is too low to determine. |

* LRR domain of laTLR14.

* TIR domain of laTLR14.

**FIGURE 2.** Phylogenetic tree for laTLR14a/b. Phylogenetic tree of puffer fish (fg), chicken (ch), mouse (mo), human (hu), and lamprey (la) TLRs. The amino acid sequences of the TIR domains were subjected to analysis. Bootstrap values (<900) are shown. Lamprey TLRs belonging to the TLR2 family are shown by bold characters.
terms of NF-κB and the IFN-β promoter at the doses at which they activate huTLRs (data not shown). The reporters did not specifically respond to these reagents even with different transfection methods (data not shown). No significant increase of reporter was observed with any bacterial extract tested (C. sporogenes and S. typhimurium) for activation of laTLR14a/b (data not shown).

Functional analysis of the CD4-laTLR14b chimera protein

We next investigated whether the reporter is activated by the chimera construction with extracellular CD4 and intracellular laTLR14a or 14b by using HEK293 human reporter analysis. The Ig-like domains of human CD4 were ligated with the transmembrane and intracellular regions of laTLR14a or 14b (Fig. 5A). Flow cytometric analysis showed that ∼3.6% of CD4-laTLR14a and 15% of CD4-laTLR14b were expressed on the surface of the transfected HEK293 cells (M1 region in Fig. 5B). Using the ELAM promoter and the p-125 luc promoter, we examined whether the chimera laTLR14a or laTLR14b elicits activation of the NF-κB and IFN-β promoter by CD4 dimerization. CD4-laTLR14b induced ELAM promoter-mediated NF-κB activation (Fig. 5C). The activation of NF-κB was suppressed by cotransfection of the MyD88 dominant negative form, suggesting that laTLR14b activates NF-κB via human MyD88. Human Mal and the Toll-IL-1R domain-containing adaptor molecule (TICAM)-1 appear not to be involved in the TLR14-mediated NF-κB activation pathway (Fig. 5C). The activation of NF-κB was suppressed by cotransfection of the MyD88 dominant negative form, suggesting that laTLR14b activates NF-κB via human MyD88. Human Mal and the Toll-IL-1R domain-containing adaptor molecule (TICAM)-1 appear not to be involved in the TLR14-mediated NF-κB activation pathway (Fig. 5C). The activation of NF-κB was completely inhibited by cotransfection with MyD88 dominant negative in HEK cells (data not shown). The minute IFN-β promoter activation appears to be induced secondarily via MyD88 and NF-κB. Thus, laTLR14b is a signaling receptor for the activation of NF-κB in the human cell system.

Even though CD4-laTLR14a was slightly expressed on the surface of HEK transfectants, it barely induced signaling in response to CD4 dimerization (data not shown).

FIGURE 3. Protein expression analysis of laTLR14a/b. A, Immunoblotting. Plasmids containing C-terminal FLAG-tagged cDNAs of laTLR14a and laTLR14b were transfected into HEK293 cells. After 24 h, cells were solubilized and lysates were collected for immunoblotting. The blots were probed with an anti-FLAG Ab or an anti-laTLR14b Ab. The control contained only the vector. Molecular mass values are shown to the left. The minor bands above the laTLR14b protein may be a secondary product generated through modification. B and C, Flow cytometric analysis for surface-expressed and intracellular TLRs. HEK293 cells were transfected with vectors that allow the expression of an N-terminal FLAG-tagged laTLR14b protein. After 24 h, cells were treated first with anti-FLAG Ab and then with FITC-labeled goat anti-rabbit IgG Ab. Isotype-matched IgG was used as a control for the first Ab. laTLR14a was barely detectable with anti-FLAG Ab (data not shown). Intracellular TLRs were detected in permeabilized cells. Similar results were obtained using C-terminal-tagged constructs (data not shown). Percentages of cells in the M region are indicated. Three independent experiments gave similar results. A representative profile using the N-terminal tagged construct is given. D, Immunohistochemical staining of lamprey gills by an Ab against laTLR14b. The specimens were stained with an anti-laTLR14b Ab and a HRP-labeled second Ab. Nonimmune rabbit IgG was used as a negative control (not shown). Left panel, Original magnification, ×80; right panel, the marked region is enlarged (original magnification, ×400).

FIGURE 4. Subcellular localization of laTLR14a/b. HeLa cells (A) or medaka (Hd-rR) OL-17 cells (B) attached to coverslips were transfected with plasmid of laTLR14a or 14b (C-terminal FLAG-tagged). Twenty-four hours after transfection, cells were incubated with organelle markers for 1 h. FLAG-tagged proteins were stained with anti-FLAG Ab and imaged by confocal microscopy. The yellow staining in the overlay indicates co-localization of laTLR14a. Examples for the co-localization analysis are shown for ER (calnexin) (upper panels) and Golgi (wheat-germ agglutinin (WGA)) (bottom panels). A predominant ER merging profile was observed in human HeLa cells as well as OL-17 fish cells (B), which were pretreated similarly to HeLa cells. Results of one of the three representative experiments is shown.
Comparison of laTLRs with VLR

Distribution of laTLR14a and 14b were determined by RT-PCR using cDNAs from various tissues as templates. For this purpose, lampreys were harvested in the Ishikari river every winter for 5 years. Individual lamprey showed similar laTLR14a/b mRNA expression profiles (Fig. 6). laTLR14a mRNA was exclusively expressed in the gills of lampreys. In contrast, laTLR14b mRNA was expressed in the skin, gills, heart, liver, gut, and leukocytes (Fig. 6). The strongest signal was obtained in the gill and gut. We could not confirm the results by immunoblotting or histochemical staining using the anti-laTLR14b Ab (data not shown), which may reflect the low protein expression levels of this protein in various organs.

It has been reported that lampreys express proteins with various sets of tandemly arranged LRRs named VLRs in their lymphocyte-like cells and that VLRs are related to the lamprey immune system (12, 13). We isolated these cells as described earlier (11), extracted RNA, and confirmed the existence of VLR messages using RT-PCR (Fig. 6). The VLR mRNAs were unequivocally detected in leukocytes but too faint to see in other organs by ethidium bromide staining. The VLR mRNA bands were detectable in the organs including the gill, heart, and gut after repetitive PCR (data not shown). It is likely that the origin of the VLR mRNAs is blood leukocytes. The results showed that leukocytes express both laTLR14b and VLR. Based on the broad PCR bands corresponding to VLR, its primary sequences appeared to be variable. To determine actual nucleotide sequences of the VLRs, we cloned the PCR amplicons and sequenced. The alignment of the representative amino acid sequences is shown in Fig. 7A.

Discussion
In this study, we demonstrated the following. First, the lamprey possesses type I membrane proteins consisting of LRRs and the TIR domain that may be classified into the TLR family based on their structure. We call these proteins laTLR14a and laTLR14b. Second, the localization analysis in human and fish cell lines revealed that the lamprey TLR-like proteins largely reside in the cytoplasmic compartment ER in human cells. In transfected fish cell lines, the laTLR14s were localized to the ER and other organelles.

FIGURE 5. laTLR14b activates NF-κB via MyD88 in human cells. A. Constructions of constitutive active forms of laTLR14a (CD4-TLR14a) and laTLR14b (CD4-TLR14b). The sequence of fused cDNAs extracellular domains of human CD4 and each TIR domain of laTLRs is shown. B. Expression of the fused proteins in HEK293 cells. Twenty-four hours after transfection of the indicated cDNAs into HEK293 cells, the protein expression levels of CD4-TLR14a and 14b on the cell surface were measured by flow cytometry using anti-CD4 Ab conjugated with PE. Protein expression is barely detectable in cells transfected with the empty vector (top) or CD4-TLR14a cDNA (center). Only CD4-TLR14b expression is detectable on the surface (bottom). C. Luciferase reporter assay was performed to measure the levels of activation of NF-κB. An empty vector and each CD4-TLR14 construct were transfected into HEK293 cells together with an ELAM-luciferase reporter plasmid. The dominant negative (DN) forms of MyD88 (MyD88 DN), Mal/TIRAP (Mal DN), or TICAM-1 (TICAM DN) were simultaneously infected into HEK293 cells together with the above proteins as described in the Materials and Methods. After 24 h, luciferase activities in cell lysates were measured. Relative luciferase activities are shown as described previously for determination of activation of NF-κB (26). Experiments were performed in triplicate. Results of one of the three experiments is shown.

FIGURE 6. Tissue distribution of laTLR14a/b in comparison with VLR. Tissue distribution of mRNA of laTLRs in L. japonica organs. Amplifications of TLR gene fragments were accomplished by the RT-PCR technique using equal numbers of PCR cycles. Cytoskeletal actin (actin) of lamprey was used for a positive control, and templates without reverse transcriptase (no RT) were used for a negative control. The control PCRs were performed with all of the primer sets indicated in the figure and no band was detected (data not shown). Results using the actin primers are shown here. cDNAs were prepared from total RNA in each tissue from 13 individual lampreys. The conditions of the PCR analysis are described in the Materials and Methods. Amplified DNA fragments were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. A representative mRNA distribution profile obtained by PCR analysis is shown here because similar results were obtained with each individual regarding the laTLR14a/b distribution profiles. VLR is exclusively expressed in lamprey leukocytes, but the VLR mRNA levels appear to be different among individuals. Examples are shown with leukocytes (animals no. 7 and no. 9). It has been reported that lampreys express proteins with various functions as described previously for determination of activation of NF-κB (26). Experiments were performed in triplicate. Results of one of the three experiments is shown.

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In this study, we demonstrated the following. First, the lamprey possesses type I membrane proteins consisting of LRRs and the TIR domain that may be classified into the TLR family based on their structure. We call these proteins laTLR14a and laTLR14b. Second, the localization analysis in human and fish cell lines revealed that the lamprey TLR-like proteins largely reside in the cytoplasmic compartment ER in human cells. In transfected fish cell lines, the laTLR14s were localized to the ER and other organelles.
Third, NF-κB and IFN-β promoter activation was promoted in human cells by artificial dimerization of the lamprey protein laTLR14b. Fourth, Lamprey TLR proteins existed structurally independent of VLR; these TLR proteins were neither variable in their primary sequences nor expected to have GPI anchors. Fifth, laTLR14b was preferentially expressed in the gills, gut, and leukocytes. Thus, the lamprey most likely has immune-related TLRs structurally distinguished from the reported VLR proteins. In TLRs, the LRR motif serves to

FIGURE 7. VLRs in the lamprey. A. Alignment of amino acid sequences of part of LRRs in VLR and TLR in the lamprey. Seventeen L. japonica VLRs were cloned from the mRNA of gill (animals no. 1 to no. 5), gut (animals no. 6 to no. 10) and leukocytes (animals no. 11 to no. 17). The VLR clones may be originated from blood leukocytes. laTLR14b DNA fragments were cloned from cDNA libraries of three individual fish (animals no. 3, no. 7, and no. 9) B, Gi, gill; WBC, white blood cells. The cloned laTLR14b fragments did not exhibit any diversity like VLRs. The identical residues are shaded by BOXSHADE program (www.ch.embnet.org/software/BOX_form.html).
recognize microbial patterns and signatures and the TIR participates in intracellular signaling. The functional results for laTLR14b over-expressed in human cells are at least compatible with those for TLRs of other species. Yet, the pattern for recognition by this laTLR remains unknown. Further investigation is needed to determine whether the lamprey TLRs are localized to putative macrophages/monocytes. These cells may differ from lamprey lymphocytes, which have been shown to be VLR-positive cells.

Our data also suggest that the lamprey protein laTLR14b is capable of activating the huTLR signal pathways. In fish and chicken TLRs, their functions can be determined by a reporter assay with human cells expressing a CD4-TIR chimera or dominant negative proteins. That is, the chicken TLR2 signals the presence of lipoproteins even in the human system (34). The fish TLR5 and TLR21 recognize flagellin and poly(I-C) (19) (A. Matsuo and T. Seya, unpublished data), respectively, to induce activation of the NF-κB and IFN-β promoter in human cells (33, 34). Thus, we currently hold that human MyD88 is associated with laTLR14b-mediated NF-κB and secondary IFN-β promoter activation according to the results of chimera and dominant negative experiments. The results show the likelihood that lamprey TLRs act as pattern recognition receptors and transmit signals to downstream adapter proteins in host cells. For final confirmation, however, we must examine the protein function in a system using lamprey cell lines (which are not yet available).

The two lamprey TLRs belong to the subfamily of TLR14. laTLR14a/b most resemble each other and their TIRs are ~50% homologous to that of huTLR2. Gene duplication of TLR14 ortholog may have resulted in the two TLR14 genes in the lamprey. TLR14 and TLR2 do not appear to be duplicated in fish. However, a TLR2 pseudogene appears upstream in tandem with the functional TLR2 in the opossum, dog, and human (6). The pseudogene is probably from a duplication event before the divergence of marsupials. Likewise, a putative duplication event may have caused the two lamprey TLRs.

T. rubripes has a set of TLRs similar to those of the human and mouse (9) and orthologs of MyD88, Mal/TIRAP, and TICAM (a single gene representative of TICAM-1 and TICAM-2) (A. Matsuo and T. Seya, unpublished data). The fish TLR system is structurally and functionally comparable with those of the human and mouse. Only minor functional variations appear to have occurred during evolution of the vertebrate TLR system, and the fundamental functional properties of the TLR system are conserved across fish and humans. Our present results may present a key to prove the lamprey possesses orthologs of the mammalian TLR system and adapters.

L. japonica cells discard part of their genome during maturation (18). Their genome properties may be changed year to year. We have surveyed the mRNAs of laTLR14b and VLR for 5 years. Leukocytes of all individual lampreys expressed laTLR14 (a/b) and VLRs for all 5 years. Distributions of laTLR14b in lampreys were similar among individual fish. More limited expression of TLR14a than TLR14b was observed in all individuals tested. Thus, the laTLR14b protein, which is the ortholog of immune-related TLR, is actually expressed in the gills of live lampreys.

TLR1, 2, 6, and 10 are members of the TLR2 subfamily. In humans and mice, TLR2 recognizes diacylated mycoplasma lipoproteins in combination with TLR6 and triacylated bacterial lipoproteins together with TLR1 (25, 35, 36). If TLR14 is a member of the TLR2 subfamily, laTLR14 might recognize some microbial patterns in concert with another TLR2 member protein. Thus, we investigated whether lamprey and fish TLR14 exerts its function with huTLR2 members. However, laTLR14a/b, as well as fish TLR14, did not activate NF-κB even by coexpression with huTLR2 or other TLR2 members under the condition of stimulation with bacterial lipoproteins or other known TLR ligands (data not shown). Only a chimera version consisting of extracellular CD4 and the TIR domain of laTLR14b activates NF-κB as previously reported for huTLR4 and mouse TLR6, which activate NF-κB via their CD4-mediated dimerization (1, 22). Because TLR14 is found in T. rubripes (fgTLR14) (6), D. rerio, and X. tropicalis but not in human, mouse and chicken, its ligand may be a component of microorganisms in water.

The genomes of Drosophila and the mosquito A. gambiae contain ~10 Toll homologues, but only a few participate in host immunity (7, 8). Others are linked to developmental functions. Likewise, immune function could not be attributed to the single Toll homologue of Caenorhabditis elegans (37) and Caenorhabditis briggsae or to the TLRs reported in the horseshoe crab Tachypleus tridentatus (1). In contrast, the genome of the sea urchin Strongylocentrotus purpuratus abounds with TLR-containing genes, presumably >300 (6, 7). The genomes of the amphioxus Branchiostoma floridae and the solitary tunicate Ciona savignyi contain >10 TLR genes (7). The protostome and deuterostome invertebrates appear to have been differentially evolved in terms of the TLR system. The differences in the TLR system may reflect the differences in the microbial environment where each species of invertebrates survives. A critical factor for the selection of the TLR system would be infection.

It has been reported that the lamprey responds to some extent to some adjuvants (38) that we have revealed as TLR agonists. In addition, the lamprey possesses a complement-like molecule that opsonizes rabbit erythrocytes (39). Later, the lamprey had been shown to have the complement system including C3 and its activation and inactivation cascades (20, 40). Major family for complement regulatory proteins consists of short consensus repeats, and the lamprey possesses a short consensus repeat-containing complement-inhibitory protein named lacrep (20). The lamprey also expresses many C-type lectins like those in Ciona intestinalis (41, 42). However, whether or not the TLR pathogen-recognition system is conserved in jawless fish has remained unclear at a molecular level. Our results regarding two lamprey TLRs of the TLR2 subfamily add some pieces of knowledge to the vertebrate innate immune system. The lamprey possesses a pattern recognition system involving the complement and its regulatory systems, C-type lectins, and the TLR system as observed in higher vertebrates, including humans.

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Disclosures

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References
