Sensing Bacterial Flagellin by Membrane and Soluble Orthologs of Toll-like Receptor 5 in Rainbow Trout (Onchorhynchus mikiss)*

Received for publication, July 7, 2004, and in revised form, August 10, 2004
Published, JBC Papers in Press, August 31, 2004, DOI 10.1074/jbc.M407634200

Tadayuki Tsujita‡§, Hironobu Tsukada‡§, Miki Nakao‡, Hiroyuki Oshiumi‡, Misako Matsumoto‡§, and Tsukasa Seya‡§**‡‡

From the ‡Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537-8511, Japan, the §Department of Molecular Immunology Nara Institute of Science and Technology, Ikoma, Nara 631-0101, Japan, the ¶Laboratory of Marine Biochemistry, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8522, Japan, and the **Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8637 Japan

Rainbow trout (Onchorhynchus mikiss) possess two genes encoding putative leucine-rich repeat (LRR)-containing proteins similar to human TLR5. Molecular cloning of these two LRR proteins suggested the presence of a TLR5-like membrane form (rtTLR5M) and a soluble form (rtTLR5S). Here we elucidated the primary structures and the unique combinatorial functions of these fish versions of TLR5. The LRR regions of rtTLR5S and rtTLR5M exhibited 81% homology and relatively high (35.6 and 33.7%) homology to the extracellular domains of human TLR5 (huTLR5). Thus, two distinct genes encode the TLR5 orthologs in fish, one of which has a consensus intracellular domain (TIR). In order to test their functions, we constructed fusion proteins with the LRR region of rtTLR5S (S-chimera) or that of rtTLR5M and the TIR of huTLR5 (M-chimera). The S- and M-chimeras expressed in HeLa or CHO cells signaled the presence of Vibrio anguillarum flagellin, resulting in NF-κB activation. rtTLR5M was ubiquitously expressed, whereas rtTLR5S was predominantly expressed in the liver. In the hepatoma cell lines of the rainbow trout RTH-149, stimulation of rtTLR5M with V. anguillarum or its flagellin allowed the up-regulation of rtTLR5S. Flagellin-mediated NF-κB activation was more significant in the presence of or simultaneous expression of rtTLR5S. Therefore, a two-step flagellin response occurred for host defense against bacterial infection in fish: (a) flagellin first induced basal activation of NF-κB via membrane TLR5, facilitating the production of soluble TLR5 and minimal acute phase proteins, and (b) the inducible soluble TLR5 amplifies membrane TLR5-mediated cellular responses in a positive feedback fashion.

Toll-like receptors (TLR)1 recognize foreign material and alert for clearance and immune responses in a systemic fashion. The NF-κB activation pathway and type I IFN-producing pathway are usually activated in response to microbial constituents, which is referred to as pathogen-associated molecular patterns (PAMPs) (2, 3). TLRs are signaling receptors that engage in an innate immune recognition together with catch-up receptors (opsonin receptor, etc.). This precedes activation of the acquired immune system, including T/B lymphocytes. Although it has been believed that the innate immune system (including TLRs) is present in all vertebrates as well as invertebrates, no functional properties of the TLR have been reported in lower vertebrates. The functional feature of TLRs has been extensively investigated in human and mice. The results of these studies are generally compared with those of Drosophila, which have a Toll family of proteins with considerably different functional properties (4).

In humans, ten members of the TLR family (TLR1–10) have been identified (1–5). All TLRs have an extracellular domain containing leucine-rich repeats (LRRs) flanked by a C-terminal region and a cytoplasmic signaling domain, called the Toll/IL-1 receptor homology domain (TIR) (1–5). Each TLR recognizes a distinct ligand(s) and elicits different, sometimes overlapping, immune responses (2, 3). A variety of immune responses are induced through TLRs in dendritic cells (DCs) (1, 2), which are major antigen-presenting cells that first encounter foreign material to mount its antigens for presentation to lymphocytes. The activation of DCs is differentially regulated by TLRs and the adaptors that bind the TIR domain of TLRs, selecting appropriate signal pathways and resulting output (2, 3).

According to the pufferfish Fugu (Fugu rubripes) genome project, the signature TIR domain has been conserved across evolution (6). Three major differences were identified in the family of the Fugu Toll-like receptors (ftTLRs) when compared with human TLRs (6): 1) There is a soluble form of the TLR5 ortholog in the fish but not in humans. 2) No TLR4 ortholog has been identified in the fish, and 3) TLRs named TLR21 and

* This work was supported in part by CREST, Japan Science and Technology Agency, grants-in-aid from the Ministry of Education, Science, and Culture (Scientific Research on Priority Areas), Zoonosis Control Project, and the Ministry of Health and Welfare, and by Center of Excellence of Nara Institute of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB092504 and AB091105.

† To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8637 Japan. Tel.: 81-11-706-5073; Fax: 81-11-706-7866; E-mail: seya-tu@med.hokkaido.ac.jp.

‡‡ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8637 Japan. Tel.: 81-11-706-5073; Fax: 81-11-706-7866; E-mail: seya-tu@med.hokkaido.ac.jp.

The abbreviations used are: TLR, Toll-like receptor; LRR, leucine-rich repeat; TM, transmembrane; TIR, Toll/IL-1 receptor homology; TICAM-1, TIR-containing adaptor molecule-1; TICAM-2, TIR-containing adaptor molecule-2; nt, nucleotides; GST, glutathione S-transferase; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; LBP, LPS-binding protein; MALP-2, macrophage-activating lipopeptide-2; ODN, oligodeoxynucleotides; PGN, peptidoglycan; RT, reverse transcription; TNF, tumor necrosis factor; UTR, untranslated region; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline; ORF, open reading frame; CHO, Chinese hamster ovary; PAMP, pathogen-associated molecular patterns; DC, dendritic cell; EST, expressed sequence tag.
Flagellin Recognition by Two TLR5 Orthologs in Fish

TLRs2 are novel and specific to fish. fgTLR2, -3, -5, -7, -8, and -9 structurally correspond to those of mammalian TLRs. A possible interpretation is that TLR1, -2, -3, -4, -5, -7, -8, -9, -21, and -22 were lost in the mammalian lineage. Solitary ascidian (Ciona intestinalis) has only three putative Toll-like proteins (7), which, like Caenorhabditis elegans Toll (8), represent primordial forms before expansion into the Toll family. Drosophila has nine Tolls, and their functional properties were mostly related to body patterning as well as host defense (9). Current evidence suggests that the development of TLR genes is earlier than that of fish, but not of the ascidian, separate from mammals.

The results of various investigations including ours conclude that the mammalian innate system was established preceding or concomitant with the assembling of the acquired immunity. This reflects selection pressure exerted by pathogens under distinct environments.

Rainbow trout has two TLR5 isoforms, membrane (rtTLR5M) and soluble (rtTLR5S) forms (10) similar to fgTLR5S (6). These two forms of TLR5 recognized Vibrio anguillarum flagellin. Here, we studied the TLR5-mediated immune response in the rainbow trout. A possible role for this unique flagellin recognition system of fish is discussed.

**MATERIALS AND METHODS**

**Cells and Reagents—**Cells were cultured in a medium containing 5–10% heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS) and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin (Invitrogen). R12-49 and RTG-2 cells (American Type Culture Collection, Manassas, VA) were cultured in minimal Eagle’s medium (MEM, Nissui, Tokyo, Japan). 0.1 mm MEM non-essential amino acid solution, and 1 mm MEM sodium pyruvate solution. Cervix adenocarcinoma HeLa cells (Japanese Cell Resource Bank, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). Chinese hamster ovary (CHO) (Japanese Cell Resource Bank) cells were cultured in Ham’s F12 medium (Nissui). SF21 cells (Invitrogen) were cultured in Sf900 II SFM (Invitrogen).

**Escherichia coli DH5α** was purchased from Invitrogen. *V. anguillarum* was a gift from Dr. Takaji Iida (National Research Institute of Aquaculture, Fisheries Research Agency, Mie, Japan). LPS was purchased from BD Biosciences (San Jose, CA). Poly(I:C) was from Amer sham Biosciences (Buckinghamshire, UK). MALP-2 was synthesized in Biologica (Nagoya, Japan) (11). Peptidoglycan (PGN) was purified from *Staphylococcus aureus*. Polymyxin B was obtained from Sigma. All nucleotide primers and oligodeoxynucleotides (ODN) containing CpG motifs (Cpg-ODN) were synthesized at Hokkaido System Sciences (Sapporo, Japan).

**Molecular Cloning of a Soluble Form of rtTLR5 (rTLR5S) —**The EST clone that contains the leucine-rich repeat (AF281346) from rainbow trout exposed to *V. anguillarum* was cloned in our laboratory (12). Full-length rTLR5S cDNA was cloned using the SMART RACE cDNA amplification kit (BD Biosciences). For this, PCR was performed with liver cDNA templates and rTLR5S gene-specific internal primers (for cloning).

| Primer name | Primer sequences |
|-------------|------------------|
| S-GSP/F      | 5′-ATTTCCTCTCTCTGTGACCCAG-3′ |
| S-SNP/F      | 5′-TGGCCTCTAACAGGGTTCGCAGTTGC-3′ |
| S-SNP/R      | 5′-TGCGCTCTCAGGGTTCGCAGTTGC-3′ |
| SF           | 5′-CGGCTCTGGATTTCTGGCAGG-3′ |
| SR           | 5′-GATTGGCTTGGATTTCTGGCAGG-3′ |
| MGSFP        | 5′-CTACAATGGGTTGAAACGGCCC-3′ |
| MNGSPF       | 5′-CTAGCTCTCTAATGGGAGGGCTCCAGAGGC-3′ |
| MNGSPR       | 5′-CCAGGCGGTCCTTGAGGAACCCTT-3′ |
| MNGSPR       | 5′-CTGCTCTCCTCCCTCCATGGACCCAG-3′ |
| SF           | 5′-GATTGGCTTGGATTTCTGGCAGG-3′ |
| SR           | 5′-GATTGGCTTGGATTTCTGGCAGG-3′ |
| fluTLR5S     | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| flu5F        | 5′-TTGCTGCCATTTGAAGGAACCCAG-3′ |
| flu5R        | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5S      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5M      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5S      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5M      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5S      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |
| rtTLR5S      | 5′-GATGGCCTGACGGTTGGAACCCAG-3′ |

* a: GSP, gene-specific primer.  
* b: NGSP, nested gene-specific primer.
Flagellin Recognition by Two TLR5 Orthologs in Fish

CATTAGG-3'). Resultant PCR products were first cloned into the PCR-Blunt vector (Invitrogen) and secondly cloned to the pGEX-2T vector (Amersham Biosciences) at the BamHI and EcoR1 sites (flaA) and BamHI and EcoR1 sites (flaB), and designated pGEX2T/FlaA and pGEX2T/FlaC. E. coli BL21(DE3)LysE cells were transformed with pGEX2T/FlaA or pGEX2T/FlaC, and GST-tagged flagellin A (GST-FlaA) and flagellin C (GST-FlaC) were purified from the solubilized cells using glutathione-Sepharose (Amersham Biosciences) according to the manufacturer's instructions. Purified GST-FlaA and GST-FlaC were digested with thrombin according to the manufacturer's recommendation and designated as recombinant FlaA (rFlaA) and flagellin C (rFlaC). Purity of the products was confirmed by SDS-PAGE (16).

Stimulation of RTH-149 and RTG-2 Cells by Flagellin or Dead Bacteria—RTH-149 and RTG-2 cells were plated in a 6-cm dish plate, and the medium was changed every 2 days. Cells (1 × 10⁶ cell) were stimulated with freeze-thaw V. anguillarum (10 colony-forming units) or rFlaA (1 µg/ml). Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). RT-PCR was performed as mentioned above. The PCR products were separated in a 1.5% (w/v) agarose gel and stained with ethidium bromide. Quantitative PCR was performed with iQ SYBER Green Supermix, and amplified PCR products were measured by iCycler iQ real-time PCR analyzing system (Bio-Rad).

Reporter Assay—The S-chimera was constructed by fusing cDNAs encoding the FLAG-tagged extracellular domain of rtTLR5S (amino acids 29–597) to the C-terminal flanking region, transmembrane, and cytoplasmic domains of huTLR5 (amino acids 574–855). The construct was cloned into the pEFBOS expression vector (17). The M-chimera was constructed to fuse the FLAG-tagged extracellular domain of rtTLR5M (amino acids 21–596) to the transmembrane and cytoplasmic domains of huTLR5 (amino acids 574–855). Full-length huTLR5 was obtained from human monocyte cDNA by RT-PCR using primers hu5F and hu5R (Table I). The cDNA of huTLR5 was placed in the pEFBOS plasmid. The promoter region of human E-selectin (ELAM) (241 to 245) was ligated between KpnI-HindIII sites of pGEM.3 (Promega) which was digested with KpnI and HindIII. The ligated plasmid was transfected in CHO or HeLa cells, which were transiently transfected with rtTLR5S cDNA (1, 10, 100 ng) and cultured for 12 h. Then the cells were treated with rFlaA (0.01, 0.1, 1 µg/ml), poly(I:C) (10 µg/ml), LPS (0.1 µg/ml), and CpG-ODN (2 µg/ml). Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) and an ECL kit (Amersham Biosciences). RT-PCR was performed as mentioned above. The PCR products were separated in a 1.5% (w/v) agarose gel and stained with ethidium bromide. Quantitative PCR was performed with iQ SYBER Green Supermix, and amplified PCR products were measured by iCycler iQ real-time PCR analyzing system (Bio-Rad).

Identification of TLR5-like Sequences from the Rainbow Trout—Based on an EST sequence obtained from rainbow trout infected with V. anguillarum (AF281346), we cloned a cDNA by 3'- and 5'-RACE that predicted the existence of LRR-containing proteins. A motif search, Kyte and Doolittle plot, and ClustalW comparison suggested that the amino acid sequence corresponds to the extracellular LRR domains of human TLR5 and surprisingly contained no transmembrane region or TIR domain (Fig. 1B). SMART analysis defined ten LRRs in this protein (Fig. 1C). Based on these results, we designated this putative protein of rainbow trout soluble TLR5 (rtTLR5S).

To identify the membrane form of TLR5 in rainbow trout, degenerate primers were designed according to the conserved sequences among the TIR domains of human, mouse, and fgTLR5 (Fig. 1A). PCR using degenerate primers (Fig. 1A) followed by the RACE method allowed us to clone additional TLR5-like cDNA. Homology search analysis suggested that this is an ortholog of human TLR5, which contained putative signal peptides, 11 LRRs (including the LRR C-terminal flanking region), and the TIR domain (Fig. 1B, C). The complete sequences of these two rainbow trout (rt) TLR5s are shown in Fig. 1B.

We have cloned seven independent RT-PCR products of rtTLR5S using mRNA and SF and SR primers (Table I). The collective deduced sequence consisted of a 1,992-bp open reading frame (ORF) and a 1,168-bp 3'-UTR. Three polyadenylation signals were found in the 3'-UTR, the last of which was followed by the poly(A) tail. The predicted amino acid sequence of this cDNA is 664 amino acids (Fig. 1). rtTLR5s was 35.6 and 37.1% homologous to the extracellular domains of human and mouse TLR5, respectively (10). rtTLR5s showed <25% homology to that of other human or mouse TLRs. Nine of the eleven cysteines in the extracellular domains of moTLR5 were conserved in rtTLR5S (Fig. 1B).

We have cloned four independent RT-PCR products for rtTLR5M using MP and MR primers (Table I). The collective deduced sequence consisted of a 2,637-bp ORF and 544-bp 3'-UTR. The polyadenylation site in the 3'-UTR was followed by the poly(A) tail. The predicted amino acid sequence of this cDNA is 799 amino acids (Fig. 1B). rtTLR5M was 40.1, 40.5, and 48.5% homologous to hu/mo/fgTLR5, respectively. rtTLR5M is <25% homologous to that of other hu/mo/fgTLRs. Ten cysteines found in the extracellular domains of mouse and human TLR5 were conserved in rtTLR5M (Fig. 1B).

The LRR regions of rtTLR5M and rtTLR5S exhibited 81.0% homology. Several tissues were tested for rtTLR5M- and rtTLR5S-specific messages using the specific primer sets shown in Table I (Fig. 1D). rtTLR5M was ubiquitously expressed in all tissues whereas rtTLR5S was predominantly present in liver. The presence of the soluble form of TLR5 is surprising since no such TLR5 has been reported either in human and mouse.
FIG. 1. Isolation of rainbow trout TLR5s. **A**, design of degenerate primers for isolation of rtTLR5M. Human, mouse, and Fugu TIR domain of TLR5 (GenBank™ accession numbers are AB060695, AF186107, and Ref. 6) were aligned by ClustalW program. Degenerate primers were designed with reference to a highly conserved region (boxed). Sequences of the relevant degenerate primers are shown below the alignment. **hu**, human; **mo**, mouse; **fg**, Fugu. **B**, alignment of the rtTLR5M and S amino acid sequences deduced from their cDNA sequences. Homology between the extracellular domains of the two types of rtTLR5 is indicated. Identical residues are indicated by asterisks. Predicted signal sequences are shown by bold underline. Location of the EST clone (AF281346.1) is underlined. **C**, schematic representation of domain organization of rtTLR5S and rtTLR5M. Each domain was predicted using the SMART program. **LRR-CT**, C-terminal flanking region that resembles one LRR domain. **D**, tissue distribution of rtTLR5S and rtTLR5M by RT-PCR. Specific primers for each type of rtTLR5 were used. Unsaturated cycles of PCR were employed for analysis. Notice, mRNA of rtTLR5S is expressed only in the liver, whereas rtTLR5M is ubiquitous.
Gene and Southern Analysis of rtTLR5S—Sequence analysis of the rtTLR5S gene revealed a 757-bp intron near the signal peptides (20 bp from the ATG start codon) (Fig. 2A). The gene of rtTLR5M had no introns. Southern blot analysis was carried out using two distinct probes covering the TIR region of rtTLR5M (probeM: rtTLR5M 1867–2707 nt, 841 bp) or the LRR region largely common to rtTLR5M and rtTLR5S (probeS: rtTLR5S 354–1314 nt, 961 bp) (Fig. 2B). Based on the nucleotide sequences of the ORFs, the intron, and 3'UTs of the DNAs, there was only one site each for EcoRI, HindIII, and PstI downstream of the probe S in rtTLR5S. Thus, based on the restriction fragment lengths, the size of the fragments must be larger than 2.4 kbp. Actual fragments obtained on Southern blots were 2.4 kbp in addition to the expected two fragments in each lane (Fig. 2B) suggesting the existence of an additional gene with a similar but different sequence.

Probe M was designed to hybridize with the downstream regions that encode TIR of rtTLR5M (Fig. 2C). ~1-kbp fragments were detected in the EcoRI- and PstI-digested lanes, which reflected the presence of the rtTLR5M gene. A 3.5-kbp fragment obtained upon HindIII digestion may correspond to the rtTLR5M gene. Additional upper bands, although faint, suggest the presence of additional sequences containing similar TIR sequences. In addition, we have also sequenced a pseudogene (third gene) with a number of stop codons in the putative coding region encoding a peptide similar to rtTLR5M (data not shown).

The genes of rtTLR5M and rtTLR5S contained two Sau3AI, AluI, and PvuII sites in their ORFs. Fragments obtained in Southern analysis are in line with these restriction sites (Fig. 2B). However, the three-band profile observed in PvuII digestion (Fig. 2C) cannot be explained. Because no message for the putative third gene of TLR5 was identified, a pseudogene similar to rtTLR5M must be present in addition to the two identified TLR5 genes.

rtTLR5S Is Induced in Response to V. anguillarum—Next we examined whether rtTLR5S is up-regulated by infection with V. anguillarum (Fig. 3). Fish cell lines of various origins were tested for this purpose. Of these, rainbow trout hepatoma cell line RTH-149 cells were used for this investigation, because rtTLR5S message induction was significant in this cell line in response to dead V. anguillarum (Fig. 3). The rtTLR5S mRNA was barely detectable in resting RTH-149 cells. The rtTLR5S mRNA was induced 4 h after bacterial stimulation. The level of mRNA became maximal at 6 h, then gradually decreased. No message was detected 48 h post-stimulation. On the other hand, the mRNA of rtTLR5M was expressed constitutively in this cell line regardless of stimulation (Fig. 3). Under the same conditions, rtIL-1β, an acute phase cytokine, was found to be up-regulated within 1 h and peaked around 3–10 h. Thus, rtTLR5S is another acute phase protein responding to stimulation. Similar induction profile of rtTLR5S was observed in a genital gland cell line RTG-2. These results were confirmed by quantitative PCR (data not shown). Hence, the acute phase response of rtIL-1β and rtTLR5S largely results from bacterial stimuli, presumably through a membrane form of rtTLR5. To
The presence of flagellin by the ectodomain and activate NF-κB via TIR in HeLa cells. A representative result using the chimera-expressing HeLa cells is shown in Fig. 5A. This chimera molecule activated the NF-κB reporter gene in HeLa cells to a degree similar to human TLR5 at similar expression levels (Fig. 5A). rFlaA and C had identical S-chimera stimulation potency (data not shown). To test the specificity, the S-chimera was stimulated with PGN (a ligand of TLR2), poly(I:C) (a ligand of TLR3), LPS (a ligand of TLR4), unmethylated CpG-ODN (a ligand of TLR9), and rFlaA (a ligand of TLR5) (Fig. 5B). The appropriate doses of these ligands were determined with human TLRs in our laboratory as described previously (19). The chimera molecule as well as human TLR5 exclusively recognized rFlaA to activate the NF-κB reporter gene (Fig. 5B). Thus, it is apparent that a functional interaction between rFlaA and rTLR5S exists.

A Chimera rTLR5M-TIR of Human TLR5 Recognizes Flagellin in Combination with rTLR5S—The function of rTLR5M was first tested by the expression of full-length rTLR5M in HeLa and CHO cells, but no NF-κB response was detected, albeit its expression was confirmed (data not shown). NF-κB activation was next determined with a chimera consisting of the LRIs of rTLR5M and the TM and TIR of huTLR5 (M-chimera) (Fig. 6). After several trials, we selected two cell lines for transfection of M-chimera. They are CHO, with a relatively high transfection efficiency, and HeLa, with a low transfection efficiency. The response of M-chimera to rFlaA (1 μg/ml) (Fig. 6A) and rFlaC (not shown) was detected as NF-κB activation in the CHO system, although the degree of the response was low. Flagellin-mediated NF-κB activation was marginally detected in M-chimera-expressing HeLa cells, probably due to its low transfection efficiency (Fig. 6A). Activation of NF-κB was specific to rFlaA in M-chimera-expressing cells, since TLR ligands, LPS (0.1 μg/ml), PGN (10 μg/ml), poly(I:C) (2 μg/ml), and CpG-ODN (2 μM) induced activation of NF-κB in these systems even at their optimal concentrations (Fig. 6B). We inferred from these that incompatibility of the TIR of rTLR5M to mammalian adapter molecules resulted in no NF-κB response.

Next the combination effect of rTLR5M and rTLR5S was studied. The experiment was designed so that the conditioned medium of rTLR5S-expressing CHO cells that contained rTLR5S was added to rTLR5M-expressing CHO cells, and the cells were treated with rFlaA (Fig. 6C). This resulted in NF-κB activation in proportion to the dose of the transfected cDNA of rTLR5S that was present in the CHO cell medium. Addition of the supernatant of M-chimera-expressing cells did not result in such up-regulation of NF-κB activation in the same system (data not shown). In M-chimera-expressing HeLa cells, flagellin-mediated NF-κB activation was much more augmented in the presence of the conditioned medium of rTLR5S-expressing HeLa cells (Fig. 6C).

To confirm this synergistic effect of rTLR5S on NF-κB activation, the rTLR5S protein was produced in the baculovirus system. Its physical binding to GST-FlaA (Fig. 7A) and functional properties (Fig. 7B) were examined. Physical binding of rTLR5S to flagellin was assessed by GST pull-down assay. The recombinant His-tagged rTLR5S (20 ng) was mixed with GST-FlaA or GST. Glutathione-Sepharose was added to the mixture and eluted with 10 mM glutathione in PBS. After extensive washing in PBS, the amounts of bound rTLR5S were checked by immunoblotting using anti-His antibody (Fig. 7A). The amounts of bound proteins were compared with that of lamprey complement 3 (AY359861) (20), which do not bind GST-FlaA. The binding assay suggested that rTLR5S has sufficient affinity to hold flagellin.
Flagellin Recognition by Two TLR5 Orthologs in Fish

In this investigation, we presented evidence for the existence of a soluble and membrane form of TLR5 in rainbow trout. The membrane form of TLR5, rtTLR5M (M stands for membrane form), was similar to human and mouse TLR5 in its overall structure. However, no soluble TLR5 has been reported in mammals. Since the soluble TLR5-like gene is homologous to the gene encoding soluble TLR5 of F. rubripes (6), we presumed this to be an ortholog of the Fugu soluble form of TLR5, and named it as rtTLR5SS (S stands for soluble form). When the LRR regions of these proteins were compared with those of huTLRs, they showed the highest similarity to huTLR5 (10). A BLAST search also showed that they are most similar to TLR5. In fish, soluble TLR5 is differentially regulated from the putative membrane form in liver to augment TLR5-mediated inflammatory responses to bacterial flagellin.

We demonstrated that rtTLR5M and S share similar ligand-recognition properties with the human and mouse TLR5 (21, 22). Hence, the flagellin recognition system is conserved across the human and fish, and in the fish lineage, this system developed into a more sophisticated one. First, bacterial flagellin stimulates membrane TLR5 in fish and NF-κB activation is increased proportionally, secondary to the induction of soluble TLR5 in the liver. Second, the induced soluble TLR5 efficiently catches-up bacterial flagellin in the circulation and brings it to membrane TLR5, which acts as a signaling receptor. In fish, robust activation of NF-κB then occurs through the combination of the two forms of TLR5. The advantage of this recognition system for flagellin is comparable to the scenario of LPS recognition by TLR5 in fish and NF-κB activation is increased proportionally, secondary to the induction of soluble TLR5 in the liver. Second, the induced soluble TLR5 efficiently catches-up bacterial flagellin in the circulation and brings it to membrane TLR5, which acts as a signaling receptor. In fish, robust activation of NF-κB then occurs through the combination of the two forms of TLR5. The advantage of this recognition system for flagellin is comparable to the scenario of LPS recognition by TLR5 in fish and NF-κB activation is increased proportionally, secondary to the induction of soluble TLR5 in the liver. Second, the induced soluble TLR5 efficiently catches-up bacterial flagellin in the circulation and brings it to membrane TLR5, which acts as a signaling receptor. In fish, robust activation of NF-κB then occurs through the combination of the two forms of TLR5.
mediated NF-κB activation in our chimera expression system (10). Flagellin activates IRAK via TLR5 (29). Recently, the TICAM-1 pathway has been shown to be responsible for IFN-β induction in TLR3 (30, 31) and TLR4 (32–34). An adapter complex, TICAM-2 and TICAM-1, plays a key role in induction of NF-κB activation in TLR4 signaling in humans (32, 33). Furthermore, in mammals, TLR5 together with TLR4 induces type I IFNs probably via TICAM-2 and TICAM-1 (35). Thus, what happens in TLR5 signaling in fish is an issue to be resolved.

Physiological significance of the soluble forms of TLR5 is a matter of interest. The basolateral expression of TLR5 serves as the flagellin receptor in epithelial cells. Indeed, TLR5 expression in the basolateral surface of intestinal epithelia detects the invasion of a large variety of microbes (36) and recruits inflammatory cells responding to the invading bacteria. The soluble TLR5 could be required in the fish to systemically amplify the inflammatory response generated by membrane TLR5. If this is the case, mammals had lost this soluble TLR5 function because the need for TLR5-mediated immune response is limited to a local environment where microbes invade, hence preventing systemic inflammatory responses induced by the bacteria (37). It has been known that fish are highly sensitive to flagellin, which induces endotoxin-like response in fish (38). Fish are highly resistant to LPS compared with mammals (39, 40). Probably because of the conserved function of TLR5 but not TLR4, fish sense flagellin rather than LPS as a major endotoxin.

The current concept is that a prototype of TLR arose for host defense before plants and animals diverged. In mammals, TLRs are pattern recognition receptors that directly recognize molecular patterns specific to microbes. In contrast, in Drosophila, Toll appears to function as “cytokine” receptors, some of which associate with development (9). The genome projects of Ciona and C. elegans suggested that Toll proteins are not involved in major part of host defense (7, 8). Thus, Drosophila Toll and aphidinae Toll and mammalian TLR families must have independently evolved (9). Although proteins with LRR motifs such as TLRs and CARD proteins (41) induce similar signal responses and functional outputs across species, the modes of microbial recognition followed by host defense response are variable among species and individual LRR protein families. These findings are interpreted to mean that the structural signature LRR does not always confer the specificity on protein recognition: the functional outputs across species, the modes of microbial recognition followed by host defense response are variable among species and individual LRR protein families. These findings are interpreted to mean that the structural signature LRR does not always confer the specificity on protein recognition.
their flagellin recognition systems. This study points to the fact that at least fish and human share a similar TLR system for the recognition of flagellin.

Our previous analysis revealed that *F. rubripes* has almost all the orthologs of the human TLRs (6). These findings suggest that TLR1, -2, -3, -5, -7, -8, -9 appeared before fish diverged from a mammalian ancestor, more than 400 million years ago (43). Our study adds the notion that the TLR system is conserved along with the NF-κB system (44) for cytokine induction across mammals and fish in not only structural but also functional features. Taken together, it is not surprising that other fish TLRs essentially conserve their functions as in TLR5. TLRs are main PAMP recognition molecules. Thus, further functional analysis of fish TLRs may enable us to show that the current mechanisms of the PAMP recognition system were already established in the human and fish common ancestor.

Recently, two reports (45, 46) mentioned the TLR family of zebrafish, which was deduced from a draft of the genome project (47). We found several inconsistent points between our results on *Fugu* TLRs and that of the zebrafish TLRs, one of which mentioned the absence of soluble TLR5 in zebrafish. We found soluble TLR5 in rainbow trout and pufferfish. At this immature stage of the genome project of zebrafish, however, one cannot conclude the absence of the soluble TLR5 in zebrafish. Once the fish system for analyzing PAMP actions is established, we will be able to clarify the signaling pathways of each TLR and determine the effect of PAMP on antibody production, CTL induction, and potentiation of NK activity. Zebrafish have been used for forward genetic screening and in vivo experiments to find new functional aspects of genes (47). Therefore, zebrafish will become a powerful tool for the analyses of TLR functions.

In summary, the prototype of the mammalian type Toll family is likely to be conserved across fish and human. Fish TLR5 signals the presence of flagellin to activate NF-κB, which enables us to interpret that human and fish share a similar flagellin recognition system and signaling pathway. The differences in the flagellin recognition system between fish and human are represented by the function of soluble TLR5 described here. Important factors for divergence between the fish and human TLR5 system would be selection pressure exerted by pathogens in distinct environments such as sea and land and the secondary adaptation of the Toll family genes to different sets of pathogens.

Acknowledgments—We thank Drs. N. Inoue, M. Tanabe, and Y. Kimura for helpful discussions. Thanks are also due to Dr. Y. Matsuura for helping establish the baculovirus expression system. Drs. F. S. Che and T. Iida kindly provided plant flagellin and *V. anguillarum*, respectively.

REFERENCES

1. Medzhitov, R. (2001) Nat. Rev. Immunol. 1, 135–145
2. Seya, T., Shingai, M., Tanabe, M., and Matsumoto, M. (2004) Rev. Med. Virol. in press
3. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
4. Hoffmann, J. A., and Reichhart, J. M. (2002) Nat. Immunol. 3, 121–126
5. Akira, S. (2003) J. Biol. Chem. 278, 38105–38108
6. Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., and Seya, T. (2003) Immunogenetics 54, 791–800
7. Azumi, K., De Santis, R., De Tomaso, A., Rigoutsos, I., Yoshizaki, F., Pinto, M. R., Marino, R., Shida, K., Ikeda, M., Ikeda, M., Araiz, M., Inoue, Y., Shimizu, T., Satoh, N., Rockash, D. S., Du Pasquier, L., Kasahara, M., Satake, M., and Nonaka, M. (2003) Immunogenetics 55, 570–581
8. Pujol, N., Link, E. M., Liu, L. X., Kurz, C. L., Alloing, G., Tan, M. W., Ray, K. P., Solari, R., Johnson, C. D., and Ewbank, J. J. (2003) Curr. Biol. 13, 809–821
9. Imler, J. L., and Hoffmann, J. A. (2001) Trends Cell Biol. 11, 304–311
10. Tsukada, H., Oshiumi, H., Fukui, A., Matsumoto, M., and Seya, T. (2001) Jpn. Soc. Mol. Biol. 16, 102 (abstr.)
11. Nishiguchi, M., Matsumoto, M., Takao, T., Hoshino, M., Shimonishi, Y., Tsuji, S., Begum, N. A., Takeuchi, O., Akira, S., Toyoshima, K., and Seya, T. (2001) J. Immunol. 166, 2610–2616
12. Bayne, C. J., Gerwick, L., Fujiki, K., Nakao, M., and Yano, T. (2001) Dev. Comp. Immunol. 25, 205–217
13. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 588–593
14. Sebastiani, G., Leveque, G., Larrivere, L., Larroche, L., Skamene, E., Gros, P., and Male, D. (2000) Genomics 64, 230–249

FIG. 7. Exogenously added recombinant rTLRSS augments rFlaA-induced NF-κB activation in M-chimera-expressing cells. A, purified His-tagged rTLRSS binds flagellin. rTLRSS (20 ng) or lamprey complement 3 (20 ng, control, data not shown) was incubated with GST-FlaA equilibrated with PBS, pH 7.5. The glutathione-Sepharose was added to the mixture and eluted the complex with 10 mM glutathione in PBS. The eluates of the proteins were analyzed on SDS-PAGE followed by immunoblotting. B, potentiation of NF-κB activation by flagellin in M-chimera-expressing cells by the addition of purified rTLRSS. M-chimera-expressing CHO (upper panel) and HeLa cells (lower panel) marginally responded to rFlaA (hatched bars, right) compared with the cells with empty vector (open bars, control). Various amounts of the purified rTLRSS (1, 10, and 100 ng/ml) were added to the cells expressing M-chimera 12 h before stimulation. At specified time intervals after rFlaA stimulation (5 h), cells were harvested, and NF-κB activity in cell lysates was determined by luciferase assay. Experiments were performed in triplicate, and the results are expressed as means ± S.D.
Sensing Bacterial Flagellin by Membrane and Soluble Orthologs of Toll-like Receptor 5 in Rainbow Trout (Onchorhynchus m. kis)
Tadayuki Tsujita, Hironobu Tsukada, Miki Nakao, Hiroyuki Oshiumi, Misako Matsumoto and Tsukasa Seya

J. Biol. Chem. 2004, 279:48588-48597.
doi: 10.1074/jbc.M407634200 originally published online August 31, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407634200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 18 of which can be accessed free at http://www.jbc.org/content/279/47/48588.full.html#ref-list-1